Original Communication

Effects of dietary nitrate and vitamin C co-ingestion on blood pressure and hand-grip strength in young adults

A pilot randomized controlled trial

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Abstract: *Background:* Co-administration of vitamin C and inorganic nitrate (NO_3^-) may reduce oxidative stress, boost the conversion of nitrite (NO_2^-) into NO and elicit positive vascular effects. *Aims:* We aimed to test the effects of oral inorganic NO_3^- and vitamin C co-supplementation on vascular function, muscular strength, and on concentrations of urinary NO_3^-, vitamin C, 8-isoprostanes and salivary NO_2^- in healthy young adults. *Methods:* Ten young healthy participants were enrolled in a randomised, double-blind (only for the NO_3^- intervention) crossover clinical trial. Participants consumed in random order: 1) nitrate-rich beetroot juice and vitamin C (N+VC), 2) nitrate-rich beetroot juice alone (N) or 3) nitrate-depleted beetroot juice alone (ND). Resting blood pressure (BP) was measured at the research centre and at home. Non-invasive, continuous measurements of BP and cardiac function parameters were performed using a Finometer device. Free-living physical activity and hand-grip strength were assessed. Salivary NO_3^- and NO_2^- and urinary NO_3^-, 8-isoprostanes and vitamin C concentrations were measured. *Results:* There were no significant differences for any of the vascular outcomes between the three interventions groups. However, analyses of within-intervention changes showed a significant lower daily systolic BP in the NO_3^-+vitamin C (N+VC) group only (P=0.04). Urinary NO_3^- (P=0.002) and salivary NO_2^- (P=0.001) were significantly higher in the N+VC group compared to the N and ND groups. *Conclusion:* These preliminary findings suggest that combining dietary NO_3^- with vitamin C could have protective effects on vascular function in young adults and could represent an effective strategy for the maintenance of healthy cardiovascular trajectories.

Keywords: nitrate, blood pressure, vitamin C, muscular strength

Introduction

Dietary nitrate (NO_3^-) intake has been linked to favourable cardiovascular and metabolic effects and lowering effects on blood pressure (BP) by improving the bioavailability of nitric oxide (NO) [1, 2]. Dietary NO_3^- supplementation may also enhance muscular efficiency [3] via an NO-mediated role in the modulation of mitochondrial coupling and insulin signalling in skeletal muscle [4]. The antioxidant properties of vitamin C have been shown to contribute to the maintenance of the systemic NO pool through a decrease in oxidative stress [5] Vitamin C supplementation has been associated with improvements in BP [6] and endothelial function [7]. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidants, influences various physiological processes [8]. Excessive ROS can damage cells, trigger inflammation, and contribute to health issues [9]. To evaluate oxidative stress in research settings, numerous biomarkers have been employed, one of which is 8-isoprostane. 8-isoprostanes are prostaglandin-like compounds that are generated as a consequence of lipid peroxidation initiated by ROS [10]. They have emerged as reliable indicators of oxidative stress due to their stability and the established association

between their levels and the degree of oxidative damage. In the context of this study, we utilized urinary 8-isoprostane concentrations as a relevant biomarker to assess oxidative stress.

The hypothesis of the study is that the combination of inorganic NO_3^- and vitamin C would produce greater improvements in vascular health (i.e., resting clinic and home BP, continuous measurement of BP using Finometer) and hand-grip strength compared to inorganic NO_3^- alone. Previous research has examined the synergistic relationship between dietary NO_3^- and vitamin C in relation to improvement of vascular function [11, 12]. Vitamin C may enhance the efficiency of both enzymatic and non-enzymatic NO pathways as potential means of preserving endothelial function in patients in whom reactive oxygen species concentrations are high and NO levels are low [13]. Ashor et al. [14] demonstrated that inorganic NO_3^- (7 mg/kg) and vitamin C (20 mg/kg) were synergistically beneficial for vascular health and decreased arterial stiffness and mean arterial BP in older adults when compared to vitamin C or NO_3^- alone. Supplemental vitamin C could therefore enhance the beneficial effects of dietary NO_3^- by augmenting NO pools and amplifying their vascular protective properties if it were introduced for a longer time period. However, there is very preliminary evidence on the combined effects of vitamin C and dietary NO_3^- from longer studies. In addition, no studies have investigated so far the combined effects of NO_3^- and vitamin C supplementation on muscle strength.

This literature gap offers the opportunity to undertake a study to: (i) test whether the co-administration of oral doses of inorganic NO_3^- and vitamin C would be associated with greater effects on BP and measures of cardiac function compared to single interventions or placebo; (ii) investigate the effects of oral inorganic NO_3^- and vitamin C, both alone and combined, on muscular strength; and (iii) assess the impact of oral inorganic NO_3^- and vitamin C alone or in combination on urinary and salivary NO_3^- concentrations and urinary concentrations of 8-isoprostanes.

Methods

This was a pilot randomised, double-blind crossover clinical trial conducted at the University of Nottingham UK between December 2021 and December 2022. The study was approved by the School of Life Sciences Ethics Committee of the University of Nottingham (Ethics approval number: A141021MS). Participants signed an informed written consent before being enrolled in the study.

Subjects and recruitment: Ten male and female young (18–35 years) normal weight (BMI between 18 and 25 kg/m^2) subjects were recruited between January and June 2022. Subjects were excluded if they were smokers, had a systolic BP>180 mmHg and/or diastolic BP>110 mmHg at the screening visit, followed a vegetarian or vegan diet, professional athletes, experienced any recent substantial weight gain or loss (>3 kg in the last month), self-reported any existing or previous medical condition that may affect the study outcomes and were unable to provide a written consent. The study was advertised using social media posters, society group chats and word of mouth. Interested participants were provided with an information sheet describing the study characteristics and, if still interested in the study, a screening phone call followed to confirm eligibility. Suitable participants were then invited to attend to the research facility to confirm the eligibility by measuring body mass index (BMI) and resting BP. If eligibility was confirmed, subjects were enrolled into the study and assigned to a randomisation pattern for the cross-over interventions.

