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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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Keywords: Exercise, Inorganic Nitrate, Nitrite, Nitric oxide, ACC, Glucose kinetics, metabolites

Running head: beetroot juice and skeletal muscle metabolism

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Abstract

Beetroot juice, which is rich in nitrate (NO_3^-), has been shown in some studies to decrease oxygen consumption (VO_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_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_{2peak}. [6,6-^{2}H] glucose was infused to determine glucose kinetics, blood samples obtained throughout exercise and skeletal muscle biopsies obtained pre and post-exercise. Plasma nitrite [NO_2^-] 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

54 **Introduction**

55 There has been much interest over recent years in the potential of inorganic NO_3^- to
56 increase exercise efficiency and exercise performance (23, 32). Based on this,
57 beetroot juice (Beet) has been used as an ergogenic aid and health supplement because
58 it contains large amounts of inorganic NO_3^-. It is now known that dietary inorganic
59 NO_3^- can be reduced to NO_2^- and nitric oxide (NO) as well as other bioactive nitrogen
60 species *in vivo* (30). The bio-activation of NO_3^- requires the formation of NO_2^- as an
61 intermediate, a reaction that is facilitated by anaerobic oral bacteria (31). The critical
62 role of oral bacteria has been supported by studies demonstrating that antiseptic
63 mouthwash abolishes the increase in plasma levels of NO_2^- after the consumption of
64 NO_3^- (16). In addition, the reduction in blood pressure and gastro protective effects
65 that have been shown with NO_3^- ingestion are abolished when an antiseptic
66 mouthwash is used prior to NO_3^- ingestion in healthy participants (24, 46).
67
68 In regards to physical exercise, some (1, 4, 28, 30, 51), but not all studies (6, 10, 26,
69 54) at a similar dose (5-8 mmol), have found that multiday Beet/ NO_3^- ingestion
70 decreases oxygen uptake (~3%) during sub-maximal and maximal exercise in healthy
71 subjects. Furthermore the ingestion of beetroot juice just prior to exercise has shown
72 similar effects (51, 54). This increase in efficiency during exercise following
73 Beet/NO_3^- ingestion has been hypothesized to be due to one or more of three possible
74 mechanisms. The first proposes that Beet/NO_3^- ingestion induces a reduction in the
75 ATP cost of force production (1). In support of this Bailey et al. (1) found using
76 phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) that there was an
77 attenuation of the decrease in PCr and estimated ATP turnover during low and high
78 intensity knee-extensor exercise after several days of Beet ingestion.

79 The second possible mechanism is related to the efficiency in which the mitochondria
80 produce ATP (28). In humans, the ratio of adenosine triphosphate (ATP) generated to
81 the oxygen consumed (P/O ratio) during exercise increased after 3 days of NO_3^-
82 supplementation in healthy subjects (28). There was also a significant decrease in
83 basal leak respiration and an increase in expression of the mitochondria protein
84 ADP/ATP translocase (ANT), one of the main proteins attributed to leak respiration
85 (28). These findings suggest that nitrate supplementation enabled the mitochondria to
86 better maintain the proton gradient across the membrane. However, an increase in
87 mitochondria efficiency can not explain comparable increases in efficiency (~3%)
88 after one dose of NO_3^- 2.5 hours prior to exercise (51, 54) and following multi-day
89 supplementation (1, 4, 28, 30). Indeed, it is unlikely that there is sufficient time for
90 changes in protein expression to occur 2.5 hr after NO_3^- ingestion which suggests that
91 the acute effects of NO_3^- ingestion on energy efficiency and/or muscle metabolism
92 may involve another mitochondria effect and/or an effect that does not involve the
93 mitochondria.
94

95 The third potential mechanism proposed to explain a decrease of oxygen consumption
96 during exercise following NO_3^- ingestion is related to substrate utilisation. One study
97 found the respiratory exchange ratio (RER) during submaximal exercise was
98 increased from 0.88 ± 0.01 to 0.91 ± 0.01 after 3 days of NO_3^- ingestion (28).
99 Although small, this indicates a 10% increase in carbohydrate oxidation, which is a
100 slightly more efficient fuel in regards to oxygen consumption per unit of ATP (11). It
101 should be noted, however, that to the best of our knowledge no other human exercise
102 study has found an effect of Beet/NO_3^- ingestion on RER during exercise (1, 4, 27, 29,
103 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 (ACCβ 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 NO2, 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 (ACCβ 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; VO_{2peak} 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 (VO_{2peak}). Total expired gas volumes were measured using a turbine flow meter (KL Engineering, Sunnyvale, California). Expired oxygen (O_2) and carbon dioxide (CO_2) fractions were continuously analysed by O_2 and CO_2 analysers (Amtek S-3A/II and Ametek CD-3A, respectively; Process Instruments, Pittsburgh, Pennsylvania, USA), which were calibrated using gases of known composition. Oxygen consumption (VO_2), CO_2 production (VCO_2) 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.

