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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Betteridge S, Bescós R, Martorell M, Pons A, Garnham AP, Stathis CC, McConnell GK. No effect of acute beetroot juice ingestion on oxygen consumption, glucose kinetics, or skeletal muscle metabolism during submaximal exercise in males. J Appl Physiol 120: 391–398, 2016. First published December 3, 2015; doi:10.1152/japplphysiol.00658.2015.—Beetroot juice, which is rich in nitrate (NO3−), has been shown in some studies to decrease oxygen consumption (VO2) for a given exercise workload, i.e., increasing efficiency and exercise tolerance. Few studies have examined the effect of beetroot juice or nitrate supplementation on exercise metabolism. Eight healthy recreationally active males participated in three trials involving ingestion of either beetroot juice (Beet; ~8 mmol NO3−), Placebo (nitrate-depleted Beet), or Beet + mouthwash (Beet+MW), all of which were performed in a randomized single-blind crossover design. Two- and-a-half hours later, participants cycled for 60 min on an ergometer at 65% of VO2 peak. [6,6-2H]glucose was infused to determine glucose kinetics, blood samples obtained throughout exercise, and skeletal muscle biopsies that were obtained pre- and postexercise. Plasma nitrate [NO3−] 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 nonesterified fatty acids, 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 phosphocreatine and the increase in muscle creatine, lactate, and phosphorylated acetyl CoA carboxylase during exercise. In conclusion, at the dose used, acute ingestion of beetroot juice had little effect on skeletal muscle metabolism during exercise.

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NO₃ ingestion (28). Although small, this indicates a 10% increase in carbohydrate oxidation, which is a slightly more efficient fuel in regard 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 NO₃ 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 Naraaha (20) found that NO₃, albeit at a supraphysiological dose, can increase force production and glucose uptake acutely in frog skeletal muscle ex vivo (20). This is interesting, as 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/NO₃ 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/NO₃ ingestion on the AMPK signaling during exercise, which is surprising given that AMPK is an energy sensor in skeletal muscle and is 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 signaling [acyl CoA carboxylase (ACC)β phosphorylation], and oxygen consumption in healthy humans during submaximal exercise. We hypothesized that a single dose of Beet would decrease oxygen consumption, increase glucose uptake, and attenuate the reduction in PCr during exercise compared with placebo. We also hypothesized that mouthwash with Beet would prevent these effects by greatly attenuating the conversion of NO₃ to NO₂, thus implying that the effects of NO₂ are not direct but via NO or NO₃.

In addition, we hypothesized that the better maintenance of skeletal muscle energy balance during exercise with Beet would result in less of an activation of AMPK signaling (ACCβ phosphorylation) during exercise.

MATERIALS AND METHODS

Participants

Eight healthy recreationally active males (means ± 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 (HERETH 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) to determine their peak pulmonary oxygen consumption during cycling (VO₂peak). Total expired gas volumes were measured using a turbine flow meter (KL Engineering, Sunnyvale, CA). Exhaled oxygen (O₂) and carbon dioxide (CO₂) fractions were continuously analyzed by O₂ and CO₂ analyzers (Ametek S-3A/II and Ametek CD-3A, respectively, Process Instruments, Pittsburgh, PA), which were calibrated using gases of known composition. Oxygen consumption (VO₂), CO₂ production (VCO₂), and RER were calculated every 15 s using Turbofit computer software (Vacumetrics, Ventura, CA). Heart rate was measured using a Polar heart rate monitor RS800cx model.

The test commenced with participants cycling in a stepwise manner at 50, 100, and 150 Watts (W) for 3 min each. The power output was then increased by 30 W·min until exhaustion. Participants cycled at a self-selected cadence between 80 and 90 rpm. The VO₂peak was determined as the average of the VO₂ over the final 30 s of exercise.

A few days following completion of the VO₂peak test, participants completed a 30-min familiarization trial to familiarize the participant with the bike setup and intensity for the experimental trial, as well as to confirm the workload estimated to elicit 65% VO₂peak for the exercise trials was correct.

