Dietary nitrate does not modify blood pressure and cardiac output at rest and during exercise in older adults : a randomised cross over study

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DIETARY NITRATE DOES NOT MODIFY BLOOD PRESSURE AND CARDIAC OUTPUT AT REST AND DURING EXERCISE IN OLDER ADULTS: A RANDOMISED CROSS OVER STUDY

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#Equal Contribution

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ABSTRACT

Dietary nitrate (NO_3^-) supplementation has been associated with improved vascular and metabolic health. We conducted a double-blind, cross-over, placebo-controlled RCT to investigate the effects of 7-day consumption of beetroot juice compared with placebo on 1) blood pressure (BP) measured in resting conditions and during exercise, 2) cardiac and peripheral vascular function and 3) biomarkers of inflammation, oxidative stress and endothelial integrity.

Twenty non-smoking healthy participants aged 60-75y and BMI 20.0-29.9kg/m^2 were recruited. Measurement were conducted before and after each 7-day intervention period. Consumption of NO_3^- had no effect on resting systolic and diastolic BP. NO_3^- consumption did not improve indexes of central and peripheral cardiac function responses during cardiopulmonary exercise testing. Dietary NO_3^- supplementation did not modify biomarkers of inflammation, oxidative stress and endothelial integrity.

This study do not support the short-term benefits of dietary NO_3^- supplementation on physiological and biochemical markers of vascular health in older healthy adults.

Trial Registration: ISRCTN19064955

INTRODUCTION

Ageing is a primary risk factor for atherosclerosis and cardiovascular diseases (CVD)(North and Sinclair 2012). Cardiac ageing is characterised by prominent changes in cardiovascular tissues including hypertrophy, altered left ventricular (LV) diastolic function and cardiac output (CO), and increased arterial stiffness. In older adults, resting CO is preserved by an increase in LV end-diastolic volume with a consequent augmentation of stroke volume (SV). With the increase in energy demands during exercise, older adults achieve people a higher SV and mean arterial blood pressure but lower heart rate and peak oxygen consumption compared to younger subjects. Therefore, SV during exercise in older adults is preserved by an increase in end-diastolic volume whereas in younger subjects is maintained by a progressive decrease in end-systolic volume(Cheitlin 2003, Houghton et al. 2016). Augmentation index was significantly higher in older than younger participants and was inversely related CO in older participants [3].

Nitric oxide (NO) appears to have pleiotropic effects on cardiac physiology(North and Sinclair 2012), being produced by all myocardial cells and is involved in the regulation of coronary vasodilation and cardiomyocyte contractility(Massion et al. 2003). NO is synthesised by vascular and endocardial endothelial nitric oxide synthases (NOS), as well as neuronal and inducible NOS(Rastaldo et al. 2007). The effects of NO on myocardial contractility appear to be mediated by the opening of sarcolemmal voltage-operated and sarcoplasmic ryanodin receptor Ca(2+) channels(Rastaldo, et al. 2007). NO is also involved in the modulating post-ischemic cardiac remodeling infarction which may be mediated by a decreased mitochondrial permeability(Di Lisa et al. 2001).

NO is involved in several other physiological functions such as maintenance of vascular tone, platelet adhesion, angiogenesis, mitochondrial oxygen consumption, muscular performance and control of immunity and inflammation signalling pathways (Kelm 1999). Inorganic NO_3^-

84 represents the final, stable end product of nitric oxide (NO) metabolism and it is mainly
85 excreted in urine (~70%). Approximately 25-30% of circulating blood NO_3^- enters a non-
86 enzymatic NO synthetic pathway involving salivary glands, oral microbiota and gastric acidic
87 environment (Lundberg et al. 2009). Inorganic NO_3^- from food can also enter the non-
88 enzymatic NO pathway, increase NO production and induce positive effects on
89 cardiovascular function and muscle performance(Lundberg, et al. 2009). The role of ageing
90 as a modifier of the effects of inorganic NO_3^- on cardiovascular outcomes remains unknown.
91 Convincing evidence on health benefits of dietary NO_3^- on cardiovascular outcomes
92 currently exists for young and middle-aged individuals (Gee and Ahluwalia 2016, Lara,
93 Ashor, et al. 2015), whereas contrasting findings have been reported in older populations(Gee
94 and Ahluwalia 2016, Lara, et al. 2015, Omar et al. 2016, Siervo et al. 2013). In addition,
95 limited information is available on the effects of inorganic NO_3^- consumption on cardiac
96 function at rest and during exercise in healthy adults (DeVan et al. 2015, Lee et al. 2015) and
97 in patients with heart disease (Eggebeen et al. 2016, Zamani et al. 2015).
98 We hypothesise that dietary NO_3^- consumption can increase systemic NO bio-availability and
99 have a positive effect on central and peripheral hemodynamic responses of healthy older
100 adults measured at rest, during different exercise intensities (low, moderate and high) and
101 post-exercise recovery. We evaluated the effects of beetroot juice, chosen as a rich source of
102 dietary NO_3^-, on blood pressure (BP), augmentation index (AIx) and hemodynamic
103 parameters of cardiac function including CO, stroke volume (SV), cardiac index (CI) and
104 heart rate (HR) measured at rest and during graded exercise on a stationary bike. We also
105 evaluated whether dietary NO_3^- consumption induced changes in circulating biomarkers of
106 inflammation, oxidative stress and endothelial integrity to provide mechanistic insights into
107 the effects of dietary NO_3^- on circulatory biomarkers closely involved in the regulation of
108 vascular function.