153 The power output was then increased by 30 $W \cdot min^{-1}$ until exhaustion. Participants
154 cycled at a self-selected cadence between 80-90 rpm. The VO_2 peak was determined
155 as the average of the VO_2 over the final 30 s of exercise.
156
157 A few days following completion of the VO_{2peak} test participants completed a 30-
158 minute familiarisation trial in order for the participant to be accustomed to the bike
159 setup and intensity for the experimental trial as well as to confirm the workload
160 estimated to elicit 65% VO_2 peak for the exercise trials was correct.
161
162 Participants were then randomly assigned in a single blind crossover design to attend
163 the laboratory for three experimental trails during which they received one of three
164 different treatments with a washout period of at least 1 week between each trial: (1)
165 140 ml of concentrated organic beetroot juice rich in NO_3^- (Beet; ~8 mMol) (Beet It,
166 James White Drinks, Ipswich, UK); (2) 140 ml of concentrated organic beetroot juice
167 depleted of NO_3^- (Placebo; ~0.01 mmol) (Beet It, James White Drinks, Ipswich, UK);
168 and (3) 140 ml of concentrated organic beetroot juice rich in NO_3^- (~8 mmol)
169 followed by rinsing mouth with mouthwash [Chlorohexidine Gluconate 2mg/ml;
170 20ml of Colgate™ Savacol] for 1 minute (Beet + MW). This dose has been shown to
171 abolish the reduction in blood pressure with NO_3^- ingestion (46). Participants were
172 provided with a list of foods rich in nitrates to refrain from in the 24 hours prior to
173 each trial. Participants were asked to complete a 24 h food diary to record what they
174 consumed prior to the first trial in order to replicate their diets as close as possible in
175 subsequent trials (food diary was photocopied and returned to them to replicate).
176

177 The participants were asked to refrain from the use of any type of mouthwash the
178 morning of the trial and attended the laboratory in an overnight fasted state. A
179 cannula (Optiva IV catheter 20GX1-1/4") was then inserted into an antecubital vein of
180 both arms, one for the infusion of the [6,6-^{2}H]-glucose isotope tracer (Cambridge
181 Isotope Laboratories, Andover, MA) for glucose kinetic determination, and the other
182 for blood sampling. After cannulas had been inserted the first blood sample was taken
183 and treatment ingested (-150 min). The [6,6-^{2}H] glucose isotope was infused using a
184 syringe pump (Terumo™ Syringe pump TE-331) and commenced 2 hours prior to
185 exercise (-120 min) with a primer dose of 54μmol.kg^{-1} that was infused over 5 min.
186 Immediately after this a continuous infusion rate of 0.62μmol.kg.min was set and
187 continued over the remaining of the experiment (120 min of rest and 60 min of
188 exercise).
189
190 The blood samples were taken at the following time points -150, -120, -60,-30,-20 and
191 0 min (just prior to exercise). Exercise involved cycling at the power output
192 determined in the preliminary testing to elicit 65% of VO_2 peak for 1 hour. During
193 exercise blood samples were obtained at 15, 30, 45 and 60 min, spun down and stored
194 for later analysis of plasma glucose, percent enrichment of [6,6-^{2}H]-glucose and
195 plasma lactate. Blood samples at -150, 0, 30 and 60 min were additionally spun down
196 and stored for later analysis of NEFA, NO_2^- and insulin. Blood for glucose and lactate
197 determination was placed in fluoride heparin tubes; blood for NEFA, and NO_2^-
198 analysis was placed in tubes containing EDTA (6); and blood for insulin was placed
199 in lithium heparin tubes. The blood samples for nitrate and NO_2^- were spun down and
200 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 NO_2^- levels were not deproteinized prior to analysis as deproteinization may be a source of NO_2^- contamination (22). NO_2^- 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}H]-glucose has been previously described (33). Briefly, 50 µl of plasma was deproteinised with $Ba(OH)_2$ and $ZnSO_4$ 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 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}H]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 precooled PCA/EDTA before the addition of precooled $KHCO_3$ 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 (Ser^{221}) (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).

296 **Muscle glycogen, lactate and metabolites**

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

300 **Cardio-respiratory measures**

301 There was no significant effect of Beet or Beet+MW on exercise VO_2, VCO_2, RER
302 (Table 1) or HR (data not shown).