Participants were then randomly assigned in a single-blind cross-over design to attend the laboratory for three experimental trials during which they received one of the three different treatments with a washout period of at least 1 wk between each trial: 1) 140 ml of concentrated organic beetroot juice rich in NO₃ (Beet; ~8 mM) (Beet It, James White Drinks, Ipswich, UK); 2) 140 ml of concentrated organic beetroot juice depleted of NO₃ (Placebo; ~0.01 mM) (Beet It, James White Drinks, Ipswich, UK); and 3) 140 ml of concentrated organic beetroot juice rich in NO₃ (~8 mM) followed by rinsing mouth with mouthwash (chlorohexidine gluconate, 2 mg/ml; 20 ml of Colgate Savacol) for 1 min (Beet+MW). This dose has been shown to abolish the reduction in blood pressure with NO₃ ingestion (46). Participants were provided with a list of foods rich in nitrates from which to refrain from eating 24 h prior to each trial. Participants were asked to complete a 24-h food diary to record what they consumed prior to the first trial 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 on the morning of the trial, and they arrived at 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 [6,6-²H₆]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 beetroot juice treatment ingested (~150 min). The [6,6-²H₆] glucose isotope was infused using a syringe pump (Terumo Syringe pump TE-331) and commenced 2 h prior to exercise (~120 min) with a primer dose of 54 µmol/kg that was infused over 5 min. Immediately after this, a continuous infusion rate of 0.62 µmol·kg⁻¹·min⁻¹ was set and continued over the remainder 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 VO₂peak for 1 h. 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 [6,6-²H₆]-glucose, and plasma lactate. Blood samples at −150, 0, 30, and 60 min were additionally spun down and stored for later analysis of nonesterified fatty acids (NEFA), NO₂⁻, and insulin. Blood for glucose and lactate determination was placed in fluoride heparin tubes; blood for NEFA and NO₃ 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 was 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 the vastus lateralis just prior to exercise and immediately (<30 s) after exercise, as described previously (38). The leg from which the sample was obtained was alternated for each trial. Muscle samples were obtained after a skin incision had been made under local anesthesia (Xylocaine 1%). Preexercise and postexercise 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 analyzed 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 analyzer (NOA 280i, Sievers, GE Power & Water, Boulder, CO), as described previously (6).

Glucose kinetics. The method to determine percent enrichment of [6,6-2H1]-glucose has been previously described (33). Briefly, 50 μl of plasma was deproteinized with Ba(OH)2 and ZnSO4 and spun. Supernatant was placed in glass vials, dehydrated overnight, and then derivatized to the pentacetate derivative with the use of pyridine and acetic anhydride. The derivatized 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 ratios of 98 and 100. Glucose kinetics were estimated using a modified one-pool nonsteady-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 V02peak, 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 [6,6-2H1]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 (−20 mg) was freeze-dried and subsequently crushed to a powder while 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 analyzed for glucosyl units using an enzymatic fluorimetric method (21). The metabolites [ATP, creatine phosphate (CrP), creatine (Cr), and lactate] were extracted firstly with precooled PCA/EDTA before the addition of precooled KHCO3 to the supernatant.

The metabolites were analyzed in triplicate using an enzymatic fluorimetric method used by Harris et al. (18). PCr, Cr, and ATP were normalized to the participant’s highest total creatine (Cr + CrP) obtained across the three trials.

Western blot analysis. The method used for Western blot analysis is similar to the method previously described (40). Briefly, a small portion (5 μg) of muscle sample was added to 200 μl of sample buffer, which was composed of 0.125 M Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 10 mM EGTA, and 0.1 M DTT. This was then left at room temperature for 1 h before being vortexed and stored at −80°C. Protein concentration was determined using the Red 660 protein assay kit (G-Biosciences, A Geno Technology, St. Louis, MO), and 2 μl of 1% bromophenol blue was added to the sample.

Samples were analyzed for total ACCβ and phosphorylated ACCβ (Ser221) (Cell Signaling Technology, Beverly, MA), a protein that is phosphorylated by AMPK (42). An optimization 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 min at 95°C. Proteins were separated on 18-well 7.5% Criterion Stainfree gels (Bio-Rad, 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 (Bio-Rad) to quantify total protein in each lane. Membranes were subsequently blocked in 5% skim milk in TBST for 1 h on a rocker at room temperature before being washed in TBST 4 times, 5 min each time.

Membranes were then cut below the 250-kDa mark on the ladder with each portion placed in the appropriate antibody to incubate overnight at 4°C on the rocker. On the next day, membranes were washed four times in TBST for 5 min before being washed in TBS for 5 min. Images were then collected following exposure to SuperSignal West Femto (Pierce, Rockford, IL) using ChemiDoc (Bio-Rad) and using Quantity One software (Bio-Rad).

