No effect of acute beetroot juice ingestion on oxygen consumption, glucose kinetics or skeletal muscle metabolism during submaximal exercise in males

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Running head: beetroot juice and skeletal muscle metabolism

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Abstract

Beetroot juice, which is rich in nitrate (NO\textsuperscript{3}\textsuperscript{-}), has been shown in some studies to decrease oxygen consumption (VO\textsubscript{2}) for a given exercise workload i.e. increasing efficiency, as well as increase exercise tolerance. Few studies have examined the effect of beetroot juice or nitrate supplementation on exercise metabolism. Eight healthy recreationally active males performed 3 trials involving ingestion of either beetroot juice (Beet; ~8 mmol NO\textsubscript{3}), Placebo (Nitrate depleted Beet), or Beet + mouthwash (Beet+MW); performed in a randomised single blind cross over design. Two and a half hours later participants cycled for 60 minutes on an ergometer at 65% of VO\textsubscript{2peak}. [6,6\textsuperscript{-2H}] glucose was infused to determine glucose kinetics, blood samples obtained throughout exercise and skeletal muscle biopsies obtained pre and post-exercise. Plasma nitrite [NO\textsubscript{2}\textsuperscript{-}] increased significantly (~130%) with Beet, and this was attenuated in MW+Beet. Beet and Beet+MW had no significant effect on oxygen consumption, blood glucose, blood lactate, plasma non esterified fatty acids (NEFA) or plasma insulin during exercise. Beet and Beet + MW also had no significant effect on the increase in glucose disposal during exercise. In addition, Beet and Beet+ MW had no significant effect on the decrease in muscle glycogen and PCr and the increase in muscle Cr, lactate and pACC during exercise. In conclusion, at the dose used acute ingestion of beetroot juice had little effect on skeletal muscle metabolism during exercise.

Keywords: beetroot, nitrate, glucose, oxygen uptake
Introduction

There has been much interest over recent years in the potential of inorganic NO$_3^-$ to increase exercise efficiency and exercise performance (23, 32). Based on this, beetroot juice (Beet) has been used as an ergogenic aid and health supplement because it contains large amounts of inorganic NO$_3^-$. It is now known that dietary inorganic NO$_3^-$ can be reduced to NO$_2^-$ and nitric oxide (NO) as well as other bioactive nitrogen species \textit{in vivo} (30). The bio-activation of NO$_3^-$ requires the formation of NO$_2^-$ as an intermediate, a reaction that is facilitated by anaerobic oral bacteria (31). The critical role of oral bacteria has been supported by studies demonstrating that antiseptic mouthwash abolishes the increase in plasma levels of NO$_2^-$ after the consumption of NO$_3^-$ (16). In addition, the reduction in blood pressure and gastro protective effects that have been shown with NO$_3^-$ ingestion are abolished when an antiseptic mouthwash is used prior to NO$_3^-$ ingestion in healthy participants (24, 46).

In regards to physical exercise, some (1, 4, 28, 30, 51), but not all studies (6, 10, 26, 54) at a similar dose (5-8 mmol), have found that multiday Beet / NO$_3^-$ ingestion decreases oxygen uptake (~3%) during sub-maximal and maximal exercise in healthy subjects. Furthermore the ingestion of beetroot juice just prior to exercise has shown similar effects (51, 54). This increase in efficiency during exercise following Beet/NO$_3^-$ ingestion has been hypothesized to be due to one or more of three possible mechanisms. The first proposes that Beet/NO$_3^-$ ingestion induces a reduction in the ATP cost of force production (1). In support of this Bailey et al. (1) found using phosphorus-31 magnetic resonance spectroscopy ($^{31}$P-MRS) that there was an attenuation of the decrease in PCr and estimated ATP turnover during low and high intensity knee-extensor exercise after several days of Beet ingestion.
The second possible mechanism is related to the efficiency in which the mitochondria produce ATP (28). In humans, the ratio of adenosine triphosphate (ATP) generated to the oxygen consumed (P/O ratio) during exercise increased after 3 days of NO₃⁻ supplementation in healthy subjects (28). There was also a significant decrease in basal leak respiration and an increase in expression of the mitochondria protein ADP/ATP translocase (ANT), one of the main proteins attributed to leak respiration (28). These findings suggest that nitrate supplementation enabled the mitochondria to better maintain the proton gradient across the membrane. However, an increase in mitochondria efficiency can not explain comparable increases in efficiency (~3%) after one dose of NO₃⁻ 2.5 hours prior to exercise (51, 54) and following multi-day supplementation (1, 4, 28, 30). Indeed, it is unlikely that there is sufficient time for changes in protein expression to occur 2.5 hr after NO₃⁻ ingestion which suggests that the acute effects of NO₃⁻ ingestion on energy efficiency and/or muscle metabolism may involve another mitochondria effect and/or an effect that does not involve the mitochondria.