Randomization: The randomisation was conducted by an independent investigator outside the research team. Codes were disclosed at the end of the trial and in case of occurrence of serious adverse events. Inorganic NO_3^- and placebo solutions had the same organoleptic characteristics and had identical volume, colour and presentation of the bottles.

Nutritional interventions: Participants were asked to consume a daily dose of 140 ml concentrated beetroot juice with 70 ml consumed in the morning and 70 ml in the evening. This quantity of beetroot juice contained approximately 800 mg of NO_3^- (~400 mg of NO_3^- per bottle), which is equivalent to eating approximately 200 g of rocket or spinach. These products are commercially available in health and food shops in the UK (Beet it, James White Company, UK). The placebo for the NO_3^- intervention was NO_3^--depleted concentrated beetroot juice (~0.3 mg of NO_3^- per 70 ml), which had the similar organoleptic characteristics of the NO_3^--rich beetroot juice and contained in the same bottles, which allowed the double-blinding of the NO_3^- intervention. Vitamin C tablets (500 mg/tablet, Vita biotics, UK) were consumed with each beetroot juice dose, providing the amount of vitamin C equivalent to eating five large oranges or drinking 1 L of orange juice. No placebo was provided for the vitamin C intervention.

Study protocol: The study involved four phases (Figure 1): (1) Recruitment and telephone screening, recruited participants were given an information sheet and contacted via telephone to confirm their eligibility, followed by an invitation for their first visit at the research centre if they were deemed eligible. (2) Screening/Baseline Visit (day 1), participants were asked to sign an informed consent and have basic anthropometric measurements (weight, height, body mass index (BMI)) and resting BP taken and if they were within the inclusion criteria, participants were included. They were then randomised to a specific order of interventions. Measurements of salivary NO_2^- concentrations were

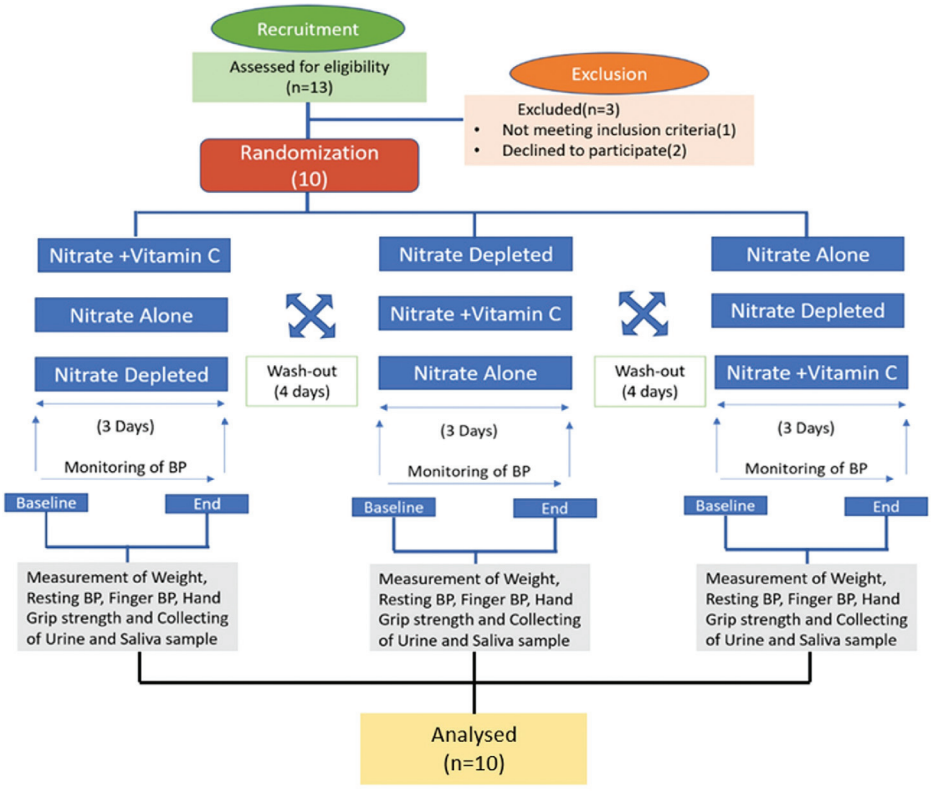

Figure 1. Flow chart and study protocol. Flow diagram of the study showing the number of participants from enrolment to inclusion in final analysis. Description of key phases of the study including randomisation, duration, wash-out and measurements is also provided. BP: blood pressure.

performed using disposable salivary strips followed by collection of spot-urine and saliva samples. Assessment of hand-grip strength by a portable dynamometer was then performed in triplicate for each arm, which was followed by an assessment of BP and cardiovascular function at rest and during isometric contraction of the upper arm using continuous finger-pulse pressure measurements. This concluded the first visit and participants were provided with the randomly allocated nitrate and vitamin C supplementations. (3) Supplementation and interventions, supplements consisted of NO_3^--rich/depleted beetroot juice and/or vitamin C which were consumed during the three days of the intervention. Participants were asked to record their resting BP at home, wear a wrist-watch accelerometer for the assessment of free-living physical activity, and fill out questionnaires for the assessments of dietary intake and physical activity. At the end of the first supplementation period participants arrived at the research centre in fasting conditions and had salivary NO_2^- measurements performed using salivary strips and urine and saliva samples taken. Resting BP, hand-grip strength and continuous finger-pulse pressure measurements were performed in the same order and using the same protocols. The accelerometers and BP devices were then collected and files checked and stored for future analyses. (4) Wash-out period and interventions, participants were then asked to return to their typical lifestyle and dietary habits during the wash-out period (4 days). Plasma concentrations of inorganic NO_3^- returns to physiological concentrations approximately after 36–48 hours from the ingestion of an oral dose [15]; similarly, plasma concentrations of vitamin C are normalized to physiological levels in about 16 hours [16]. Participants returned to the research centre to complete the two remaining interventions, dispensed according to the randomisation sequence, and measurements were repeated in the same order throughout the study. At the end of the study, participants filled out a brief feedback questionnaire on the intervention and measurement protocols. Participants were asked to collect saliva and spot urine samples at the beginning and end of each intervention.