303 **Acetyl Co carboxylase (ACC)**

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

307 **Discussion**

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

313

314 At first we were surprised that we observed no effect of acute ingestion of Beet on
315 oxygen consumption during exercise since two previous studies found lower VO_2
316 during exercise after an acute dose of beetroot juice (51, 54). However, several
317 studies have also found no acute effect of either beetroot juice (7) or NO_3^- in
318 pharmacological form (5, 6, 43) on VO_2 during exercise although these studies were

319 performed in well-trained individuals. The concentration of plasma NO_2^- in our study
320 increased to a similar if not greater extent than previous studies that have observed
321 reductions in oxygen consumption during exercise following Beet/ NO_3^-
322 supplementation (28-30, 51, 52). For example, Vanhatalo et al. (51) found that 2.5
323 hours after beetroot juice ingestion (~5.2 mmol NO_3^-) plasma values of NO_2^- were
324 raised by approximately 160 nM above baseline and this was associated with a
325 significant increase in exercise efficiency (~4%), but we found no effect on exercise
326 efficiency despite an almost identical increase in plasma NO_2^- after ~8 mmol of nitrate
327 ingestion in beetroot juice. The study by Vanhatalo et al. (51) did report significantly
328 higher baseline plasma levels of NO_2^- (~450 nM) compared with the current and other
329 previous studies (100-200 nM) (1, 3, 27, 30, 41). These values reported by Vanhatalo
330 et al. (51) are difficult to explain by differences in the methodology given all the
331 studies used a similar approach (chemiluminescence) to assess plasma NO_2^-.
332 However in the study by Vanhatalo et al. (51) plasma was deproteinized prior to NO_2^-
333 being measured which may be a source of NO_2^- contamination (22). The variation in
334 baseline plasma levels of NO_2^- may also be a result of seasonal differences across
335 investigations as it has been reported that plasma concentrations of NO_2^- and their
336 bioactivity can be augmented by exposure to UVA radiation (39). Furthermore in
337 regards to absolute plasma [NO_2^-] the levels of plasma nitrite achieved with the 8 mM
338 dose used in our study is comparable to the levels of nitrite achieved previously where
339 an effect on VO_2 during exercise has been shown after multiday supplementation (28,
340 30). For example in the study by Larsen et al. (30), the first study to find a reduction
341 in VO_2 during sub-maximal exercise with NO_3^- ingestion, a mean peak plasma NO_2^-
342 of 226 ± 87 nM was achieved compared with the placebo group of 124 ± 87 nM.

343 These levels are similar to the mean peak plasma nitrite of 261 ± 31 and placebo
344 group levels of ~125 nM achieved in our study**.** However in spite of this we recognise
345 the possibility that the lack of physiological effects in our study may be due to the
346 nitrate dose supplemented (~ 8 mmol) and/or the bioconversion to NO_2^-. In regards to
347 this Wylie et al. (54) found that 16 mmol of NO_3^- resulted in a greater peak plasma
348 NO_2^- concentration (653 ± 356 nM) and this was related with an enhancement in
349 exercise efficiency, with no significant physiological effects reported when the same
350 participants consumed 8 mmol of NO_3^-. Thus, it remains unclear whether a higher
351 dose and/or longer period of supplementation of inorganic nitrate might have a
352 significant impact on the parameters analysed in this study. Therefore the dose used
353 and the fact only an acute dose was adminstered is a feasible limitation of this study.
354 Thus, further research is needed using a high dose acutely and/or chronically to clarify
355 the effect of beetroot juice supplementation on VO_2 and muscle metabolism during
356 exercise in healthy humans.

357 There is evidence from NOS inhibition studies that the generation of NO in skeletal
358 muscle during contraction plays a key role in skeletal muscle glucose disposal during
359 contraction in rodents and during exercise in humans (35, 36, 47-49). Given this and
360 the fact that Holloszy and Narahara (20) found that nitrate increased glucose uptake in
361 isolated frog sartorius muscles during contraction, we predicted that Beet would
362 increase glucose disposal during exercise. However, Beet had no effect on glucose
363 disposal during sub-maximal exercise. This does not mean that NO is not important
364 for glucose uptake during exercise, since increasing levels of NO from NO_3^- above the
365 normal level of NO produced during contraction from NOS may be in excess of
366 requirements.

367 In addition this study does not discard the possibility that NO_3^- supplementation may
368 have an effect on glucose disposal during high intensity exercise as it has been
369 previously shown that NO_3^- supplementation lowered mean plasma glucose
370 concentrations during high intensity intermittent exercise compared to placebo Wylie
371 et al. (55). Future studies should examine if nitrate can increase or normalize skeletal
372 muscle glucose uptake during contraction or exercise in situations where skeletal
373 muscle NOS levels are reduced, such as in mdx mice (44) or in diabetes (8, 25, 45). It
374 should also be noted that the study finding that NO_3^- increased isolated frog muscle
375 glucose uptake during contraction (20) used a dose of NO_3^- that was 3 orders of
376 magnitude higher than found after NO_3^- supplementation.