The third potential mechanism proposed to explain a decrease of oxygen consumption during exercise following NO₃⁻ ingestion is related to substrate utilisation. One study found the respiratory exchange ratio (RER) during submaximal exercise was increased from 0.88 ± 0.01 to 0.91 ± 0.01 after 3 days of NO₃⁻ ingestion (28). Although small, this indicates a 10% increase in carbohydrate oxidation, which is a slightly more efficient fuel in regards to oxygen consumption per unit of ATP (11). It should be noted, however, that to the best of our knowledge no other human exercise study has found an effect of Beet/NO₃⁻ ingestion on RER during exercise (1, 4, 27, 29, 51, 54). Wylie et al. (55) found lower plasma glucose concentration throughout high
intensity intermittent exercise after NO$_3^-$ compared with placebo which may suggest alterations in carbohydrate use. In addition there is evidence in animals that nitrate can alter substrate oxidation during isolated muscle contraction (20). Holloszy and Narahara (20) found that NO$_3$, albeit at a supra-physiological dose, can increase force production and glucose uptake acutely in frog skeletal muscle *ex-vivo* (20). This is interesting since there is also evidence showing that NO plays a key role in glucose uptake during contraction in skeletal muscle (9, 34, 35, 37). However, to the best of our knowledge, no previous study has thoroughly investigated the effect of Beet/NO$_3^-$ ingestion on glucose kinetics during exercise in humans. The analysis of glucose kinetics after nitrate ingestion would provide important insight into determining the effects of nitrate on glucose metabolism during exercise in humans. Additionally, no studies to date have examined the effects of Beet/NO$_3^-$ ingestion on the AMP kinase (AMPK) signalling during exercise, which is surprising given that AMPK is an energy sensor in skeletal muscle and activated by exercise (17, 53). Therefore, we examined the effect of a single dose of Beet with, and without mouthwash on glucose kinetics, muscle metabolism, AMPK signalling (ACC$\beta$ phosphorylation) and oxygen consumption in healthy humans during submaximal exercise. We hypothesised that a single dose of Beet would decrease oxygen consumption, increase glucose uptake and attenuate the reduction in PCr during exercise in comparison with placebo. We also hypothesised that mouth wash with Beet would prevent these effects by greatly attenuating the conversion of NO$_3$ to NO$_2$, thus implying that the effects of NO$_3$ are not direct but via NO$_2$ or NO. In addition, we hypothesised that the better maintenance of skeletal muscle energy balance during exercise with Beet would result in less of an activation of AMPK signalling (ACC$\beta$ phosphorylation) during exercise.
Material and Methods

Participants

Eight healthy recreationally active males (mean ± SE, age 27±1 years, height 178±2 cm, body mass 77±6 kg; VO2peak 46±3 ml·kg⁻¹·min⁻¹) volunteered to participate in this study. The procedures carried out in this study were approved by Victoria University Human Ethics Committee (HRETH 11/292) in accordance with the Declaration of Helsinki. Before commencing the study participants were informed about the associated risks and potential benefits of participation, and they gave their written informed consent.

Procedures

Participants were required to report to the laboratory on five occasions. During the first visit participants performed a ramp incremental exercise test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) in order to determine their peak pulmonary oxygen consumption during cycling (VO2peak). Total expired gas volumes were measured using a turbine flow meter (KL Engineering, Sunnyvale, California). Expired oxygen (O2) and carbon dioxide (CO2) fractions were continuously analysed by O2 and CO2 analysers (Amtek S-3A/II and Amtek CD-3A, respectively; Process Instruments, Pittsburgh, Pennsylvania, USA), which were calibrated using gases of known composition. Oxygen consumption (VO2), CO2 production (VCO2) and RER were calculated every 15 sec using Turbofit computer software (Vacumetrics Inc., Ventura, California, USA). Heart rate was measured using a Polar hear rate monitor RS800cx model.