Blood pressure: BP measurements were conducted using three different protocols. (1) Resting: BP was measured triplicate with an automated BP device, using an appropriate cuff size for each subject (Omron M3, Omron Healthcare, The Netherlands); (2) Home-BP monitoring: The same BP portable device (Omron M3) was provided for resting BP measurements at home. Participants were asked to take measurements in duplicate (2 measurement in the

morning and 2 at night) after resting in a seated position for at least 5 minutes; (3) Finger-pulse pressure measurements (Finapres): A portable, non-invasive device (Finapres® device, TNO Biomedical Instrumentation, Amsterdam, Netherlands) was used to monitor BP and estimate cardiovascular parameters. Measurements were conducted after participants had rested for at least 15 minutes and were in a supine position. A small, inflatable cuff was fitted to the participants' index finger of the dominant arm and measurement were conducted in resting conditions (three-minutes), during isometric muscle contraction of the dominant form performed by holding a hand-held dynamometer at a fixed power (5kg) for three minutes using their dominant arm, followed by another three-minute rest (recovery phase). All measurements conducted at the research centre were performed in a quiet and temperature-controlled room (~22–24°C).

Hand-grip strength: Hand-grip strength was assessed in both arms immediately after the resting BP measurements using a portable digital dynamometer (Takei 5401, Takei, Japan). Measurements were performed in triplicate for each arm (6 measurements) and the average of the measurements was used in the analysis.

Free-living physical activity: After the baseline visit, subjects were invited to wear a wrist-worn accelerometer GENEActiv for 3 days and return it at the end of each intervention. The GENEActiv (Activinsights Ltd., Cambridgeshire, UK) is a tri-axial, ±6 g seismic acceleration sensor, which is small (36 cm×30 cm×12 cm), lightweight (16 g), waterproof, and offers a near body temperature sensor to help improve the confirmation of wear and non-wear time. The raw accelerometer data was then inputted independently into the wrist or thigh "Sedentary Sphere" open access Macros [17]. These Excel Macro's classified minutes of physical activity (Sedentary, Light, Moderate, Vigorous) as well as posture classification (Sitting or Standing) based off accelerometer movement patterns and positioning.

Salivary Strips: Participants were asked to apply a NO Test Strip (Berkeley Test®, CA, USA). The single-use strip was applied on the participant's dorsal tongue for 30 seconds, with the colour developing and read against a concentration chart to determine NO_2^- concentrations.

Nitrate, nitrite, vitamin C and 8-isoprostanes concentrations: Colorimetric assays based on the Griess reaction (Cayman Chemical, Cambridge Bioscience, Cambridge, UK) were used to measure NO_3^- and NO_2^- concentrations in saliva and urine (nitrate only) samples. A colorometric assay kit was used to measure vitamin C in urine samples (Abcam ab65656, Abcam-UK, Cambridge, UK). An ELISA Kit was used to analyse the amount of 8-isoprostane in the urine samples as a measure of oxidative stress (Abcam ab175819, Abcam-UK, Cambridge, UK). The kit was a competitive immuno-enzymatic assay for quantitatively measuring 8-isoprostane in biological samples. Urine samples were diluted 4-fold prior to analysis. The assay's sensitivity and precision (CV%) are 1 pg/ml and 1.75 %, respectively.

Dietary assessment and physical activity: The 9-item short form of the International Physical Activity Questionnaire was used to record the duration of 4 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 metabolic equivalent−minutes/week [18]. The European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire was administered at baseline, and FETA software was used to extract dietary (energy and nutrient) information [19]. They were both administered at the first visit. Participants were adviced to maintain their habitual dietary habits while they were enrolled in the study.

Sample size: This was an exploratory, pilot study with the aim to provide preliminary information on the feasibility of the interventions and measurement protocols and overall magnitude and direction of the effects on primary and secondary outcomes. The sample size of this study was decided by taking into consideration (1) previous nitrate interventions showing positive effects on BP in healthy adults [20, 21] and (2) predicted effect size estimates recommended by Whitehead et al. [22] to calculate sample size for pilot, feasibility trials. The recommendations suggest that a minimum pilot trial sample size per arm of 10 participants to detect a medium effect size ($0.3 \leq \delta < 0.7$) for the difference between the interventions [22].

Statistical analysis: Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (version 28, IBM.Inc, USA). Data are presented as means± standard error of the mean (SEM) and percentages (%) for the categorical variables. We assessed the normality of the relevant variables using Shapiro-Wilk test. For those variables that were not normally distributed, we applied a log transformation to address issues of non-normality and stabilize variance. After careful evaluation, only the distribution of urinary vitamin C concentrations was skewed and it was log-transformed (Tables E1–E2 of the Electronic Supplementary Material [ESM] 1). The primary analyses focused on the investigation of between-intervention differences, which were conducted using repeated-measure ANOVA was used to evaluate differences in responses to the interventions. Time (baseline and end values, T) was entered as a within-subject factor and intervention (I) groups as a between-subject factor and the interaction between the two terms was derived (T×I). Absolute changes (Δ) from baseline for the outcomes were also calculated. As secondary, exploratory analyses, we also investigated within-group changes in outcomes in line with the

pilot design of the study. Paired t-test was used to compare baseline and 3-day measurement in each intervention. Spearman's correlation was used to evaluate the association between salivary NO_3^- and NO_2^- concentrations measured by Griess assay and salivary test strips. A p value less 0.05 was considered as significant.

