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DIETARY NITRATE DOES NOT HAVE AN EFFECT ON PHYSICAL ACTIVITY OUTCOMES IN HEALTHY OLDER ADULTS: A RANDOMIZED, CROSS-OVER TRIAL

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Abbreviations

BMI= body mass index; HGS= hand-grip strength; TUG= time-up-and-go; RCRT=repeated-chair-rising-test; WLS=10m walking speed; NO= nitric oxide; ATP= Adenosine triphosphate; PAD= peripheral arterial disease; COPD= chronic obstructive pulmonary disease; BP= blood pressure; eNOS= endothelial Nitric Oxide Synthase; ROS= reactive oxygen species; ECG = electrocardiography; CHO= carbohydrate; PRO= protein; FAT= fat; BIA= Bioelectrical impedance analyses; FM= fat mass; FFM= fat free mass; WC= waist circumference; IPAQ= International Physical Activity Questionnaire; EPIC= European Prospective Investigation into Cancer and Nutrition; FFQ= Food Frequency Questionnaire; RER= respiratory exchange ratio; GC-MS= gas chromatography mass spectrometry; GLM= General Linear Models; HOMA-IR= Homeostatic Model of Insulin Resistance; MET=Metabolic Equivalent of Task.

ABSTRACT

Dietary nitrate (NO_3^-) ingestion appears to enhance exercise capacity and performance in young individuals whereas inconclusive findings have been reported in older people. We conducted a double-blind, cross-over randomized clinical trial in older normal weight and overweight healthy participants testing whether beetroot juice (a rich source of NO_3^-) for one week may increase nitric oxide bioavailability via the non-enzymatic pathway and enhance 1) exercise capacity during an incremental exercise test, 2) physical capability and 3) free-living physical activity.

Twenty non-smoking healthy participants aged 60-75y and BMI 20.0-29.9kg/m^2 were included. Pre and post supplementation resting, sub-maximal, maximal and recovery gas exchanges were measured. Physical capability was measured by hand-grip strength (HGS), time-up-and-go (TUG), repeated-chair-rising-test (RCRT), and 10m walking speed (WLS). Free-living physical activity was assessed by triaxal accelerometry. Changes in urinary and plasma NO_3^- concentrations were measured by gas chromatography mass spectrometry.

Nineteen participants (M/F=9/10) completed the study. Beetroot juice increased significantly both plasma and urinary NO_3^- concentrations ($p<0.001$) compared to placebo. Beetroot juice did not influence resting, sub-maximal and maximal oxygen consumption during the incremental exercise test. In addition, measures of physical capability and physical activity levels measured in free-living conditions were not modified by beetroot juice ingestion.

The positive effects of beetroot juice ingestion on exercise performance seen in young individuals were not replicated in healthy, older adults. Whether aging represents a modifier of the effects of dietary NO_3^- on muscular performance is not known and mechanistic studies and larger trials are needed to test this hypothesis.

Keywords: inorganic nitrate, nitric oxide, exercise, oxygen consumption, aging

1. INTRODUCTION

Aging is characterized by a progressive decline in muscle mass and strength which are risk factors for physical disability[1]. Aging is also associated with modifications of mitochondrial bioenergetics with consequent effects on muscular performance[2]. Dietary nitrate (NO_3^-) supplementation enhances muscular efficiency in humans[3, 4], a finding which can be explained by increased nitric oxide (NO) bioavailability and the role of NO in modulating mitochondrial coupling and bioenergetics of muscular activity[5, 6]. However, the majority of NO_3^- supplementation studies have been conducted in healthy, physically active young adults[7, 8] and few studies have evaluated the effects of dietary NO_3^- on physical or muscular function in older people[9-11]. Larsen et al in 2007[12] was the first to report reduced sub-maximal O_2 uptake in young healthy adults after three-day oral supplementation with potassium NO_3^-. Kenjale et al[10] observed delayed onset of claudication after three days of oral NO_3^- supplementation in older patients with peripheral arterial disease (PAD). However, subsequent studies reported contrasting results for the effects of dietary NO_3^- on exercise performance in healthy older people[9, 13] as well as in at risk populations (i.e., those with diabetes[11], heart failure[14], and chronic obstructive pulmonary disease (COPD)[15, 16]). All studies employed a double-blind randomized cross-over study design and administered beetroot juice to increase NO_3^- intake. However, differences in study duration, NO_3^- dose or assessment of exercise capability likely contributed to the observed heterogeneous responses. For example, outcomes have included sub-maximal [12, 15, 17] or maximal oxygen (O_2) uptake [18-20] assessed with incremental standardised tests [12, 15, 21] as well as time trials [22-24] or physical capability tests [9, 25], all of which were performed in controlled settings. No study has investigated the effects of dietary NO_3^- supplementation on free living physical activity.