The test commenced with participants cycling in a stepwise manner at 50, 100 and 150 Watts (W) for 3 minutes each.
The power output was then increased by 30 W·min$^{-1}$ until exhaustion. Participants cycled at a self-selected cadence between 80-90 rpm. The VO$_2$ peak was determined as the average of the VO$_2$ over the final 30 s of exercise.

A few days following completion of the VO$_2$peak test participants completed a 30-minute familiarisation trial in order for the participant to be accustomed to the bike setup and intensity for the experimental trial as well as to confirm the workload estimated to elicit 65% VO$_2$ peak for the exercise trials was correct.

Participants were then randomly assigned in a single blind crossover design to attend the laboratory for three experimental trials during which they received one of three different treatments with a washout period of at least 1 week between each trial: (1) 140 ml of concentrated organic beetroot juice rich in NO$_3^-$ (Beet; ~8 mMol) (Beet It, James White Drinks, Ipswich, UK); (2) 140 ml of concentrated organic beetroot juice depleted of NO$_3^-$ (Placebo; ~0.01 mmol) (Beet It, James White Drinks, Ipswich, UK); and (3) 140 ml of concentrated organic beetroot juice rich in NO$_3^-$ (~8 mmol) followed by rinsing mouth with mouthwash [Chlorohexidine Gluconate 2mg/ml; 20ml of Colgate™ Savacol] for 1 minute (Beet + MW). This dose has been shown to abolish the reduction in blood pressure with NO$_3^-$ ingestion (46). Participants were provided with a list of foods rich in nitrates to refrain from in the 24 hours prior to each trial. Participants were asked to complete a 24 h food diary to record what they consumed prior to the first trial in order to replicate their diets as close as possible in subsequent trials (food diary was photocopied and returned to them to replicate).
The participants were asked to refrain from the use of any type of mouthwash the morning of the trial and attended the laboratory in an overnight fasted state. A cannula (Optiva IV catheter 20GX1-1/4") was then inserted into an antecubital vein of both arms, one for the infusion of the [6,6-2H]-glucose isotope tracer (Cambridge Isotope Laboratories, Andover, MA) for glucose kinetic determination, and the other for blood sampling. After cannulas had been inserted the first blood sample was taken and treatment ingested (-150 min). The [6,6-2H] glucose isotope was infused using a syringe pump (Terumo™ Syringe pump TE-331) and commenced 2 hours prior to exercise (-120 min) with a primer dose of 54µmol.kg⁻¹ that was infused over 5 min. Immediately after this a continuous infusion rate of 0.62µmol.kg.min was set and continued over the remaining of the experiment (120 min of rest and 60 min of exercise).

The blood samples were taken at the following time points -150, -120, -60,-30,-20 and 0 min (just prior to exercise). Exercise involved cycling at the power output determined in the preliminary testing to elicit 65% of VO₂ peak for 1 hour. During exercise blood samples were obtained at 15, 30, 45 and 60 min, spun down and stored for later analysis of plasma glucose, percent enrichment of [6,6-²H]-glucose and plasma lactate. Blood samples at -150, 0, 30 and 60 min were additionally spun down and stored for later analysis of NEFA, NO₂⁻ and insulin. Blood for glucose and lactate determination was placed in fluoride heparin tubes; blood for NEFA, and NO₂⁻ analysis was placed in tubes containing EDTA (6); and blood for insulin was placed in lithium heparin tubes. The blood samples for nitrate and NO₂⁻ were spun down and plasma extracted within 10 min of collection due to its rapid degradation (4).
The remaining blood samples were spun down at the end of the trial. Respiratory gas analysis was performed at the time points 10-15 and 45-50 min during exercise.

Skeletal muscle biopsy samples (~150 mg) were obtained from vastus lateralis just prior to exercise and immediately (<30 sec) after exercise as described previously (38). The leg which the sample obtained taken was alternated for each trial. Muscle samples were obtained after a skin incision had been made under local anaesthesia (Xylocaine™ 1%). Pre and Post exercise muscle biopsy incisions were prepared at the same time. Muscle samples were immediately frozen in the needle in liquid nitrogen and were later transferred to cryotubes for storage at -80°C.

**Analytical Techniques**

**Blood analysis**

Plasma glucose and lactate concentration were determined in duplicate using an automated glucose oxidase and L-lactate oxidase method, respectively (model YSI 2300 Stat, Yellow Springs Instrument, Yellow Springs, OH). Plasma NEFA content was analysed in duplicate using an enzymatic colorimetric assay (NEFA-C test, Wako, Osaka, Japan).