Results

Participants: Of the 13 subjects who were enrolled in the study, 5 females and 5 males were eligible, and completed all the interventions and study visits (Figure E1 of the ESM 1). The mean age of the subjects was 24.8±1.6 years; their mean BMI was 22.6±0.7 kg/m^2. Their baseline characteristics are presented in Table 1.

Resting blood pressure: There were no differences in resting systolic or diastolic BP values between the three groups at the end of the intervention (P=0.55) (Table 2). Also, there were no differences in BP for the within-group comparisons.

Home-BP monitoring: No differences between systolic and diastolic BP values were noted when the groups were compared post intervention (Table 3). However, upon analysing the changes within each group, a non-significant decrease in systolic BP was observed in the N group (–4.85±4.6 mmHg, P=0.30) whereas a significant reduction in home systolic BP was detected in the N+VC group (–6.62 ±2.8 mmHg, P=0.04) (Table 3).

Table 1. Baseline characteristics

Variables	Total (n=10)
M/F	6/4
Age (years)	24.8±1.5
Weight (kg)	67.0±12.5
Height (cm)	170±.10
BMI (kg/m^2)	22.6±0.7
Energy (MJ/day)	13.66±2.01
CHO (g/day)	405±68
Fat (g/day)	123±16
Protein (g/day)	123±20
Vitamin C (mg/day)	181±29
Systolic BP (mmHg)	116.8±5.2
Diastolic BP (mmHg)	67.8±1.7
HR (beats/min)	72.8±4.3
IPAQ (METs/week)	4691.45±815.0

Notes. The values of the continuous variables were presented as mean and standard deviation. BMI: body mass index; CHO: carbohydrate; BP: blood pressure; HR: heart rate; HGS: hand-grip strength; IPAQ: international physical activity questionnaire; METs, metabolic equivalent of tasks.

Table 2. Changes in resting blood pressure, heart rate, and hand-grip strength following 3-day supplementation with nitrate-rich beetroot juice alone (N), nitrate-depleted beetroot juice (ND) and nitrate-rich beetroot juice plus vitamin C (N+VC)

Time point	N	ND	N+VC	P
Resting systolic BP (mmHg)				
Baseline	119.0±4.3	118.5±4.2	117.9±5.3	T=0.56
Endpoint	120.1±4.1	118.0±4.4	114.9±5.0	I=0.88
Δ	1.1±2.4	−0.5±2.0	−3.0±2.7	T×I=0.49
P*	0.68	0.80	0.29	
Resting diastolic BP (mmHg)				
Baseline	120.1±2.0	67.1±.8	68.7±2.6	T=0.93
Endpoint	68.8±4.8	65.7±3.0	66.1±3.0	I=0.28
Δ	4.4±3.5	−1.3±2.7	−2.6±2.7	T×I=0.28
P*	0.24	0.62	0.48	
Resting HR (bpm)				
Baseline	70.0±3.1	72.6±3.4	74.3±3.9	T=0.81
Endpoint	72.1±3.8	71.4±4.7	74.9±2.9	I=0.72
Δ	2.1±4.4	−1.2±3.2	0.6±2.5	T×G=0.79
P*	0.65	0.70	0.81	
HGS (kg)				
Baseline	35.76±5.0	35.4±4.8	35.2±4.5	T=0.06
Endpoint	34.5±4.6	33.4±5.0	35.1±84.7	I=0.98
Δ	−0.8±1.0	−1.9±0.06	−0.04±0.7	T×I=0.27
P*	0.45	0.01*	0.95	

Notes. BP: blood pressure; HR: heart rate; HGS: hand-grip strength. **Δ**: mean difference between baseline and endpoint within intervention group; values are reported as mean±SEM. Paired t-tests were used to compare baseline and 3-day measurement within each intervention (P* value). Repeated-measure ANOVA was used to evaluate differences in responses to the interventions (P value). Time (baseline and end values, T) was entered as a within-subject factor, intervention (I) groups as a between-subject factor and the interaction between the two terms was derived (T×I).

Table 3. Changes in home resting blood pressure and heart rate following a 3-day supplementation with nitrate-rich beetroot juice alone (N), nitrate-depleted beetroot juice (ND) and nitrate-rich beetroot juice plus vitamin C (N+VC)

Day	N	ND	N+VC	Comparison P
Systolic blood pressure (mmHg)				
D0	123.7±5.3	112.6±12.4	122.5±4.6	T=0.69
D1	121.7±4.6	117.9±3.3	122.0±4.8	I=0.55
D2	122.1±4.2	113.7±4.4	117.7±4.2	T×I=0.29
D3	118.9±2.9	119.9±4.2	115.8±4.5	
Δ_{D3-D0}	−4.8±4.6	7.3±9.5	−6.6±2.8	
P*	0.32	0.46	0.04*	
Diastolic blood pressure (mmHg)				
D0	67.90±1.99	72.60±3.44	68.70±2.10	T=0.45
D1	69.05±2.10	69.76±1.90	67.94±1.79	I=0.43
D2	70.22±1.65	67.61±1.58	65.80±.92	T×I=0.31
D3	69.90±1.81	68.95±2.19	67.06±1.45	
Δ_{D3-D0}	2.0±2.4	−3.6±3.6	−1.6±2.2	
P*	0.43	0.34	0.48	
Heart rate (beats/min)				
D0	65.8±3.9	72.1±3.7	72.0±3.6	T=0.79
D1	69.3±4.0	70.9±3.3	75.0±3.9	I=0.80
D2	71.0±3.6	69.0±3.4	70.9±4.0	T×I=0.24
D3	69.2±4.4	69.3±5.4	71.5±3.8	
Δ_{D3-D0}	−0.55±3.0	−2.75±2.9	3.35±1.76	
P*	0.90	0.37	0.86	