We hypothesized that dietary NO_3^- supplementation would increase NO bioavailability, muscular energetics and exercise performance – with significant changes expected in sub-maximal, maximal and recovery O_2 uptake – which may translate into beneficial effects on physical capability and free living physical activity. To test these hypotheses, we conducted a double-blind, cross-over, placebo controlled RCT in older healthy adults to investigate the effects of beetroot juice, chosen as a rich source of dietary NO_3^-, on physical activity outcomes measured in research (O_2 uptake during incremental cycle ergometer exercise, walking speed, time-up-and-go, repeated chair rising test and hand grip strength) and free living (accelerometry) settings.

2. METHODS and MATERIALS

The trial was approved by the North of Scotland Research Ethics committee (14/NS/0061) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was a double-blind, cross-over, placebo-controlled RCT which took place between May and August 2014 across two sites (Newcastle upon Tyne and Sheffield). The duration of the each intervention was one week with a wash-out period between treatments of at least one week. This trial was registered in the International Standard Randomized Controlled Trial Number Register (ISRCTN19064955).

2.1 Participants: Twenty male and female, older (60-75 y) non-obese adults (BMI range: 18.5 - 29.9 kg/m^2) were enrolled in the study. Participants were non-smokers and weight stable. Participants were included in the study if they did not have medical conditions or were not taking medications that might influence the study outcomes. A full list of the inclusion and exclusion criteria is provided in the **Online Supplementary Material**. Participants were asked to maintain their habitual diet and to avoid using chewing gum or mouth wash for at least 48 prior to the baseline visits (first and third visit) and during each of the one-week supplementation periods.

2.2 Randomization: A randomization list for each site was generated by a member of staff not involved in the study using www.sealedenvelopes.com. Each participant was randomized to the cross-over interventions (i.e., placebo ➔ NO_3^- or NO_3^- ➔ placebo). Intervention agents were dispensed at each baseline visit by two members of staff not involved in the study who had access to the stored beetroot juice and ensured the correct treatment allocation.

2.3 Study Overview: A telephone screening was performed to check eligibility according to the trial inclusion and exclusion criteria. Eligible participants were invited for a further screening visit at the research facilities including measurement of BMI, resting BP and resting 12-lead electrocardiography (ECG). Participants were asked to arrive after a 12-hour overnight fast and having avoided strenuous physical activity for three days preceding the visit. If eligible, participants were randomized to a cross-over intervention and the baseline assessment continued with the measurement of body composition, collection of blood and urine samples and assessment of physical capability. Participants then rested for one hour and consumed a meal providing approximately 300kcal (CHO=85%, PRO=3%, FAT=12%). In addition, during this one-hour rest period, participants completed a series of questionnaires to assess dietary intake and physical activity. After the one-hour rest, participants were explained the exercise test while they accustomized to the ergometer. The exercise protocol is described in **Figure S1 of the Online Supplementary Material**. After the exercise test, instructions were provided for self-administration of the nutritional intervention (14 bottles of either NO_3^- -rich or NO_3^- -depleted beetroot juice; 70ml x 2/day; Beet It, James White Ltd, UK) and asked to consume one bottle of beetroot juice each morning and evening for the subsequent 7 days. The daily dose of NO_3^- -rich (intervention) or NO_3^- -depleted (placebo) beetroot juice contained ~12mmol and ~0.003mmol of NO_3^-, respectively. Participants were provided with instructions and forms for recording wearing time of the accelerometer. This concluded Visit 1 of the trial. Participants returned to the research facilities in the morning of day eight after they had completed a seven-day supplementation period. A detailed medical interview was conducted to ascertain any side effects experienced during the supplementation period. A resting 12-lead ECG was performed and, if normal, the study visit was completed by repeating the same assessments as performed during Visit 1. At the end of the second visit, participants were asked to resume their habitual diet and physical activity. After a wash out period of at least seven days the second phase (including Visits 3 and 4) was conducted similar to the first phase with the exception that participants crossed-over experimental arms i.e. consumed the other intervention agent.

2.4 Body Composition: Bioelectrical impedance analyses (BIA) (Newcastle: TANITA 418MA, Tanita Ltd, Japan; Sheffield: InBody 720 Analyser, InBody Bldg, Korea) was used to assess fat mass (FM) and fat free mass (FFM). Body weight, height and waist circumference (WC) were measured using standardized protocols.

2.5 Resting Blood Pressure: Resting BP was measured in triplicate using an automated BP monitor (Omron M3, Omron Healthcare, UK) with the participant seated comfortably for 15 min prior to measurement and the arm supported at the level of the heart. The recorded value was calculated as the mean of the three measurements.