377

378 We also found no effect of Beet on RER during exercise which fits with the lack of
379 effect of Beet on glucose disposal and muscle glycogen use during exercise. Other
380 studies have also found no effect of acute Beet/ NO_3^- ingestion of RER during
381 exercise. Larsen et al. (28) found an increase in carbohydrate oxidation (higher RER)
382 during exercise after 3 days of NO_3^- supplementation but as far as we are aware this is
383 the only study to find an effect of NO_3^- ingestion on RER during exercise. Therefore,
384 taken together, the lack of effect of acute Beet/ NO_3^- supplementation on glucose
385 disposal, muscle glycogen use and RER suggests that acute NO_3^- supplementation
386 does not affect carbohydrate metabolism during exercise at the dose given. Future
387 studies should examine these parameters during exercise at higher dose and after
388 several days of Beet/ NO_3^- supplementation where there is more evidence to suggest
389 that Beet/NO_3^- supplementation may affect exercise metabolism.

390 Bailey et al (2) found using ^{31}P-MRS that 6 days of Beet ingestion attenuated the
391 reduction of skeletal muscle PCr content and estimated ATP turnover during both low
392 and high intensity exercise compared with placebo. It also increased the mean force
393 per unit of PCr depletion. Rodent studies suggest that NO_3^- feeding effects skeletal
394 muscle blood flow (12) and force production (19) only in fast-twitch skeletal muscles.
395 Therefore, it would appear that the attenuated reduction in PCr with Beet ingestion
396 observed by Bailey et al. (2) during low intensity exercise, which predominantly
397 recruits slow-twitch fibres (15), are likely independent of blood flow and force. To
398 further explore this, we analysed PCr ,Cr and ATP content in skeletal muscle biopsies
399 performed pre and post exercise. In contrast with the *in vivo* data by Bailey et al (2),
400 and in line with the lack of effect of Beet on exercise VO_2, we found that Beet did not
401 induce any effect on these parameters. This is consistent with our finding of no effect
402 of Beet on the increase in p-ACC during exercise, a protein phosphorylated by the
403 energy-sensing enzyme AMPK (42).

404

405 Although the limitations with measuring metabolites via muscle biopsy due to rapid
406 PCr recovery kinetics (13) and the time (~30 sec) it takes to obtain and freeze the
407 muscle sample is acknowledged, it is in agreement with the recent study by Fulford et
408 al (14). They found that the ingestion of beetroot juice did not significantly reduce
409 mean PCr cost after a series of maximum voluntary contractions in the Beet trials
410 compared to placebo despite a daily dose (~10.2 mmol) approximately double that
411 used by Bailey et al. (2).

412

413 The reason for the varying results is unclear and cannot be explained by the differing
414 intensities of exercise as Bailey et al (1) investigated both a low and high intensity
415 protocol and found a reduction in PCr attenuation with Beet in both. In addition, the
416 daily dose of NO_3^- used is unlikely to explain the lack of effect by Fulford et al. (14)
417 as a far higher dose was used compared with Bailey et al. (2).

418

419 In summary, despite a similar increase in plasma NO_2^- as previous acute Beet/NO_3^-
420 ingestion studies, we found no effect of beetroot juice ingestion on oxygen
421 consumption, glucose disposal, muscle metabolites (glycogen, PCr, ATP, lactate) or
422 AMPK signalling during submaximal exercise. Further research is required to
423 investigate whether chronic supplementation of beetroot juice or a higher acute dose
424 might have an impact on any of these parameters.

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432

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624 **Figure captions**

625

626 Figure 1: Plasma NO_2^- at rest and during 60 min of cycling at ~65%VO_{2peak} after
627 ingestion of either Beet, Beet + MW or placebo. A significant treatment and
628 treatment by time interaction was found ($p<0.05$). Values are means ± SE, n=8. †
629 Significant ($p<0.05$) difference between Beet vs. placebo.