Plasma insulin was determined in duplicate using an ultrasensitive ELISA assay (Mercodia AB, Uppsala, Sweden). Plasma samples to assess NO₂⁻ levels were not deproteinized prior to analysis as deproteinization may be a source of NO₂⁻ contamination (22). NO₂⁻ levels were determined in duplicate by detecting liberated NO in a gas-phase chemiluminescence reaction with ozone using a NO analyser (NOA 280i; Sievers, GE Power & Water, Boulder, CO) as described previously (6).
**Glucose kinetics**

The method to determine percent enrichment of \([6,6-^2\text{H}]-\text{glucose}\) has been previously described (33). Briefly, 50 µl of plasma was deproteinised with \(\text{Ba(OH)}_2\) and \(\text{ZnSO}_4\) and spun. Supernatant was placed in glass vials, dehydrated overnight then derivatised to the pentacetate derivative with the use of pyridine and acetic anhydride. The derivatised glucose was measured with a gas chromatography mass spectrometer (Shimadzu Model GMS-QP2010 Plus, Kyoto, Japan) using a selected ion-monitoring mode to determine the relative abundance of the selected ions with mass-to-valence rations of 98 and 100. Glucose kinetics were estimated using a modified one-pool non steady-state model proposed by Steele et al. (50) with the assumption of 0.65 as the rapidly mixing portion of the glucose pool, and estimating the apparent glucose space as 25% of body weight. During cycling at 60% of \(\text{VO}_2\) peak, 80-85% of tracer-determined whole-body glucose uptake is attributed to uptake by the legs (21). Rates of plasma glucose appearance (Ra) and glucose disappearance (Rd) were calculated from the change in percent enrichment of \([6,6-^2\text{H}]-\text{glucose}\) and the glucose concentration. The glucose clearance rate (GCR) was calculated by dividing Rd by the plasma glucose concentration.

**Muscle analysis**

A portion of each muscle sample (~20mg) was freeze-dried and subsequently crushed to a powder whilst any visible connective tissue was removed. The extraction of muscle glycogen commenced by incubating the sample in HCL before being neutralized with NaOH and subsequently analysed for glucosyl units using an enzymatic flurometric method (21). The metabolites (ATP, CrP, Cr, and lactate) were extracted firstly with precool ed PCA/EDTA before the addition of precool ed KHCO\(_3\) to the supernatant.
The metabolites were analysed in triplicate using an enzymatic flurometric method used by Harris et al. (18). PCr, Cr and ATP were normalised to the participant’s highest total creatine (Cr + CrP) obtained across the 3 trials.

**Western blotting**

The method used for western blotting is similar to method previously described (40). Briefly, a small portion (5μg) of muscle sample was added to 200ul of sample buffer which was composed of 0.125 M TRIS-HCL (pH 6.8), 4% SDS, 10% Glycerol, 10mM EGTA and 0.1M DTT. This was then left at room temperature for 1hr before being vortexed and stored at -80°C. Protein concentration was determined using the Red 660 protein assay kit (G-Biosciences, A Geno technology, Inc, USA) 2uL of 1% bromophenol blue was added to the sample.

Samples were analysed for total acetyl CoA carboxylase (ACCβ) and phosphorylated ACCβ (Ser221) (Cell Signalling Technology, USA), a protein that is phosphorylated by AMPK (42). An optimisation gel was carried out for each protein to determine the optimal protein to load. For the determination of total and phosphorylated ACC samples were heated for 5 minutes at 95°C. Proteins were separated on 18 well 7.5% Criterion Stainfree gels (BioRad, Hercules, CA). Following electrophoresis, proteins in gels were transferred to nitrocellulose using the Trans-Blot® Turbo™ transfer packs and system. After transfer membranes were imaged following UV activation using a Stainfree Chemidoc (BioRad™) to quantify total protein in each lane. Membranes were subsequently blocked in 5% skim milk in TBST for 1 hour on a rocker at room temperature before being washed in TBST 4 times, 5 minutes each time.
Membranes were then cut below the 250kD mark on the ladder with each portion placed in the appropriate antibody to incubate overnight at 4°C on the rocker. The next day membranes were washed 4 times in TBST for 5 minutes before being washed in TBS for 5 minutes. Images were then collected following exposure to SuperSignal West Femto (Pierce) using ChemiDoc (BioRad™) and using Quantity One software (BioRad™).