2.6 Physical Capability: A battery of tests (hand grip strength (HGS), timed up and go (TUG), repeated chair rise test (RCRT) and 10m walking speed (WLS)), performed in the same order at each visit, was completed at baseline and at the end visit of each phase. Triplicate measurements of HGS were performed in both arms at baseline and after intervention using a digital dynamometer (Takei 5401, Takei, Japan). The average of six measurements was calculated. To complete the TUG, participants were asked to stand up from a chair, walk three meters at a self-selected comfortable speed, cross a line on the floor, turn around, walk back, and sit down again. The RCRT was completed using a standard chair without armrests. Participants had both arms crossed against the chest, starting from the seated position and standing up (legs straight) and sitting down (full weight on the chair) and the test calculates the time required (in seconds) to complete five repeated chair stands. For the WLS, a 10-m path with a flying start was used to avoid acceleration/deceleration effects associated with starting and stopping during this assessment. The middle 6-m of this path were used for the measurement. Patients were instructed to “walk as fast as they can” and the time (in seconds) to complete the 6-m path was recorded.

2.7 Objective Measurement of Free Living Physical Activity: Participants were asked to wear a triaxial accelerometer (GT3X ActiGraph accelerometer (Pensacola, FL, USA)) above the right hip for eight consecutive days during waking hours and to remove it only for water activities (for example, swimming or bathing). Accelerometery data were collected in one-minute epochs. Non-wear time was defined as 60 min or more of consecutive zero counts. One participant experienced a device malfunction and data were excluded from subsequent analysis. Counts per minute were converted into minutes of sedentary time (less than or equal to 100 counts per min), light (100-759 counts per min), moderate (1952–5724 counts per min) and vigorous-intensity (5725+ counts per min) physical activity[26]. Physical activity energy expenditure was calculated using the Freedson approach[26].

2.8 Dietary and Lifestyle Questionnaires: The 9-item short form of the International Physical Activity Questionnaire (IPAQ) was used to record duration of four intensity levels of physical activity: 1) vigorous-intensity activity, 2) moderate-intensity activity, 3) walking, and 4) sitting. A combined total physical activity score was calculated and expressed in MET-minutes/week[27]. The EPIC Food Frequency Questionnaire (FFQ) was administered at baseline and the FETA software used to extract dietary (energy and nutrient) information[28].

2.9 Exercise Test: An incremental exercise test was performed at baseline and at the end of each intervention period to assess pulmonary gas exchange variables at rest, during sub-maximal and maximal intensities and in the post-exercise recovery phase. Briefly, each participant underwent cardiopulmonary exercise testing on an electronically-braked cycle ergometer. The protocol included a five-minute resting phase followed by a 20 watts stepwise increase in workload every three minutes while they were invited to maintain a stable pedalling rate (60-70 rpm). After reaching 80 watts, participants were asked to exercise until exhaustion (ramp protocol: 10 watts/minute), which was followed by a five-minute passive recovery period. A graphical description of the protocol is described in **Figure S1 of the Online Supplementary Material**. Pulmonary gas exchange and ventilation were measured (Newcastle: MetaMax 3B, Cortex Biophysik, Leipzig, Germany; Ultima CardiO2, Medgraphics, St Paul, MN, USA). Heart rate (HR) was measured during all tests using cardio-thoracic impedance. Oxygen uptake ($\dot{V}O_2$), minute ventilation ($\dot{V}E$), carbon dioxide excretion rate ($\dot{V}CO_2$), and respiratory exchange ratio (RER) were assessed. $\dot{V}O_2$ assessed during the last minute of the incremental exercise test was recorded as $\dot{V}O_{2peak}$. Ventilatory threshold was calculated using the V-slope method[29].

2.10 Blood and Urine Collection: Fasting blood samples were collected at the beginning of each visit and centrifuged at 3,000rpm for 10 min at 4 °C within 30min of collection. Aliquots of plasma and serum were frozen and stored at –80 °C for subsequent analyses. Mid-stream urine samples were collected, in fasting conditions, into sterile containers and stored at –20 °C for subsequent analyses.

2.11 Biomarker Analysis: A modified version of the gas chromatography mass spectrometry (GC-MS) method proposed by Tsikas et al[30] was used to determine NO_3^- concentrations in urine and plasma samples. The protocol and validation of the modified GC-MS method have been described elsewhere[31]. This method showed good repeatability, with coefficients of variation for replicate analyses of 7.8%, 8.6% and 12.0% for saliva, urine and plasma samples, respectively.