Notes. D: day. Δ: Difference, the mean difference between baseline and endpoint within intervention group; values are reported as means±SEM. Paired t-tests were used to compare baseline and 3-day measurement in each intervention, (P* value ≤0.05). Repeated-measure ANOVA was used to evaluate differences in responses to the interventions. Time (baseline and end values, T) was entered as a within-subject factor and intervention (I) groups as a between-subject factor and the interaction between the two terms was derived (T×I).

Finger-pulse pressure measurements (Finapres): No differences in systolic and diastolic BP values were identified during resting, exercise and recovery phases in all intervention arms using the Finometer (Table 4). Also, there were no differences in BP for the within-group comparisons. The change in resting and exercise total peripheral resistance (TPR) saw no significant results. Recovery TPR remained constant within all interventions (Table 4).

Hand-grip strength and free-living physical activity: No significant changes were observed in the hand-grip strength within or between groups post interventions (T×I, P=0.24) (Table 2). Across the interventions, no changes in the amount of time spent within the different measurable activity thresholds were identified (Table E3 in the ESM 1).

Salivary Strips: There was a significant increase in mean changes in salivary NO_2^- concentrations between the intervention groups: N (Δ=190.0±40.8 μmol/L, P=0.004), N+VC (387.5±99.6 μmol/L, P=0.001) (Table 5). Comparisons between the intervention groups showed that salivary NO^-_2 concentrations were significantly higher at the endpoint than at baseline (T×I, P=0.014). The Spearman's correlation analysis revealed a strong correlation between salivary NO_3^- and salivary NO_2^- (ρ=0.63, p<0.001), and a moderate correlation between salivary strip NO_2^- and salivary NO_3^- (ρ=0.36, p<0.004).

Nitrate, nitrite, vitamin C and 8-isoprostanes concentrations: Significant differences were observed in the mean changes in salivary NO_3^- and NO_2^- concentrations, respectively, within both NO_3^- intervention groups: N (NO_3^-, Δ=10.7±6.4 μmol/L, P=<0.001; NO_2^-, Δ=1.7±1.2 μmol/L, P=0.002), N+VC (NO_3^-, Δ=8.7±10.4 μmol/L, P=0.026; NO_2^-, Δ=1.2±1.0 μmol/L, P=0.005) (Table 5). For all the intervention groups, endpoint saliva NO_3^- concentrations were increased compared to baseline (T×I, P=0.007) (Table 5). Also, endpoint saliva NO_2^- concentrations were elevated compared to baseline (T×I, P=0.009). Changes in urinary NO_3^- concentrations were higher in the N+VC (Δ=4.2±3.0 μmol/L, P=0.002) and N intervention (Δ=5.1±4.8 μmol/L, P=0.009) cohorts more than ND group (Table 5). Within intervention group, comparisons demonstrated that urine vitamin C concentrations increased significantly over time following 3 days of N +VC administration (Δ=821.6±416.7 mg/dL, p<0.001) (Table 5). There was also a significant difference in urine vitamin C change between the interventions (T×I, P<0.001). No significant differences in urinary 8-isoprostanes were observed.

Table 4. Changes is cardiovascular parameters measured by Finapres at rest and during isometric muscle contraction following a 3-day supplementation with nitrate-rich beetroot juice alone (N), nitrate-depleted beetroot juice (ND) and nitrate-rich beetroot juice plus vitamin C (N+VC)

Time point	N	ND	N+VC	Comparison P
Resting systolic BP (mmHg)				
Baseline	138.40±33.18	138.4±10.4	125.7±9.8	T=0.52
Endpoint	136.60±25.21	136.6±7.9	125.7±5.9	I=0.62
Δ	−1.8±33.1	8.4±14.7	0.0±21.5	T×I=0.61
P*	0.86	0.10	1.00	
Resting diastolic BP (mmHg)				
Baseline	71.50±20.79	71.5±6.5	59.5±5.1	T=0.17
Endpoint	68.80±15.41	68.8±4.8	58.9±3.9	I=0.75
Δ	−2.7±19.4	5.9±13.0	−0.6±11.4	T×I=0.42
P*	0.67	0.18	0.87	
Resting HR (beats/min)				
Baseline	66.80±7.56	66.8±2.3	68.6±3.1	T=0.98
Endpoint	70.70±13.54	70.7±4.2	70.6±4.0	I=0.09
Δ	10.4±28.9	2.6±9.1	2.0±6.7	T×I=0.89
P*	0.27	0.39	0.37	
Resting SV (ml)				
Baseline	80.90±18.75	80.9±5.9	86.9±4.8	T=0.78
Endpoint	85.90±18.95	85.9±5.9	88.2±5.6	I=0.84
Δ	5.0±15.9	−4.8±12.5	1.3±12.3	T×I=0.28
P*	0.43	0.25	0.74	
Resting CO (ml/min)				
Baseline	5.65±1.27	5.6±0.4	5.8±0.2	T=0.74
Endpoint	5.90±.78	5.9±0.2	6.2±0.4	G=0.50
Δ	0.2±1.1	−0.1±0.8	0.3±1.3	T×G=0.51
P*	0.51	0.49	0.46	
Resting TPR (mm Hg·min/L)				
Baseline	1.11±0.72	1.1±0.2	0.8±0.06	T=0.28
Endpoint	1.34±0.54	1.3±0.1	1.7±0.5	I=0.77
Δ	−0.1±0.60	0.1±0.2	−0.0±0.2	T×I=0.36
P*	0.43	0.15	0.92	
HG systolic BP (mmHg)				
Baseline	120.70±18.2	120.7±5.7	115.7±9.6	T=0.49
Endpoint	118.50±25.5	118.5±8.0	106.5±5.1	I=0.89
Δ	−2.2±24.8	10.0±13.2	−9.2±17.2	T×I=0.09
P*	0.78	0.04*	0.12	
HG diastolic BP (mmHg)				
Baseline	75.30±14.4	75.3±4.5	66.8±4.0	T=0.15
Endpoint	74.70±14.0	74.7±4.4	63.9±2.2	I=0.67
Δ	−0.6±14.2	6.6±14.4	−2.9±11.0	T×I=0.26
P*	0.89	0.18	0.42	
HG HR (beats/min)				
Baseline	67.50±8.3	67.5±2.6	69.6±3.8	T=0.98
Endpoint	73.70±13.7	73.7±4.3	70.1±3.5	I=0.09
Δ	6.2±10.6	2.2±10.3	0.5±6.3	T×I=0.38
P*	0.09	0.52	0.80	