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

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

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

642

Figure 1

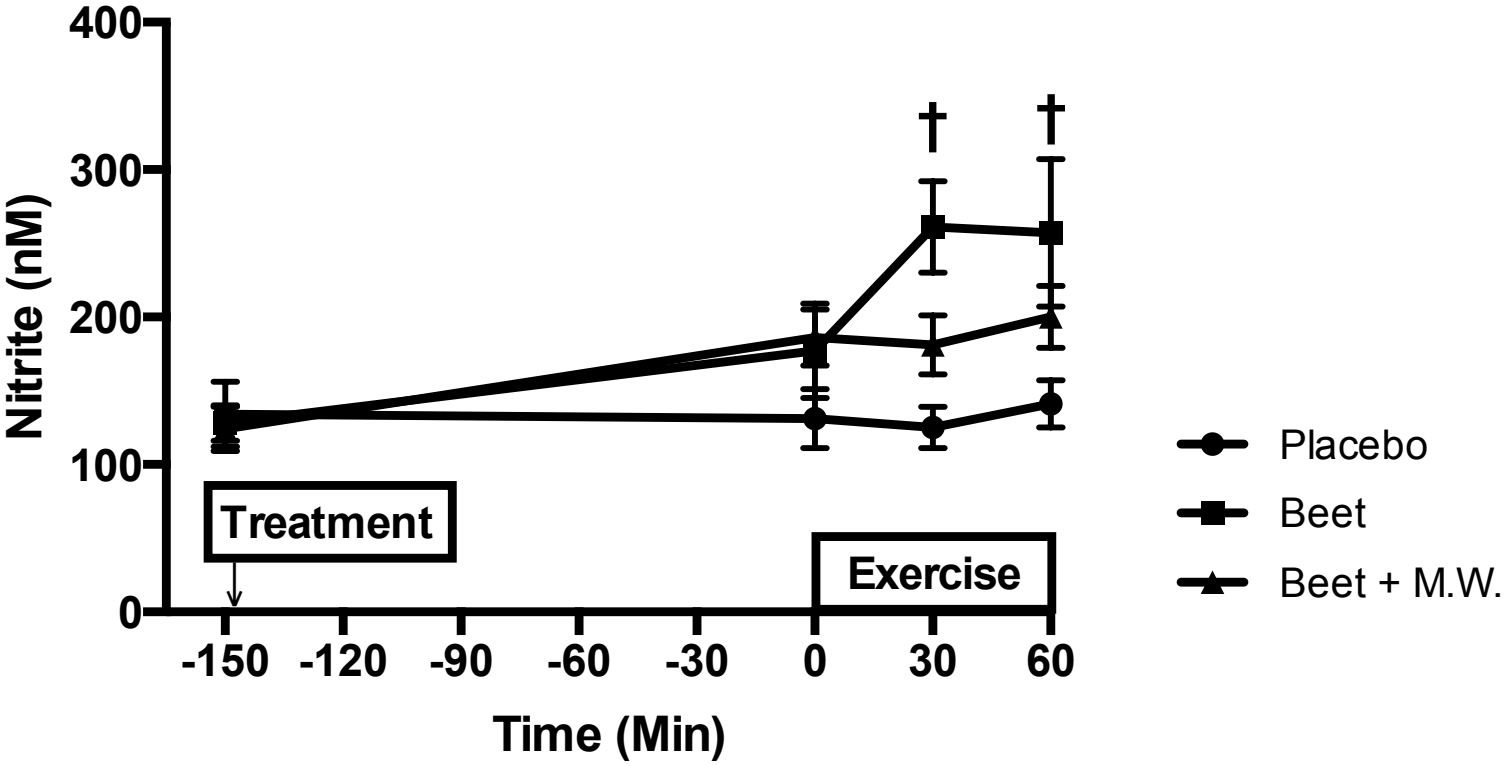

400
300
200
100
0
Nitrite (nM)
†
†
Treatment
Exercise
-150
-120
-90
-60
-30
0
30
60
Time (Min)
Placebo
Beet
Beet + M.W.