2.12 Sample size: The primary outcome of the study was the effect of NO_3^- supplementation on VO_2 consumption during sub-maximal exercise. Data on the expected effect size were obtained from a previous cross-over design study testing the effects of incremental exercise on sub-maximal and maximal O_2 consumption in young adults after a six-day nitrate supplementation[32] which showed that $\dot{V}O_2$ during moderate exercise was 1.53±0.12 $L \cdot min^{-1}$ and 1.45±0.12 $L \cdot min^{-1}$ in the placebo and nitrate groups respectively. On this basis, 20 participants were needed in a cross-over randomized trial to detect a difference of 0.08±0.12 $L \cdot min^{-1}$ with a power of 0.80 and alpha of 0.05.

2.13 Statistical Analyses: Repeated-Measures General Linear Models (GLM) were used to test the effect at the end of each intervention of NO_3^- supplementation on measures of exercise performance and physical capability. Treatment (NO_3^- vs placebo) was entered as a group factor (Tr) and the time points of the incremental exercise test as the repeated factor (Ti). Post-hoc comparison between treatment groups at each time point was performed using the Fisher LSD test. The area under the curve (AUC) for $\dot{V}O_2$ consumption during the incremental exercise test was calculated at baseline and end of study using the trapezoidal method. A paired t test was used to compare differences between the two interventions for the AUCs and free living physical activity outcomes. Data were presented as means ± SD or means ± 95% confidence intervals (95%CI). Analyses were conducted using Statistica 10 for Windows (StatSoft.Inc, Tulsa, OK, USA). Statistical significance was set at <0.05.

3. RESULTS

3.1 *Participants' characteristics, s*afety and Compliance with Interventions: Twenty participants were randomized to the intervention. One person developed an ischemic event during the physical exercise testing performed at the second visit and he was excluded from the study (**Figure 1**). The remaining 19 participants (mean age 64.7±3.0 years (range: 60 - 75 years)) reported no side effects apart for the expected urine discoloration related to the excretion of beetroot juice pigment (beeturia). All participants reported that they consumed all the intervention drinks provided and all of them completed all the measurements included in the study protocol. This included high compliance with wearing of the accelerometer (total wear time: ~7.5-8.0 days out of maximum 8 days).

3.2 Dietary Intake and Self-Reported Physical Activity: Energy intake was 2728±1430 kcal/day with 47±8%, 35±7% and 18±4% of energy provided by carbohydrates, fats and protein respectively. Self-reported physical activity was again not different between the placebo and the NO_3^- arms as participants in both groups reported an average increase in total physical activity of approximately 300 METs/week (p=0.99) (**Table 1 and Table S2 of the Online Supplementary Material**).

3.3 Body Composition: Mean baseline BMI was 25.6±3.4 kg/m^2 with 12 participants being in the overweight category (25≤BMI<30kg/m^2). Body weight was stable across the study with changes of 0.01±0.85 kg in the placebo and -0.16±0.57 kg in the intervention group (p=0.51). Similarly, no statistically significant between-treatment differences were found for FFM (0.02±1.00 kg vs 0.11±0.77 kg, p=0.65) and FM (-0.03±0.79 kg vs 0.27±0.75 kg, p=0.86) (**Table 1 and Table S2 of the Online Supplementary Material**).

3.4 Resting Blood Pressure: Baseline resting systolic and diastolic BP ranged from 100.0 to 168.0 mmHg and 62.0 to 97.0 mmHg, respectively. The decrease in systolic BP (-5.05±9.45 mmHg) with NO_3^- supplementation was approximately double that observed with the placebo (-2.64±9.04 mmHg) but this difference was not significant (p=0.48). Both interventions produced similar falls in diastolic BP (-3.70±5.59 vs -3.49±6.42 mmHg, p=0.90) (**Table 1 and Table S2 of the Online Supplementary Material**).

3.5 Laboratory biomarkers: Concentrations of nitrite plus nitrate (NO_2^-+NO_3^-, NOx) in plasma and urine increased substantially after NO_3^- supplementation by 150±77% and 979±488% but not after the placebo intervention (-9±33% and -13±34%, respectively).