METHODS

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 Randomised Controlled Trial Number Register (ISRCTN19064955).

Participants: Twenty older healthy people (10 male, 10 female) were recruited (10 participants per site). 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.

Study Overview: A telephone screening was performed to check eligibility to the trial's inclusion and exclusion criteria. Eligible participants were asked to arrive at the research facilities after a 12-hour overnight fast and having avoided strenuous physical activity for three days preceding the visit. Eligibility to the study was confirmed by measuring BMI, resting blood pressure and conducting a resting 12 lead electrocardiogram. Participants were randomised to a cross-over intervention and the assessment continued with the measurement of body composition and collection of blood and urine samples and the assessment of physical capability (reported elsewhere). Participants then rested for one hour and consumed a meal providing approximately 300kcal (CHO=85%, PRO=3%, FAT=12%). After the 1 hour rest period the exercise test was explained to the participants and they and they became

accustomised to the bicycle ergometer. The exercise protocol is described in **Figure S1 of the Online Supplementary Material**. After the vascular measurements and 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. 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. Measurements were conducted approximately after 12 hours from drinking the beetroot juice as participants were asked to fast overnight before arriving to the research centre. The resting 12 lead ECG was performed and if normal the visit continued with a repeat of the assessments performed at 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.

Resting and Daily Blood Pressure: Resting BP was measured in triplicate using an automated BP monitor (Omron M3, Omron Healthcare, UK) at each clinic visit with the participant seated comfortably for 15 min prior to the measurement and the arm supported at the level of the heart. The same BP monitor (Omron M3, Omron Healthcare, UK) was provided to each participant for the measurements of daily resting BP at home. Participants were asked to conduct duplicate measurements in a seated position in the morning before drinking the juice and in the evening before going to bed. Agreement of the daily BP monitoring was verified against the BP recordings obtained from the 24-hr ABPM (systolic BP, $r=0.71$, $p<0.001$, $n=84$; diastolic BP, $r=0.80$, $p<0.001$, $n=84$)(Jajja et al. 2014).

Resting and exercise central hemodynamics: All subjects performed a maximal graded cardiopulmonary exercise test using an electro-magnetically controlled bicycle ergometer (Corival, Lode, Groningen, Netherlands) with online gas exchange measurements (Metalyzer 3B, Cortex, Leipzig, Germany). The maximal progressive exercise test included cycling with 10-watt increments every minute until volitional exhaustion. The 12-lead ECG (Custo, CustoMed GmbH, Ottobrunn, Germany) was continuously monitored and blood pressure (Tango, SunTech Medical, Morrisville, NS, USA) recorded at rest, during exercise and recovery (Newcastle Centre only, N=10). The test was terminated when the subject was unable to pedal at a cadence of 50 revolutions per minute or they reached maximal oxygen consumption. Peak oxygen consumption was defined as the average oxygen uptake during the last minute of exercise. Non-invasive central hemodynamics parameters (SV, CO and CI) were measured by bioreactance method (NICOM, Cheetah Medical, Deleware, USA).(Jakovljevic 2014) CO was estimated under resting and exercise stress testing conditions using the bio-reactance method which analysis the frequency of relative phase shifts of electrical current applied across the thorax using four dual-surface electrodes. Signals were applied to and recorded from the left and right sides of the thorax; these signals are processed separately and averaged after digital processing. The signal processing unit of the system determines the relative phase shift between the input signal relative to the output signal. The phase shift occurs due to instantaneous changes in blood flow in the aorta. Cardiac output is subsequently estimated as the product of stroke volume and heart rate. Cardiac Index (CI) is calculated by adjusting the CO for body surface area. A graphical description of the protocol is described in **Figure S2 of the Online Supplementary Material**.