Figure 2

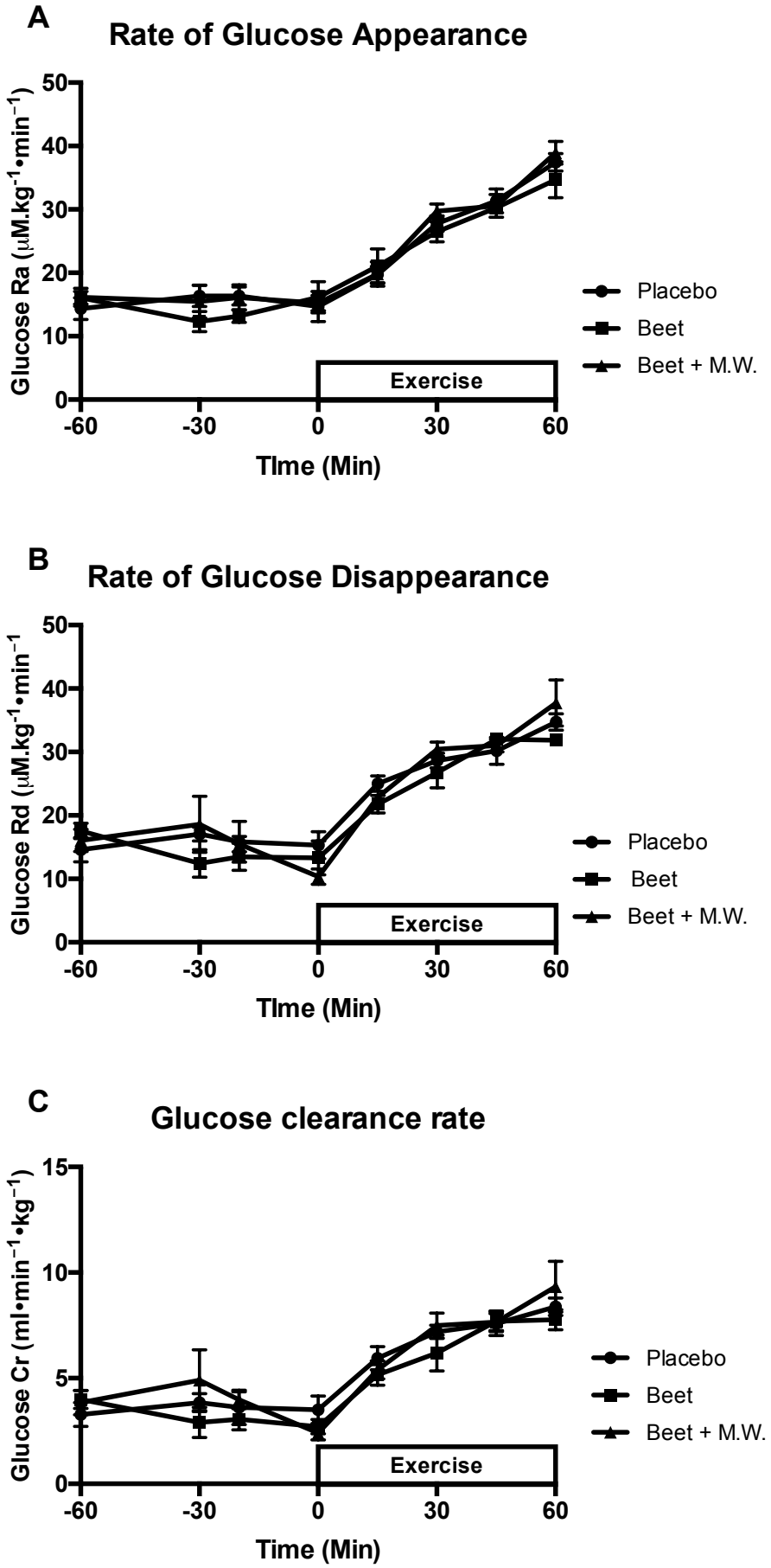
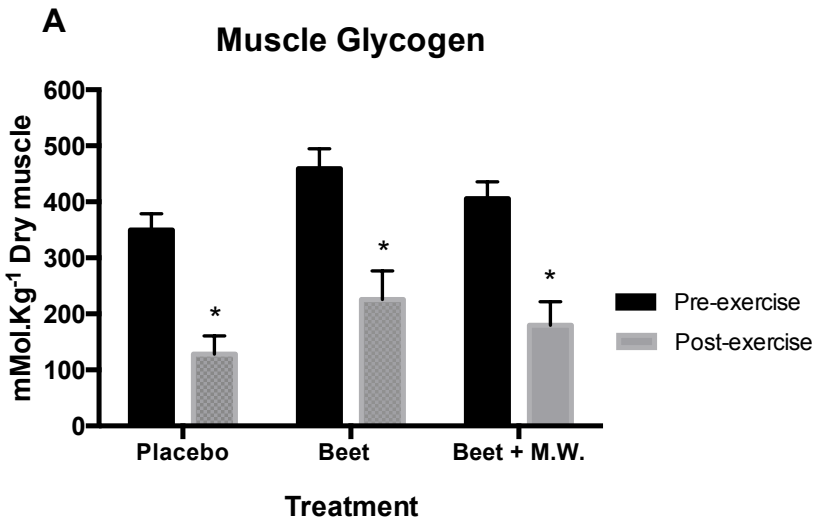
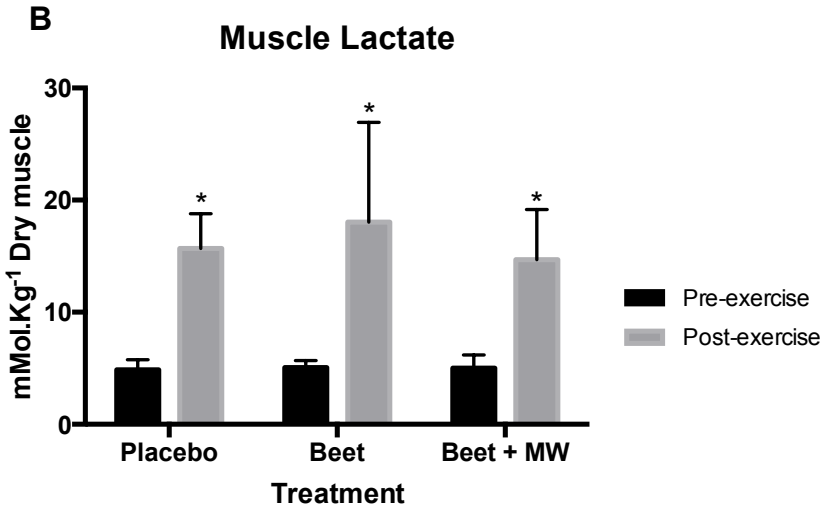