3.6 Gas-Exchange during Standardized Exercise: Nitrate supplementation had no significant effect on pulmonary gas exchange (O_2 and CO_2) measured during resting, sub-maximal, maximal and recovery phases of the incremental exercise test. O_2 consumption increased linearly with the intensity of the workload and O_2 consumption at exhaustion was 1.67±0.51 and 1.64±0.55 $L \cdot min^{-1}$ following NO_3^- and placebo interventions (p=0.86), respectively. There was a steady and comparable decline in O_2 consumption during the 5-minute recovery phase with return to baseline resting values for both interventions (**Figure 3A**). The AUCs for O_2 consumption for both treatments were similar (p=0.89, data not showed). Similarly, weight-adjusted O_2 consumption did not significantly different between the NO_3^- and placebo groups (p=0.99, **Figure S2 of the Online Supplementary Material**). O_2 consumption at ventilatory threshold was similar for the NO_3^- (0.90±0.39 $L \cdot min^{-1}$) and placebo (0.91±0.39 $L*min^{-1}$) treatments (p=0.35) and no differences between the two interventions were observed for CO_2 production, RER, $\dot{V}E$ and HR (**Figure 3B to 3E**). Time to exhaustion was shorter following the NO_3^- intervention but the difference was not significant (p=0.10, **Figure 3F**). The adjustment of the analyses for baseline values of gas exchanges did not modify the results (data not showed). A summary of the data for each time point is provided in **Table S3 of the Online Supplementary Material**.

3.7 Physical Capability and Objective Assessment of Free Living Physical Activity: Physical performance was assessed using a battery of tests measuring strength, performance and balance. NO_3^- supplementation produced small improvements in performance for all tests but the effects were not statistically significant (**Table 2**). Similarly, NO_3^- supplementation had no significant effect on total energy physical activity or on each type of physical activity (i.e., sedentary, light, moderate, vigorous) (**Table 3**).

4. DISCUSSION

4.1 Summary of Research Findings: This is the first study to evaluate the effects of dietary NO_3^- supplementation on physical performance assessed in research settings and free-living conditions in healthy older participants. Contrary to the large body of evidence supporting a positive effect of dietary NO_3^- supplementation on exercise performance, our study showed no effects of NO_3^- supplementation on O_2 consumption during sub-maximal and maximal exercise performance in older healthy participants. In addition, there were no significant effects of dietary NO_3^- supplementation on measures of physical capability and free-living physical activity.

4.2 Comparison with Body of Evidence: Research into the effects of dietary NO_3^- on exercise performance has been influenced by two significant events: 1) first paper published by Larsen et al in 2007[12] reporting a reduced sub-maximal O_2 consumption after three-day oral NO_3^- supplementation and 2) development of a NO_3^--depleted and NO_3^--enriched concentrated beetroot juice which has allowed the design of robust double-blind, randomized nutritional interventions[11]. Since 2007, several RCTs have tested the effects of dietary NO_3^- on exercise performance in humans. A small number of these trials supplemented participants with pharmacological preparation (sodium or potassium NO_3^-)[3, 12, 19, 21, 33-35] whereas the majority of the trials used beetroot juice as a way to increase dietary NO_3^- intake[9-11, 16, 23, 25, 36]. Most of the studies recruited mainly young, physically fit participants and only a few trials [9-11, 13, 15-17, 37, 38] have tested the effects of dietary NO_3^- in older participants (mean age range: 63 – 70 years). The first study in older participants was conducted in eight patients with PAD who received 3.5 hours before the exercise testing either 500ml of beetroot juice or orange juice[10]. The study found an increased exercise time before onset of claudication pain and time to exhaustion. The remaining studies in older participants have reported contrasting results, which may be explained by differences in the duration of supplementation (range: 2.5 hours[15] to 14 days[11]), type of population (healthy[9, 13], PAD[11], COPD[15, 16], type 2 diabetes[11], heart failure[14, 17]), dose of NO_3^- (range: ~ 300 – ~ 700mg) or exercise test (walking test[9, 10, 16, 25], incremental exercise[10, 14], forearm exercise[13]). Overall, the results have showed a reduced responsiveness of older participants to dietary NO_3^- supplementation. Negative results were seen in healthy older participants[9] and patients with diabetes[25] and COPD[16], whereas improved exercise performance was observed in patients with heart failure[14] and PAD[10]. Our study confirmed that dietary NO_3^- supplementation for one week in older adults produced no beneficial effects on physical capability or exercise performance measured in standardized clinical settings. In addition, we reported for the first time a lack of effect of NO_3^- supplementation on free living physical activity, which may entail a re-examination of the usefulness of dietary NO_3^- supplementation as a viable nutritional population strategy to enhance physical performance.