Data analyses. All data are expressed as means ± SE. The data were analyzed using the statistical software SPSS Version 21 (IBM) using a two-factor repeated-measures ANOVA. When a significant interaction (time × 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 NO2 (Fig. 1) increased significantly (P < 0.05) by 130% above baseline during exercise at time points 30, and 60 min in beetroot juice (Beet) with no changes from baseline in placebo and MW + Beet.

Glucose Kinetics, Plasma Glucose, Insulin, NEFA, and Lactate

Glucose appearance (Ra), glucose disappearance (Rd), and 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).

![Figure 1. Plasma NO2 at rest and during 60 min of cycling at −65% V02peak after ingestion of either Beet, Beet + mouthwash (MW), or placebo. A significant treatment and treatment × time interaction was found (P < 0.05). Values are expressed as means ± SE; n = 8. *Significant (P < 0.05) difference between Beet vs. Placebo group.](http://jap.physiology.org/DownloadedFrom/japp.physiology.org/10220_34.on.July.31.2017)
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).

Cardiorespiratory Measures

There was no significant effect of Beet or Beet+MW on exercise VO₂, VCO₂, RER (Table 1), or HR (data not shown).

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 VO₂ 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 NO₃ in pharmacological form (5, 6, 43) on VO₂ during exercise, although these studies were performed in well-trained individuals. The concentration of plasma NO₂ 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/NO₃ supplementation (28–30, 51, 52). For example, Vanhatalo et al. (51) found that 2.5 h after beetroot juice ingestion (~5.2 mmol NO₃), plasma values of NO₂ rose by ~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 NO₂ after ~8 mmol of nitrate ingestion in beetroot juice. The study by Vanhatalo et al. (51) did report significantly higher baseline plasma levels of NO₂ (~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 of the studies used a similar approach (chemiluminescence) to assess plasma NO₂. However, in the study by Vanhatalo et al. (51), plasma was deproteinized prior to NO₂ being measured, which may be a source of NO₂ contamination (22). The variation in baseline plasma levels of NO₂ may also be a result of seasonal differences across investigations, as it has been reported that plasma concentrations of NO₂ and its bioactivity can be augmented by exposure to UVA radiation (39). Furthermore, in regard to absolute plasma [NO₂⁻], the levels of plasma nitrite achieved with the 8-mM dose used in our study is comparable to the levels of nitrite achieved previously, in which an effect on VO₂ during exercise has been shown after multiday supple-

Fig. 2. A: rate of glucose appearance (Ra). B: rate of glucose disappearance (Rd). C: mean glucose clearance rate (Cr) (Rd/plasma glucose) at rest and during 60 min of cycling at ~65% VO₂peak after ingestion of either Beet, Beet +MW, or placebo. All increased significantly (P < 0.05) during exercise. Values are expressed as means ± SE; n = 8.
A. Muscle Glycogen

B. Muscle Lactate

C. ATP, PCr & Cr

Fig. 3. Muscle glycogen (A), lactate (B), ATP, phosphocreatine (PCr), and creatine (Cr) (C) at rest and immediately following 60 min of cycling at ~65% \( V_{\text{O2\ peak}} \) after acute ingestion of either Beet, Beet + MW, or placebo. Values are expressed as means ± SE; \( n = 6–8 \). *Significant (\( P < 0.05 \)) difference from preexercise.

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 evidence 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 submaximal exercise. This does not mean that NO is not important for glucose uptake during exercise, since increasing levels of NO from NO\(_3\) 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 with placebo (55). Future studies should examine whether nitrate can increase or normalize skeletal muscle glucose uptake during contraction or exercise in situations in which skeletal muscle NOS levels are reduced, such as in \( mdx \) mice (44) or in patients with 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 three 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 attenuation in PCr with Beet ingestion observed by Bailey et al. (2) during low-intensity exercise, which predominantly recruits slow-twitch fibers (15), are likely independent of blood flow and force. To further explore this, we analyzed PCr, Cr, and ATP content in skeletal muscle biopsies performed preexercise and postexercise. In contrast with the in vivo data by Bailey et al. (2), and in line with the lack of effect of Beet on exercise $V_O_2$, 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 pACC 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 s) it takes to obtain and freeze the muscle sample is acknowledged, the results here are 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 with 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 (glucose, PCr, ATP, and lactate), or AMPK signaling 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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**DISCLOSURES**

Conflict of interest statement: No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