**Data analyses**

All data are expressed as mean ± SEM. The data was analysed using the statistical software SPSS Version 21 (IBM™) using a two-factor repeated measures ANOVA. When a significant interaction (Time x Treatment) was found post-hoc analysis was performed using Tukey post-hoc test. The level of significance was set at p<0.05.

**Results**

**Nitrite**

Plasma levels of NO$_2^-$ (Fig. 1) increased significantly (P<0.05) by ~130% above baseline during exercise at time points 30, and 60 minutes in beetroot juice (Beet) with no changes from baseline in placebo and MW+Beet.

**Glucose kinetics, plasma glucose, insulin, non-esterified free fatty acids (NEFA) and lactate**

Glucose appearance (Ra), glucose disappearance (Rd) and glucose clearance rate (GCR) increased similarly during exercise in the three trials (Fig. 2). In addition, changes in plasma levels of glucose, insulin and NEFA during exercise were similar in the three trials (data not shown).
Muscle glycogen, lactate and metabolites

Muscle contents of glycogen (Fig. 3A) and PCr (Fig. 3C) decreased and muscle lactate increased with exercise similarly in the three trials (Fig. 3). Muscle ATP content did not change significantly during exercise in any trials (Fig. 3).

Cardio-respiratory measures

There was no significant effect of Beet or Beet+MW on exercise VO$_2$, VCO$_2$, RER (Table 1) or HR (data not shown).

Acetyl Co carboxylase (ACC)

Total ACC protein content was unchanged with treatment and exercise (data not shown). Exercise significantly increased phosphorylated ACC relative to total ACC, with no difference between trials (Fig. 4).

Discussion

The main finding of this study was that contrary to our hypothesis acute ingestion of beetroot juice (Beet) had no significant effect on glucose disposal, muscle metabolism, ACCβ phosphorylation, oxygen consumption or RER during moderate exercise in healthy males. In addition, the combination of Beet and MW also had no effects on any of the parameters.

At first we were surprised that we observed no effect of acute ingestion of Beet on oxygen consumption during exercise since two previous studies found lower VO$_2$ during exercise after an acute dose of beetroot juice (51, 54). However, several studies have also found no acute effect of either beetroot juice (7) or NO$_3^-$ in pharmacological form (5, 6, 43) on VO$_2$ during exercise although these studies were
performed in well-trained individuals. The concentration of plasma \( \text{NO}_2^- \) in our study increased to a similar if not greater extent than previous studies that have observed reductions in oxygen consumption during exercise following Beet/\( \text{NO}_3^- \) supplementation (28-30, 51, 52). For example, Vanhatalo et al. (51) found that 2.5 hours after beetroot juice ingestion (~5.2 mmol \( \text{NO}_3^- \)) plasma values of \( \text{NO}_2^- \) were raised by approximately 160 nM above baseline and this was associated with a significant increase in exercise efficiency (~4%), but we found no effect on exercise efficiency despite an almost identical increase in plasma \( \text{NO}_2^- \) after ~8 mmol of nitrate ingestion in beetroot juice. The study by Vanhatalo et al. (51) did report significantly higher baseline plasma levels of \( \text{NO}_2^- \) (~450 nM) compared with the current and other previous studies (100-200 nM) (1, 3, 27, 30, 41). These values reported by Vanhatalo et al. (51) are difficult to explain by differences in the methodology given all the studies used a similar approach (chemiluminescence) to assess plasma \( \text{NO}_2^- \). However in the study by Vanhatalo et al. (51) plasma was deproteinized prior to \( \text{NO}_2^- \) being measured which may be a source of \( \text{NO}_2^- \) contamination (22). The variation in baseline plasma levels of \( \text{NO}_2^- \) may also be a result of seasonal differences across investigations as it has been reported that plasma concentrations of \( \text{NO}_2^- \) and their bioactivity can be augmented by exposure to UVA radiation (39). Furthermore in regards to absolute plasma \([\text{NO}_2^-]\) the levels of plasma nitrite achieved with the 8 mM dose used in our study is comparable to the levels of nitrite achieved previously where an effect on \( \text{VO}_2 \) during exercise has been shown after multiday supplementation (28, 30). For example in the study by Larsen et al. (30), the first study to find a reduction in \( \text{VO}_2 \) during sub-maximal exercise with \( \text{NO}_3^- \) ingestion, a mean peak plasma \( \text{NO}_2^- \) of 226 ± 87 nM was achieved compared with the placebo group of 124 ± 87 nM.
These levels are similar to the mean peak plasma nitrite of 261 ± 31 and placebo group levels of ~125 nM achieved in our study. However in spite of this we recognise the possibility that the lack of physiological effects in our study may be due to the nitrate dose supplemented (~ 8 mmol) and/or the bioconversion to NO₂⁻. In regards to this Wylie et al. (54) found that 16 mmol of NO₃⁻ resulted in a greater peak plasma NO₂⁻ concentration (653 ± 356 nM) and this was related with an enhancement in exercise efficiency, with no significant physiological effects reported when the same participants consumed 8 mmol of NO₃⁻. Thus, it remains unclear whether a higher dose and/or longer period of supplementation of inorganic nitrate might have a significant impact on the parameters analysed in this study. Therefore the dose used and the fact only an acute dose was administered is a feasible limitation of this study.