4.3 Biological Mechanisms: Dietary NO_3^- is converted to NO in a two-step reduction process proceeding via the intermediate formation of NO_2^-. The first step is performed by saprophytic bacteria with reductase activity colonizing the dorsal area of the tongue. NO_2^- is then either converted to NO in the acidic gastric environment or transported in blood and reduced enzymatically in areas of tissues with lower oxygen tension and pH where metabolic demands are higher[39]. The latter conditions are frequently encountered in areas of contracting muscles, which favour the NO_2^- conversion into NO to enhance coupling between muscle perfusion and metabolic activities[5]. The improved metabolic activity reported in previous studies appears to be related to an increased mitochondrial efficiency and/or reduction of the energetic cost of muscle contractions[6]. This raises important questions about why NO_3^- supplementation does not improve physical capability or function in older people and stimulate future studies to investigate mechanisms that may explain the reduce effects of NO_3^- supplementation on muscular performance with aging. Putative mechanisms may involve altered reductase capacity to convert NO_3^- into NO or reduced effects of NO on skeletal muscle mediated by age-related changes in mitochondrial function and contractile efficiency. Whether higher doses or longer supplementation periods may overcome the alleged age-related decline in muscular response to dietary NO_3^- supplementation is currently not known.

4.4 Limitations: The small sample size and the relatively short duration of the intervention are important limitations of this study and therefore the results may require a careful interpretation. While we measured plasma NO_2^- concentrations using GCMS, due to logistic constraints it was not possible to process the samples immediately after collection to minimise NO_2^- degradation. These results are therefore unavailable. However, previous studies involving dietary NO_3^- supplementation in older participants where plasma NO_2^- concentration was measured, an increase in plasma NO_3^- concentrations similar to the amount observed in this study occurred alongside a significant rise in plasma NO_2^- concentrations [40].

5. CONCLUSIONS

We tested for the first time the ergogenic effects of dietary NO_3^- supplementation in older participants on exercise performance and free-living physical activity and found that, overall, dietary NO_3^- supplementation had no effects. The results seem to indicate that aging may modify the muscular response to dietary NO_3^- supplementation. However, these results await confirmation in future studies with larger samples size and in targeted populations with impaired muscular performance.

Author contributions

M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M.S. and E.W. designed the study. M.S. wrote the manuscript and researched data; C.O., D.J., D.H., C.C., A.W.A., A.R., M.R., M.K., E.W. researched data. All authors contributed to discussion and reviewed/edited manuscript.

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REFERENCES

[1] Kalyani RR, Corriere M, Ferrucci L. Age-related and disease-related muscle loss: the effect of diabetes, obesity, and other diseases. The Lancet Diabetes & Endocrinology.2:819-29.

[2] Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, et al. Decline in skeletal muscle mitochondrial function with aging in humans. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:5618-23.

[3] Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell metabolism. 2011;13:149-59.

[4] Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. Journal of Applied Physiology. 2010;109:135-48.

[5] Jones AM. Dietary nitrate supplementation and exercise performance. Sports medicine (Auckland, NZ). 2014;44 Suppl 1:S35-45.

[6] Larsen FJ, Schiffer TA, Weitzberg E, Lundberg JO. Regulation of mitochondrial function and energetics by reactive nitrogen oxides. Free radical biology & medicine. 2012;53:1919-28.

[7] Hoon MW, Johnson NA, Chapman PG, Burke LM. The effect of nitrate supplementation on exercise performance in healthy individuals: a systematic review and meta-analysis. International journal of sport nutrition and exercise metabolism. 2013;23:522-32.

[8] Jones AM. Influence of dietary nitrate on the physiological determinants of exercise performance: a critical review. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme. 2014;39:1019-28.

[9] Kelly J, Fulford J, Vanhatalo A, Blackwell JR, French O, Bailey SJ, et al. Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. American journal of physiology Regulatory, integrative and comparative physiology. 2013;304:R73-83.

[10] Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, et al. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. J Appl Physiol (1985). 2011;110:1582-91.

[11] Gilchrist M, Winyard PG, Fulford J, Anning C, Shore AC, Benjamin N. Dietary nitrate supplementation improves reaction time in type 2 diabetes: development and application of a novel nitrate-depleted beetroot juice placebo. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2014;40:67-74.

[12] Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiologica. 2007;191:59-66.

[13] Casey DP, Treichler DP, Ganger CTt, Schneider AC, Ueda K. Acute dietary nitrate supplementation enhances compensatory vasodilation during hypoxic exercise in older adults. J Appl Physiol (1985). 2015;118:178-86.

[14] Zamani P, Rawat D, Shiva-Kumar P, Geraci S, Bhuva R, Konda P, et al. Effect of inorganic nitrate on exercise capacity in heart failure with preserved ejection fraction. Circulation. 2015;131:371-80; discussion 80.

[15] Berry MJ, Justus NW, Hauser JI, Case AH, Helms CC, Basu S, et al. Dietary nitrate supplementation improves exercise performance and decreases blood pressure in COPD patients. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2015;48:22-30.