A
Rate of Glucose Appearance
Glucose Ra (μM.kg-1•min-1)
50
40
30
20
10
0
-60
-30
0
30
60
TIme (Min)
Exercise
Placebo
Beet
Beet + M.W.
B
Rate of Glucose Disappearance
Glucose Rd (μM.kg-1•min-1
50
40
30
20
10
0
-60
-30
0
30
60
TIme (Min)
Exercise
Placebo
Beet
Beet + M.W.
C
Glucose clearance rate
Glucose Cr (ml•min-1•kg-1)
15
10
5
0
-60
-30
0
30
60
Time (Min)
Exercise
Placebo
Beet
Beet + M.W.

Figure 3


A
Muscle Glycogen
mMol.Kg-1 Dry muscle
600
500
400
300
200
100
0
*
*
*
Placebo
Beet
Beet + M.W.
Treatment
Pre-exercise
Post-exercise


B
Muscle Lactate
mMol.Kg-1 Dry muscle
30
20
10
0
*
*
*
Placebo
Beet
Beet + MW
Treatment
Pre-exercise
Post-exercise

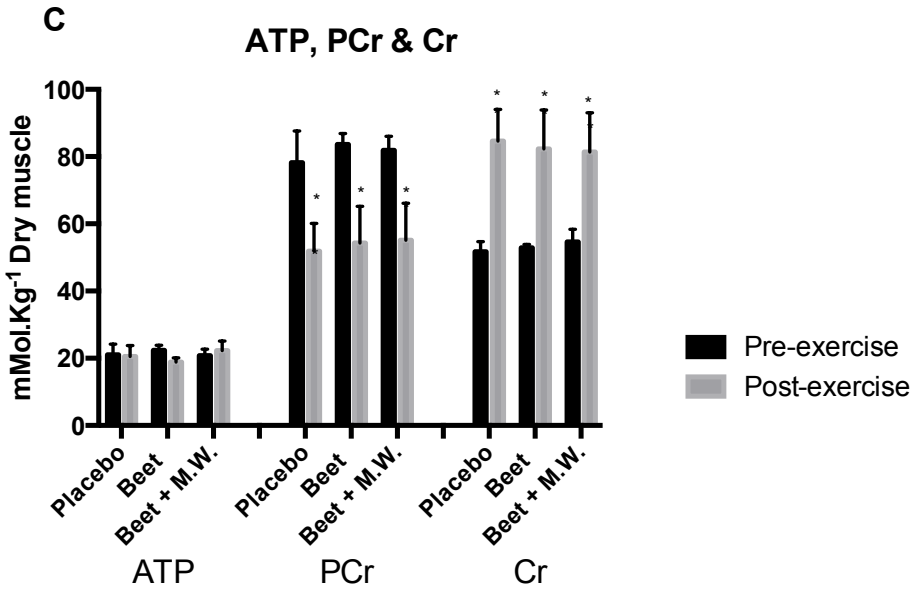

C
ATP, PCr & Cr
mMol.Kg-1 Dry muscle
100
80
60
40
20
0
Placebo
Beet
Beet + M.W.
Placebo
Beet
Beet + M.W.
Placebo
Beet
Beet + M.W.
ATP
PCr
Cr
Pre-exercise
Post-exercise