(Continued on next page)

Table 4. (Continued)

Time point	N	ND	N+VC	Comparison P
HG SV (ml)				
Baseline	70.30±20.9	70.3±6.6	75.3±7.7	T=0.34
Endpoint	63.70±19.7	63.7±6.2	71.3±5.9	I=0.52
Δ	−6.6±21.2	3.0±17.4	−4.0±24.6	T×I=0.58
P*	0.35	0.59	0.62	
HG CO (ml/min)				
Baseline	4.88±1.6	4.8±0.5	4.9±0.5	T=0.46
Endpoint	4.58±1.2	4.5±0.4	5.0±0.4	I=0.96
Δ	−0.3±1.5	0.3±1.4	0.02±1.8	T×I=0.69
P*	0.56	0.49	0.96	
HG TPR (mm Hg·min/L)				
Baseline	1.46±1.04	1.4±0.3	1.1±0.1	T=0.39
Endpoint	1.34±.54	1.3±0.1	1.7±0.5	I=0.30
Δ	−0.1±.9	16.9±53.2	0.5±1.9	T×I=0.38
P*	0.69	0.33	0.36	
Recovery systolic BP (mmHg)				
Baseline	130.60±23.29	130.6±7.3	120.3±7.9	T=0.32
Endpoint	131.40±28.26	131.4±8.9	114.5±5.0	I=1.0
Δ	0.8±25.4	5.0±10.7	−5.8±18.9	T×I=0.46
P*	0.92	0.17	0.35	
Recovery diastolic BP (mmHg)				
Baseline	69.80±14.19	69.8±4.4	60.1±4.1	T=0.06
Endpoint	70.30±15.07	70.3±4.7	65.1±3.0	I=0.98
Δ	0.5±10.6	3.4±10.9	−4.0±12.0	T×I=0.34
P*	0.88	0.35	0.32	
Recovery HR (beats/min)				
Baseline	67.70±8.53	67.7±2.7	66.5±3.1	T=0.93
Endpoint	71.00±12.61	71.0±3.9	71.0±2.9	I=0.05
Δ	3.3±10.2	3.6±8.9	2.5±3.8	T×I=0.95
P*	0.33	0.23	0.07	
Recovery SV (ml)				
Baseline	84.40±14.37	84.4±4.5	84.4±5.3	T=0.71
Endpoint	80.40±17.34	80.4±5.4	80.4±4.8	I=0.19
Δ	−4.0±12.1	−1.5±17.7	−5.2±13.3	T×I=0.84
P*	0.32	0.69	0.24	
Recovery CO (ml/min)				
Baseline	5.73±0.99	5.7±0.3	5.7±0.2	T=0.67
Endpoint	5.49±67	5.4±0.2	5.4±0.3	I=0.75
Δ	−0.2±0.8	0.1±1.02	−0.0±1.0	T×I=0.74
P*	0.39	0.76	0.90	
Recovery TPR (mm Hg·min/L)				
Baseline	1.01±0.44	1.0±0.1	1.0±0.05	T=0.15
Endpoint	1.01±0.29	1.0±0.09	1.0±0.06	I=0.93
Δ	−0.03±0.3	0.01±0.2	−0.0±0.2	T×I=0.93
P*	0.97	0.79	0.72	

Notes. N: nitrate alone; ND: nitrate depleted; N+VC: nitrate with vitamin C; BP: blood pressure; HR: heart rate; HGS, hand grips strength; Δ: difference, the mean difference between baseline and endpoint within intervention group; MAP: mean arterial pressure; SV: stroke volume; CO: cardiac output; TPR: total peripheral resistance. Values are reported as means±SEM. Paired t-tests were used to compare baseline and 3-day measurement in each intervention, (P* value $\leq$0.05). Repeated-measure ANOVA was used to evaluate differences in responses to the interventions. Time (baseline and end values, T) was entered as a within-subject factor and intervention (I) groups as a between-subject factor and the interaction between the two terms was derived (T×I).