Augmentation Index: A high fidelity micro-manometer was used to apply a gentle pressure and therefore flatten the radial artery in the non-dominant hand at the wrist under resting

condition using the SphygmoCor (AtCor Medical, NSW, Australia). Central aortic pressure and augmentation index was then calculated automatically using the SphygmoCor software. AIx was calculated as the difference between the first systolic peak and the second systolic peak of the central arterial waveform, which was expressed as a percentage of pulse pressure.

Anthropometry, Dietary and Lifestyle Questionnaires: Body weight and height were measured to the nearest 0.1 kg and 0.5 cm, respectively. The 9-item short form of the International Physical Activity Questionnaire (IPAQ) was used to record 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(CRAIG et al. 2003). The EPIC Food Frequency Questionnaire (FFQ) was administered at baseline and the FETA software used to extract dietary (energy and nutrient) information(Mulligan et al. 2014).

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.

Biomarker Analysis: A modified version of the gas chromatography mass spectrometry (GC-MS) method proposed by Tsikas et al(Tsikas 2000) was used to determine NO_3^- and NO_2^- concentrations in urine and plasma samples and sum of NO_3^- and NO_2^- (NOx) was calculated. However, blood samples were not immediately processed (~30-45 minutes) to preserve NO_2^- and therefore NO_3^- is the main contributor to the total concentration of NOx. The protocol and validation of the modified GC-MS method have been described elsewhere(Qadir et al. 2013). Methods for the measurement of glucose, insulin, IL-6, 3-NT, cGMP, ET-1, P-

selectin, E-Selectin, intercellular adhesion molecule-3 (ICAM-3) and thrombomodulin are reported in the **Online Supplementary Material**.

Statistical Analysis: Repeated-Measures General Linear Models (GLM) were used to test the effect of NO_3^- consumption on measures of vascular function and blood biomarkers. Treatment (nitrate 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. Analyses were conducted using Statistica 10 for Windows (StatSoft.Inc, Tulsa, OK, USA). Statistical significance was set at <0.05.

RESULTS

Participants' characteristics and safety: Twenty participants were randomised to the interventions. 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) reported no side effects apart for the expected urine discoloration related to the excretion of beetroot juice pigment (beeturia). Baseline VO_2 max of participants was 23.6±5.8 ml/kg/min for men and 20.5±2.8 ml/kg/min for women.

Body weight, dietary Intake and self-reported physical activity: Mean baseline BMI was 25.6±3.4 kg/m^2. Body weight did not change during the study in either groups (p=0.51) (**Table S1 of the Online Supplementary Material**). Changes in self-reported physical activity were again not different between the placebo and the NO_3^- arms (p=0.99) (**Table S1 of the Online Supplementary Material**).

Resting Clinic and Daily Blood Pressure: Baseline resting systolic and diastolic BP were 127.4 ± 16.1 mmHg (range: 100.0 – 168.0 mmHg) and 76.2 ± 9.6 mmHg (range: 61.6 – 95.7 mmHg), respectively. Clinic systolic BP were not significant after NO_3^- consumption compared to placebo (-5.05±9.45 vs -2.64±9.04 mmHg respectively, p=0.42) (**Figure 2a**).

Similarly, daily BP was not significant for both systolic (p=0.75) and diastolic (p=0.63) readings measured over the 7-day period (**Figure 2b**).

Augmentation Index: NO_3^- consumption did not have a significant effect on AIx (p=0.87, **Figure S3**).

Blood Pressure and Cardiac Function during Standardised Exercise: NO_3^- consumption did not influence systolic BP response (p=0.92, **Figure 3a**) during exercise whereas a non-significant trend for lower diastolic BP after NO_3^- supplementation (p=0.08, **Figure 3b**). Specifically, lower diastolic BP readings were recorded during moderate sub-maximal exercise intensities (work rate: 40W, 60W and 80W). Dietary NO_3^- consumption did not modify parameters of cardiac function (CO, HR, SV and CI) measured at rest, during exercise and post-exercise recovery (**Figure 4a-d**). In addition, one week dietary NO_3^- consumption did not modify the association between CO and oxygen consumption (VO_2) (nitrate, B±SE= 6.04±0.34, R^2=0.60, p<0.001; placebo, B±SE= 6.68±0.42, R^2=0.55, p<0.001) measured at different levels of exercise intensities (**Figure S4 of the Online Supplementary Material**).