Thus, further research is needed using a high dose acutely and/or chronically to clarify the effect of beetroot juice supplementation on VO₂ and muscle metabolism during exercise in healthy humans.

There is evidence from NOS inhibition studies that the generation of NO in skeletal muscle during contraction plays a key role in skeletal muscle glucose disposal during contraction in rodents and during exercise in humans (35, 36, 47-49). Given this and the fact that Holloszy and Narahara (20) found that nitrate increased glucose uptake in isolated frog sartorius muscles during contraction, we predicted that Beet would increase glucose disposal during exercise. However, Beet had no effect on glucose disposal during sub-maximal exercise. This does not mean that NO is not important for glucose uptake during exercise, since increasing levels of NO from NO₃⁻ above the normal level of NO produced during contraction from NOS may be in excess of requirements.
In addition this study does not discard the possibility that NO$_3^-$ supplementation may have an effect on glucose disposal during high intensity exercise as it has been previously shown that NO$_3^-$ supplementation lowered mean plasma glucose concentrations during high intensity intermittent exercise compared to placebo Wylie et al. (55). Future studies should examine if nitrate can increase or normalize skeletal muscle glucose uptake during contraction or exercise in situations where skeletal muscle NOS levels are reduced, such as in mdx mice (44) or in diabetes (8, 25, 45). It should also be noted that the study finding that NO$_3^-$ increased isolated frog muscle glucose uptake during contraction (20) used a dose of NO$_3^-$ that was 3 orders of magnitude higher than found after NO$_3^-$ supplementation.

We also found no effect of Beet on RER during exercise which fits with the lack of effect of Beet on glucose disposal and muscle glycogen use during exercise. Other studies have also found no effect of acute Beet/ NO$_3^-$ ingestion of RER during exercise. Larsen et al. (28) found an increase in carbohydrate oxidation (higher RER) during exercise after 3 days of NO$_3^-$ supplementation but as far as we are aware this is the only study to find an effect of NO$_3^-$ ingestion on RER during exercise. Therefore, taken together, the lack of effect of acute Beet/ NO$_3^-$ supplementation on glucose disposal, muscle glycogen use and RER suggests that acute NO$_3^-$ supplementation does not affect carbohydrate metabolism during exercise at the dose given. Future studies should examine these parameters during exercise at higher dose and after several days of Beet/ NO$_3^-$ supplementation where there is more evidence to suggest that Beet/NO$_3^-$ supplementation may affect exercise metabolism.
Bailey et al (2) found using $^{31}$P-MRS that 6 days of Beet ingestion attenuated the reduction of skeletal muscle PCr content and estimated ATP turnover during both low and high intensity exercise compared with placebo. It also increased the mean force per unit of PCr depletion. Rodent studies suggest that NO$_3^-$ feeding effects skeletal muscle blood flow (12) and force production (19) only in fast-twitch skeletal muscles. Therefore, it would appear that the attenuated reduction in PCr with Beet ingestion observed by Bailey et al. (2) during low intensity exercise, which predominantly recruits slow-twitch fibres (15), are likely independent of blood flow and force. To further explore this, we analysed PCr, Cr and ATP content in skeletal muscle biopsies performed pre and post exercise. In contrast with the in vivo data by Bailey et al (2), and in line with the lack of effect of Beet on exercise VO$_2$, we found that Beet did not induce any effect on these parameters. This is consistent with our finding of no effect of Beet on the increase in p-ACC during exercise, a protein phosphorylated by the energy-sensing enzyme AMPK (42).