Table 5. Changes in nitrate, nitrite, vitamin C, and 8-isoprostanes concentrations following a 3-day of supplementation with nitrate-rich beetroot juice alone (N), nitrate-depleted beetroot juice (ND) and nitrate-rich beetroot juice plus vitamin C (N+VC)

Time point	N	ND	N+VC	Comparison P
Saliva NO_2^- (μmol/L)				
Baseline	0.32±0.05*	0.34±0.05	0.45±0.10*	T=0.08
Endpoint	2.08±0.43*	0.83±0.29	1.6±0.36*	I=<0.001
Δ	1.7±1.2	0.46±0.9	1.2±1.0	T×I=0.04
P*	0.002*	0.14	0.005*	
Saliva NO_3^- (μmol/L)				
Baseline	1.33±0.36*	1.50±0.24	3.6±1.6*	T=0.002
Endpoint	12.04±2.0*	3.57±1.45	12.3±2.3*	I=<0.001
Δ	10.7±6.4	2.0±4.7	8.7±10.4	T×I=0.04
P*	<0.001*	0.203	0.02*	
Urine NO_3^- (μmol/L)				
Baseline	0.23±2.06*	0.36±0.07	0.59±0.19*	T=0.008
Endpoint	5.3±1.5*	0.32±0.07	4.8±1.0*	I=<0.001
Δ	5.1±4.8	−0.03±0.27	4.2±3.0	T×I=0.004
P*	0.009*	0.715	0.002*	
Urine Vitamin C# (mg/dL)				
Baseline	137.1±53.9	196.7±98.7	108±42.2*	T=<0.001
Endpoint	79.0±13.8	206.3±63.2	929.8±132.0*	I=<0.001
Δ	−58.1±143.9	9.6±403.7	821.6±416.7	T×I=<0.001
P*	0.23	0.94	<0.001*	
8-isoprostanes (pg/ml)				
Baseline	876.63±182.0	856.4±168.9	887.8±195.3	T=0.42
Endpoint	814.24±194.0	900.4±284.9	601.5±112.0	I=0.83
Δ	−62.3±159	19.4±306.4	−286.3±146.7	T×I=0.55
P*	0.70	0.88	−70	
Saliva NO_2^- by strip (μmol/L)				
Baseline	29.0±9.0*	38.0±12.0	85.0±20.6*	T=<0.001
Endpoint	416.5±103.6*	137.5±40.3	275.0±47.9*	I=0.05
Δ	387.5± 99.6	99.5±37.9	190.0±40.8	T×I=0.01
P*	0.004*	0.028*	0.004*	

Notes: N: nitrate alone; ND: nitrate depleted; N+VC: nitrate with vitamin C; NO_2^- : nitrite; NO_3^-: nitrate. Values are reported as means±SEM. paired t-tests were used to compare baseline and 3-day measurement in each intervention, (P* value ≤0.05). Repeated-measure ANOVA was used to evaluate differences in responses to the interventions. Time (baseline and end values, T) was entered as a within-subject factor and intervention (I) groups as a between-subject factor and the interaction between the two terms was derived (T×I). #Urinary vitamin C concentrations were log-transformed for the conduction of the repeated measure ANOVA. However, raw values are showed in the table for an easier interpretation of the results.

Discussion

The results of this pilot study indicate that the potential synergy between NO_3^- and vitamin C had no effect on participants' resting BP. Interestingly, this synergy had a significant effect on lowering the daily systolic BP within the combined NO_3^- and vitamin C intervention. The protocol results failed to achieve any significant results in relation to muscular strength. Within the N+VC group, there was a significant increase in urine vitamin C concentrations, salivary NO_2^- concentration but no changes in urinary 8-isoprostanes.

An important finding of this study was that there was a beneficial interaction between inorganic NO_3^- and vitamin C supplementation on daily systolic BP, i.e. the combination of agents resulted in a −6.6 mmHg reduction (P=0.04) but this was only seed in the within-group analyses. There was also a trend towards a gradual decrease in diastolic BP (−1.63±2.2 mmHg, P=0.24). Plausible mechanisms for this interaction could reflect: (i) enhanced conversion of NO_2^- to NO in the presence of supplemental vitamin C and the extra NO resulting in a lower BP; and (ii) a decrease in oxidative stress given a non-significant reduction in urinary 8-isoprostane concentrations noted in the N+VC group. 8-isoprostanes are prostaglandin-like compounds and are commonly used as biomarkers of oxidative stress. Increased levels have been associated with a greater risk of cardiovascular and neurodegenerative diseases in animal and human experiments [23, 24].

In the present investigation, there was no evidence that co-supplementation with inorganic NO_3^- and vitamin C

altered resting BP (systolic, P=0.55; diastolic, P=0.47). This result was considered unexpected in view of previous studies and meta-analyses, which demonstrated a decrease in BP with NO_3^- [14, 25, 26, 27] and vitamin C supplementations [6]. Ashor et al. [14] observed that the BPs of participants were reduced following inorganic NO_3^- supplementation in an inverse relationship to their baseline BP. It is, therefore, possible that the effect of NO_3^- supplementation on lowering BP was diminished in the current study's participants due to their relatively low, normotensive BP prior to supplementation. Additionally, nearly half of the studies included in the meta-analysis publishedby Juraschek et al. [6] supplemented vitamin C with other micronutrients or pharmacological agents; in this study, subjects received inorganic NO_3^- supplementation alone.