[16] Shepherd AI, Wilkerson DP, Dobson L, Kelly J, Winyard PG, Jones AM, et al. The effect of dietary nitrate supplementation on the oxygen cost of cycling, walking performance and resting blood pressure in individuals with chronic obstructive pulmonary disease: A double blind placebo controlled, randomised control trial. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2015;48:31-7.
[17] Eggebeen J, Kim-Shapiro DB, Haykowsky M, Morgan TM, Basu S, Brubaker P, et al. One Week of Daily Dosing With Beetroot Juice Improves Submaximal Endurance and Blood Pressure in Older Patients With Heart Failure and Preserved Ejection Fraction. JACC Heart failure. 2016.
[18] Masschelein E, Van Thienen R, Wang X, Van Schepdael A, Thomis M, Hespel P. Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. J Appl Physiol (1985). 2012;113:736-45.
[19] Peacock O, Tjonna AE, James P, Wisloff U, Welde B, Bohlke N, et al. Dietary nitrate does not enhance running performance in elite cross-country skiers. Medicine and science in sports and exercise. 2012;44:2213-9.
[20] Thompson KG, Turner L, Prichard J, Dodd F, Kennedy DO, Haskell C, et al. Influence of dietary nitrate supplementation on physiological and cognitive responses to incremental cycle exercise. Respiratory physiology & neurobiology. 2014;193:11-20.
[21] Bescos R, Rodriguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. Acute administration of inorganic nitrate reduces VO(2peak) in endurance athletes. Medicine and science in sports and exercise. 2011;43:1979-86.
[22] Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, et al. Acute dietary nitrate supplementation improves cycling time trial performance. Medicine and science in sports and exercise. 2011;43:1125-31.
[23] Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, Easton C. The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers. International journal of sport nutrition and exercise metabolism. 2013;23:498-506.
[24] Porcelli S, Ramaglia M, Bellistri G, Pavei G, Pugliese L, Montorsi M, et al. Aerobic Fitness Affects the Exercise Performance Responses to Nitrate Supplementation. Medicine and science in sports and exercise. 2015;47:1643-51.
[25] Shepherd AI, Gilchrist M, Winyard PG, Jones AM, Hallmann E, Kazimierczak R, et al. Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomized, double-blind, placebo-controlled crossover trial. Free radical biology & medicine. 2015;86:200-8.
[26] Freedson PS, Melanson E, Sirard J. Calibration of the Computer Science and Applications, Inc. accelerometer. Medicine and science in sports and exercise. 1998;30:777-81.
[27] CRAIG CL, MARSHALL AL, SJÖSTRÖM M, BAUMAN AE, BOOTH ML, AINSWORTH BE, et al. International Physical Activity Questionnaire: 12-Country Reliability and Validity. Medicine & Science in Sports & Exercise. 2003;35:1381-95.
[28] Mulligan AA, Luben RN, Bhaniani A, Parry-Smith DJ, O'Connor L, Khawaja AP, et al. A new tool for converting food frequency questionnaire data into nutrient and food group values: FETA research methods and availability. BMJ open. 2014;4:e004503.
[29] Schneider DA, Phillips SE, Stoffolano S. The simplified V-slope method of detecting the gas exchange threshold. Medicine and science in sports and exercise. 1993;25:1180-4.
[30] Tsikas D. Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. Anal Chem. 2000;72:4064-72.
[31] Qadir OK, Teh J, Siervo M, Seal CJ, Brandt K. Method using gas chromatography mass spectrometry (GC-MS) for analysis of nitrate and nitrite in vegetables. In: D’Haene Karoline,

Vandecasteele Bart , De Vis Raf, Crappé Sara, Callens Danny, Mechant Els, et al., editors. NUTRIHORT : Nutrient management, innovative techniques and nutrient legislation in intensive horticulture for an improved water quality. Ghent, Belgium: Institute for Agricultural and Fisheries Research; 2013.

[32] Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol. 2009;107:1144-55.

[33] Bescos R, Ferrer-Roca V, Galilea PA, Roig A, Drobnic F, Sureda A, et al. Sodium nitrate supplementation does not enhance performance of endurance athletes. Medicine and science in sports and exercise. 2012;44:2400-9.

[34] Sandbakk SB, Sandbakk O, Peacock O, James P, Welde B, Stokes K, et al. Effects of acute supplementation of L-arginine and nitrate on endurance and sprint performance in elite athletes. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2015;48:10-5.

[35] Bourdillon N, Fan JL, Uva B, Muller H, Meyer P, Kayser B. Effect of oral nitrate supplementation on pulmonary hemodynamics during exercise and time trial performance in normoxia and hypoxia: a randomized controlled trial. Frontiers in physiology. 2015;6:288.

[36] Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, et al. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. American journal of physiology Regulatory, integrative and comparative physiology. 2010;299:R1121-31.