Although the limitations with measuring metabolites via muscle biopsy due to rapid PCr recovery kinetics (13) and the time (~30 sec) it takes to obtain and freeze the muscle sample is acknowledged, it is in agreement with the recent study by Fulford et al (14). They found that the ingestion of beetroot juice did not significantly reduce mean PCr cost after a series of maximum voluntary contractions in the Beet trials compared to placebo despite a daily dose (~10.2 mmol) approximately double that used by Bailey et al. (2).
The reason for the varying results is unclear and cannot be explained by the differing intensities of exercise as Bailey et al. (1) investigated both a low and high intensity protocol and found a reduction in PCr attenuation with Beet in both. In addition, the daily dose of NO$_3^-$ used is unlikely to explain the lack of effect by Fulford et al. (14) as a far higher dose was used compared with Bailey et al. (2).

In summary, despite a similar increase in plasma NO$_2^-$ as previous acute Beet/NO$_3^-$ ingestion studies, we found no effect of beetroot juice ingestion on oxygen consumption, glucose disposal, muscle metabolites (glycogen, PCr, ATP, lactate) or AMPK signalling during submaximal exercise. Further research is required to investigate whether chronic supplementation of beetroot juice or a higher acute dose might have an impact on any of these parameters.

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References


Figure captions

Figure 1: Plasma NO\textsuperscript{2+} at rest and during 60 min of cycling at ~65% VO\textsubscript{2peak} after ingestion of either Beet, Beet + MW or placebo. A significant treatment and treatment by time interaction was found (p<0.05). Values are means ± SE, n=8. † Significant (p<0.05) difference between Beet vs. placebo.

Figure 2: (A) Rate of glucose appearance (B) Rate of glucose disappearance and (C) Mean glucose clearance rate (glucose Rd/plasma glucose) at rest and during 60 min of cycling at ~65% VO\textsubscript{2peak} after ingestion of either Beet, Beet +MW or placebo. All increased significantly (p<0.05) during exercise. Values are means ± SE. n=8.

Figure 3: Muscle glycogen (A), lactate (B) adenosine triphosphate (ATP), phosphocreatine (PCr) and creatine (Cr) at rest and immediately following 60 min of cycling at ~65% VO\textsubscript{2peak} after acute ingestion of either Beet, Beet +MW or placebo. Values are means ± SE. n=6-8. * Significant (p<0.05) difference from pre-exercise.

Figure 4: Phosphorylated ACC (Ser221) relative to total ACC protein at rest and immediately following 60 min of cycling at ~65% VO\textsubscript{2peak} after acute ingestion of either Beet, Beet +MW or placebo. Values are means ± SE. n=7. *Significantly different (p<0.05) from pre-exercise.
Figure 1

![Graph showing nitrite levels over time with treatment and exercise conditions. The x-axis represents time in minutes, ranging from -150 to 60. The y-axis represents nitrite levels in nM, ranging from 0 to 400. Legend includes Placebo, Beet, and Beet + M.W. treatments.](image-url)
Figure 2

A  Rate of Glucose Appearance

B  Rate of Glucose Disappearance

C  Glucose clearance rate
Figure 3

A. Muscle Glycogen

B. Muscle Lactate

C. ATP, PCr & Cr
Figure 4

ACC pSer^{221}  ACC

p-ACC/ACC (AU)

Pre-exercise  Beet  Beet + M.W.

Placebo  Beet  Beet + M.W.
Table 1: Respiratory response to exercise and treatments (n=8).

<table>
<thead>
<tr>
<th>Exercise Time</th>
<th>10-15 min</th>
<th>45-50 min</th>
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<tr>
<td>Treatment</td>
<td>Placebo</td>
<td>Beet</td>
</tr>
<tr>
<td><strong>VO$_2$ (L·min$^{-1}$)</strong></td>
<td>2.20 ± 0.06</td>
<td>2.21 ± 0.07</td>
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<tr>
<td><strong>VCO$_2$ (L·min$^{-1}$)</strong></td>
<td>2.14 ± 0.06</td>
<td>2.11 ± 0.07</td>
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<tr>
<td><strong>RER</strong></td>
<td>0.97 ± 0.01</td>
<td>0.96 ± 0.01</td>
</tr>
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VO$_2$, oxygen consumption; VCO$_2$, carbon dioxide production; RER, respiratory exchange ratio. Values are means ± SE.