Laboratory Biomarkers: Concentrations of nitrite plus nitrate (NO_2^-+NO_3^-, NOx) in plasma and urine increased after NO_3^- consumption by 150±77% and 979±488% compared to placebo (-9±33% and -13±34%, respectively). NO_3^- consumption did not modify concentrations of fasting glucose (p=0.41), insulin (p=0.95) and HOMA-IR (p=0.88). NO_3^- consumption also did not induce any changes in biomarkers of endothelial function (cGMP, endothelin-1, E-Selectin, P-Selectin, thrombomodulin and ICAM-3), inflammation (IL-6) and oxidative stress (3-NT) (**Table 1**).

DISCUSSION

This study does not support a beneficial effect in the short-term of dietary NO_3^- ingestion on cardiac and peripheral vascular health in older healthy adults. In particular, a lack of effect

was observed for BP and central hemodynamic responses measured both at rest and during increased metabolic demands. These physiological measurements were complemented by a panel of circulating biomarkers of metabolic control, oxidative stress and endothelial integrity. None of these measurements were altered by one-week dietary NO_3^- ingestion, which stimulate further discussion on uncovering the factors that may explain the lack of efficacy in older populations and the contrast with the more consistent beneficial effects observed in younger populations.

Extensive work has been conducted in the last decade to test the effects of dietary NO_3^- on BP but, despite numerous trials, the evidence is still limited due to the small sample size and short duration of completed trials(Gee and Ahluwalia 2016, Khatri et al. 2016, Mills et al. 2016, Siervo, et al. 2013). Further research is especially needed in older populations, although patients with comorbidities such as peripheral arterial disease or heart failure (HF) appear to receive greater health benefits from dietary NO_3^- consumption(Eggebeen, et al. 2016, Kenjale et al. 2011, Zamani, et al. 2015). However, DeVan et al(DeVan, et al. 2015) have recently reported that sodium nitrite supplementation was well-tolerated and improved endothelial function and lessens carotid artery stiffening in middle-aged and older adults. Conversely, our group has recently reported non-significant effects of dietary NO_3^- consumption on endothelial function and on 24-hr ambulatory blood pressure (BP) in older adults with and without type 2 diabetes (>60years)(Lara, Ogbonmwan, et al. 2015, Siervo et al. 2015). These results have also recently been corroborated by Bondonno et al who found no effect of seven-day dietary NO_3^- consumption on home and 24-hr ambulatory BP in patients with raised BP (age range: 30-70y)(Bondonno et al. 2015, Bondonno et al. 2014). However, Kapil et al showed that a four-week intervention in drug-naïve hypertensive subjects (age range: 18-85y) significantly reduced clinic, home and 24-hr ambulatory BP and improved endothelial function(Kapil et al. 2015). The divergence of results is again part of

283 the discussion around the effects of dietary NO_3^- on health outcomes and priority is now
284 being assigned to the identification of factors accounting for the mixed findings. These
285 factors may include phenotypic characteristics (i.e., age, BMI, health status) of the
286 populations, dose of dietary nitrate and duration of supplementation, study design,
287 measurement protocols of BP an vascular health, type of cardiopulmonary fitness test
288 protocols. The dynamic BP responses during exercise have been investigated in young,
289 healthy populations(Bond et al. 2014, Lee, et al. 2015) and in patients with COPD(Berry et
290 al. 2015) and HF(Coggan et al. 2015, Coggan and Peterson 2016, Eggebeen, et al. 2016). In
291 healthy young populations, two studies reported a decline of sub-maximal systolic BP after
292 acute (single dose) and short-term (15 days) dietary NO_3^- consumption (Bond, et al. 2014,
293 Lee, et al. 2015). In older COPD patients, dietary NO_3^- decreased systolic BP at rest whereas
294 only diastolic BP showed a significant decline during 5W pedaling and 75% of maximal
295 work rate(Berry, et al. 2015). In patients with HF, acute dietary NO_3^- did not improve BP
296 responses during knee extension or cycle ergometry tests at sub-maximal and maximal
297 efforts(Coggan and Peterson 2016). However, one week of daily dosing with dietary NO_3^-
298 significantly improved submaximal BP in elderly patients with HF with preserved ejection
299 fraction(Eggebeen, et al. 2016). Our study is the first trial to investigate resting and dynamic
300 BP responses in older healthy adults and our results do not support a beneficial effect of
301 dietaryNO_3^-on oxygen consumption (data not shown) as well as vascular responses during
302 exercise. Precisely why there is a lack of response to dietary NO_3^- in our study is not known
303 since we have supplemented subjects for one week and administered a NO_3^- dose
304 considerably higher compared to other studies (>700mg/day). The factors explaining these
305 divergent age-dependent responses are still largely undetermined, which could potentially be
306 related to a decline in the reducing capacity to convert NO_3^- into NO_2^- or sensitivity of
307 cellular targets to NO.