[37] Kerley CP, Cahill K, Bolger K, McGowan A, Burke C, Faul J, et al. Dietary nitrate supplementation in COPD: an acute, double-blind, randomized, placebo-controlled, crossover trial. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2015;44:105-11.

[38] Leong P, Joosten S, Hamilton G, Bardin PG. Dynamic laryngeal narrowing in COPD may have effects on the trachea. Thorax. 2015;70:693.

[39] Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, et al. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol. 2009;5:865-9.

[40] Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, Benjamin N. Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. Free radical biology & medicine. 2013;60:89-97.

FIGURE LEGENDS

Figure 1: Description of recruitment phases

Figure 2: Changes in plasma and urinary nitrate after either one-week supplementation of nitrate-rich or nitrate-depleted beetroot juice in 19 older healthy adults. Data presented as means±95%CI. A paired t test was applied to test differences between the two interventions at baseline and end of the study.

Figure 3: Differences in gas exchanges and heart rate after one-week supplementation with either nitrate-rich or nitrate-depleted (placebo) beetroot juice in 19 older healthy adults. Data presented as means±95%CI. A repeated-measure ANOVA model was applied to test differences between the two interventions at the end of each intervention. $\dot{V}O_2$ = oxygen volume; $\dot{V}CO_2$ = carbon dioxide volume; RER= respiratory exchange ratio; $\dot{V}E$= pulmonary ventilation; HR= heart rate.

Table 1: Baseline characteristics (N=19)

	Means	SD
M/F	9/10	
Age (years)	64.7	3.0
BMI (kg/m^2)	25.6	3.4
WC (cm)	88.5	13.9
FM (kg)	22.0	6.3
FFM(kg)	50.2	11.5
Resting Systolic BP (mmHg)	127.4	16.1
Resting Diastolic BP (mmHg)	76.2	9.6
Energy Intake (Kcal/day)	2728	1431
CHO (g/day)	308	152
FAT (g/day)	107	73
PRO (g/day)	103	57
Saturated Fat (g/day)	35.6	26.5
Unsaturated Fat (g/day)	14.1	10.4
Fibre (g/day)	23.9	13.0

N= number of participants; M= Male; F= Female; Body mass index= body mass index; WC= waist circumference; FM= fat mass; FFM= fat free mass; BP= blood pressure; CHO= carbohydrate; FAT= fat; PRO= protein;

Table 2: Measures of physical capability before and after supplementation with either nitrate-rich or nitrate-depleted (placebo) beetroot juice for one week.					
	Placebo		**Nitrate**		
	Baseline	**End**	**Baseline**	**End**	**Main Effect**
Hand-Grip Strength (kg)	28.92±9.09	29.49±9.26	29.24±9.34	29.51±9.92	0.53
Time Up and Go (seconds)	5.44±0.76	5.62±0.76	5.67±1.07	5.58±1.00	0.53
Repeated Chair Standing (seconds)	8.03±2.24	7.65±1.73	7.73±1.77	7.60±1.73	0.41
10m Walking Test (seconds)	2.83±0.60	2.80±0.44	2.94±0.53	2.84±0.54	0.79

Data presented as means±SD. A repeated-measure ANOVA model was applied to test differences between the two interventions at the end of each intervention in 19 older healthy adults.

Table 3: Measures of free living physical activity after supplementation with either nitrate-rich or nitrate-depleted (placebo) beetroot juice measured over each one-week intervention with either placebo or nitrate.

	Placebo	**Nitrate**	**Δ**	**P**
Total Physical Activity (kcal)	3378.66±1615.62	3066.11±1274.17	-312.55 ± 904.17	0.14
Average Length of Sedentary Bouts (minutes)	170.15±41.57	175.73±68.76	5.57±68.73	0.72
Daily Average of Sedentary Bouts (minutes)	184.10 ± 194.84	136.10 ± 155.60	-48.01 ± 85.25	0.40
Average Length of Sedentary Breaks (minutes)	110.31±42.81	129.10±86.42	18.78±100.36	0.42
Daily Average of Sedentary Breaks (minutes)	331.05 ± 102.62	322.68 ± 94.62	-8.38 ± 56.28	0.79
Time in Sedentary Activity (minutes)	8993.68±984.47	8473.15±2139.85	-520.52±1782.42	0.21
Time in Light Activity (minutes)	2690.31±1194.68	2520.63±1171.47	-169.68±806.39	0.37
Time in Moderate Activity (minutes)	249.42±149.13	222.42±144.92	-26.94±97.83	0.24
Time in Vigorous Activity (minutes)	32.94±94.86	20.52±56.40	-12.42±54.82	0.19

Data presented as means±SD. Δ= difference between placebo and beetroot juice groups. A paired t test was used to compare differences between the two interventions for free living physical activity outcomes in 19 older healthy adults.