Figure 4

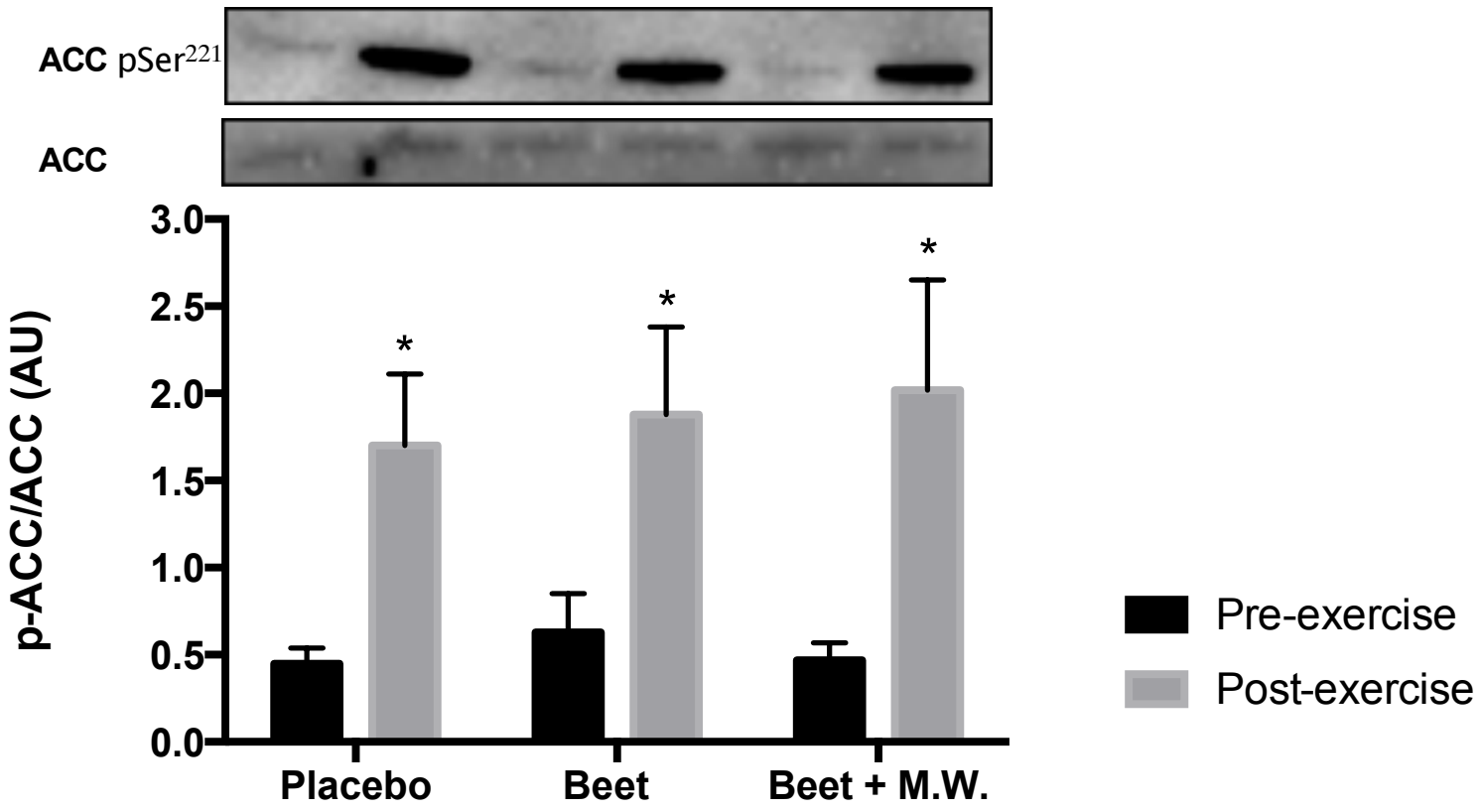

ACC pSer221
ACC
p-ACC/ACC (AU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Placebo
Beet
Beet + M.W.
Pre-exercise
Post-exercise

Table 1: Respiratory response to exercise and treatments (n=8).

Exercise Time	**10-15 min**			**45-50 min**		
Treatment	Placebo	Beet	Beet + M.W.	Placebo	Beet	Beet + M.W.
VO_2 ($L \cdot min^{-1}$)	2.20 ± 0.06	2.21 ± 0.07	2.20 ± 0.07	2.33 ± 0.07	2.35 ± 0.08	2.30 ± 0.07
VCO_2 ($L \cdot min^{-1}$)	2.14 ± 0.06	2.11 ± 0.07	2.12 ± 0.06	2.14 ± 0.06	2.10 ± 0.08	2.01 ± 0.07
RER	0.97 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	0.92 ± 0.01	0.90 ± 0.01	0.92 ± 0.01

VO_2, oxygen consumption; VCO_2, carbon dioxide production; RER, respiratory exchange ratio. Values are means ± SE.