In mice, NO_3^- consumption have been showed to increase the expression of calcium handling proteins in the heart, resulting in increased cardiomyocyte calcium signaling and improved left ventricular contractile function(Pironti et al. 2016). These findings have provided preliminary support to the role of dietary NO_3^- as a cardiac modulator, which have then been translated into clinical interventions in populations without and with impaired cardiac function. In healthy populations, acute dietary NO_3^- consumption did not modify resting or sub-maximal CO(Bond, et al. 2014) whereas an improvement of CO and SV was observed after a one-week dietary NO_3^- consumption(Lee, et al. 2015). The only study testing the acute effects of dietary NO_3^- on cardiac function in HF patients with preserved ejection fraction found greater reductions in systemic vascular resistance, aortic augmentation index and increased CO during exercise(Zamani, et al. 2015). We tested whether dietary NO_3^- could minimise the age-related decline in myocardial contractility and ejection fraction, which could prompt compensatory myocardial cardiac hypertrophy(North and Sinclair 2012). This enhances in the short-term CO but the long-term effect of LV hypertrophy are known as represents an important step in the development of HF and coronary syndromes(Gosse 2005). Our scope was to evaluate whether dietary NO_3^- could represent an effective and simple nutritional intervention that may minimise age-related changes in cardiac function and impact, from a primary prevention perspective, on the risk for HF. However, these preliminary results may not support the beneficial effects of dietary NO_3^- on cardiac function in older healthy populations but, if confirmed in more robust trials, dietary NO_3^- may still represent a promising nutritional strategy in patients with impaired cardiac function.

The small sample size and the short duration are important limitations of this trial and a cautious interpretation of the results is needed; nevertheless, our study is to date one of the longest trials testing the effects of dietary NO_3^- on resting and exercise vascular responses in older participants. We did not assess daily dietary intake during the trial. However,

participants were asked to maintain their habitual dietary intake during the study and the differences in nitrate intake between intervention and placebo groups were clearly demonstrated by the large differences in plasma and urinary nitrate concentrations. Plasma NO_2^- concentrations were not measured since it was not possible due to logistic constraints to process the samples immediately after collection to minimise the immediate NO_2^- degradation (half-life: ~5 minutes). However, in previous studies testing the effects of dietary NO_3^- consumption in older participants where plasma NO_2^- concentration was measured, an increase in plasma NO_3^- and NOx concentrations similar to the amount observed in this study occurred alongside a significant rise in plasma NO_2^- concentrations(Gilchrist et al. 2013). In addition, the measurement of NOx was critical to assess the compliance to the interventions as well as the attainment of a significant rise in plasma NO_3^- to enable an increased NO generation via the NO non-enzymatic pathway. Finally, the limitations of bio-reactance for the assessment of cardiac hemodynamic profiles have to be taken into account for the interpretation of the results.

Testing the efficacy of dietary NO_3^- consumption on cardiovascular health is an attractive research area due to the potential use of natural products to increase NO_3^- intake and applicability in long-term dosing. However, this short term intervention showed that dietary NO_3^- consumption did not modify physiological and biochemical markers of vascular health in healthy older adults. However, these findings are preliminary and require corroboration in studies with longer duration and larger samples of healthy older individuals as well as in older patients with increased cardiovascular risk.

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., C.C., A.W.A., A.R., M.R., M.K., E.W. collected the data. All authors contributed to discussion and reviewed/edited manuscript.

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Conflicts of interest

None to report

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FIGURE LEGENDS

Figure 1: Plasma and urinary nitrate after either 7-day consumption of nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±1SE.

Figure 2: Resting (Panel A) and daily (Panel B) systolic and diastolic blood pressure (BP) measured during a one week oral consumption (End) with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM. SBP= systolic blood pressure; DBP= diastolic blood pressure.

Figure 3: Systolic (Panel A) and diastolic (Panel B) blood pressure (BP) at rest, during incremental exercise and post-exercise recovery after one week oral consumption with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM.

Figure 4: Heart rate (Panel A), stroke volume (Panel B), cardiac output (Panel C) and cardiac index (Panel D) at rest, during incremental exercise and post-exercise recovery after one week oral consumption with either nitrate-rich or nitrate-depleted beetroot juice. Data presented as mean±SEM.