The results from this protocol, which was aimed towards the identification of any benefits in muscular performance across the differing supplementation interventions, did not achieve any levels of significance. The theory behind muscular activation through inorganic NO_3^- supplementation is that inorganic NO_3^- has the ability to promote pathways leading to the production of NO. NO is a signalling molecule that induces smooth muscle relaxation and takes part in processes that could enhance muscle contraction [28], although it has been proven in a systematic review that NO_3^- supplementation seems to improve muscle efficiency [29]. Only a slight change was noticed within interventions, regardless of the intervention type (N, Δ=−0.80 kg; ND, Δ=−1.9 kg; N+VC, Δ=−0.04 kg). In a recent systematic review [29], 2 out of 12 studies used hand-grip strength to assess muscular strength [30, 31]. In both studies, the participants were different from the current study's subjects. Gustavo Oliveira et al. studied athletes; Sim et al. investigated an elderly cohort. It therefore appears that the beneficial effects of dietary NO_3^- supplementation on forearm muscle strength have been observed in physically active subjects [32] and in older adults [33].

In this study, salivary NO_2^- was assessed using a salivary strip kit and a significant increase in salivary NO_2^- concentration with the N+VC intervention was observed. This is in keeping with previous studies suggesting that vitamin C acts as a potent reducing agent to support NO_2^- reduction from the exogenous NO pathway [34, 35]. Urinary concentrations of vitamin C increased significantly after the N+VC intervention.

While our study cohort comprised individuals without clinically diagnosed hypertension, the potential implications of even minor BP reductions in this population are noteworthy. These include the prevention of future hypertension, and the recognition of lifestyle interventions as valuable tools for promoting cardiovascular health at an early stage of adulthood.

Key limitations of this study include its small sample size and the short duration of the interventions. Additionally, home BP was self-measured, which could affect the accuracy of the results. While efforts were made to provide participants with clear instructions and guidance, the absence of direct supervision during home BP monitoring sessions could have resulted in variations in measurement techniques. Furthermore, the study exclusively focused on young adults of normal weight. Consequently, the generalizability of the findings to other populations may be limited. Another limitation pertains to the potential variability in the vitamin C concentration of the beverages used during the intervention. Although we assessed the vitamin C content of the concentrated lemon juice (BRJ) brand as reported in the article, it should be noted that actual vitamin C content may exhibit variations between different batches or sources, which could introduce variability into the study results. Additionally, we did not employ a placebo for the vitamin C tablets administered to the participants. The absence of a placebo to blind participants regarding the vitamin C tablets may have introduced the possibility of participant bias. Future studies may consider incorporating a placebo to address this potential source of bias.

Conclusions

In this randomized controlled trial, resting systolic and diastolic BP values did not significantly differ among the three study groups post interventions. The within-group analyses showed that the 3-day co-supplementation of dietary NO_3^- and vitamin C significantly decreased home daily systolic BP. The protocol used in this study did not produce any significant change in muscular strength. The combination of vitamin C with dietary NO_3^- could be a promising strategy to improve vascular health. Further investigation using a more robust study design is necessary to confirm these preliminary results.

Electronic supplementary material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000799

ESM 1. Normality test for baseline and endpoint variables of resting SBP, DBP, HR, HGS, saliva NO , NO and urine NO , Vitamin C (Table E1); Normality test for baseline Finapres variables (Table E2); Accelerometer measurements of free living physical activity following 3-day supplementation with N, ND, and N+VC Interventions (Table E3); Flow chart showing recruitment, allocation to treatment and compliance to study (Figure E1).

References

1. Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am J Clin Nutr. 2009;90(1):1–10.
2. Bailey JC, Feelisch M, Horowitz JD, Frenneaux MP, Madhani M. Pharmacology and therapeutic role of inorganic nitrite and nitrate in vasodilatation. Pharmacol Ther. 2014;144(3):303–20.
3. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab. 2011;13(2):149–59.
4. Siervo M, Oggioni C, Jakovljevic DG, Trenell M, Mathers JC, Houghton D, et al. Dietary nitrate does not affect physical activity or outcomes in healthy older adults in a randomized, cross-over trial. Nutr Res. 2016;36(12):1361–9.
5. May JM, Harrison FE. Role of vitamin C in the function of the vascular endothelium. Antioxid Redox Signal. 2013;19(17):2068–83.
6. Juraschek SP, Guallar E, Appel LJ, Miller ER 3rd. Effects of vitamin C supplementation on blood pressure: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2012;95(5):1079–88.
7. Ashor AW, Siervo M, Lara J, Oggioni C, Afshar S, Mathers JC. Effect of vitamin C and vitamin E supplementation on endothelial function: a systematic review and meta-analysis of randomised controlled trials. Br J Nutr. 2015;113(8):1182–94.
8. Chen SJ, Allam JP, Duan YG, Haidl G. Influence of reactive oxygen species on human sperm functions and fertilizing capacity including therapeutical approaches. Arch Gynecol Obstet. 2013;288(1):191–9.
9. Caliri AW, Tommasi S, Besaratinia A. Relationships among smoking, oxidative stress, inflammation, macromolecular damage, and cancer. Mutat Res Rev Mutat Res. 2021;787.
10. Santilli F, D'Ardes D, Davì G. Oxidative stress in chronic vascular disease: from prediction to prevention. Vascul Pharmacol. 2015;74:23–37.
11. Siervo M, Lara J, Ogbonmwan I, Mathers JC. Inorganic nitrate and beetroot juice supplementation reduces blood pressure in adults: a systematic review and meta-analysis. J Nutr. 2013;143(6):818–26.
12. Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, et al. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2008;88(1):38–50.
13. Ashor AW, Lara J, Mathers JC, Siervo M. Effect of vitamin C on endothelial function in health and disease: a systematic review and meta-analysis of randomised controlled trials. Atherosclerosis. 2014;235(1):9–20.
14. Ashor AW, Shannon OM, Werner AD, Scialo F, Gilliard CN, Cassel KS, et al. Effects of inorganic nitrate and vitamin C co-supplementation on blood pressure and vascular function in younger and older healthy adults: a randomised double-blind crossover trial. Clin Nutr. 2020;39(3):708–17.
15. Kelm M. Nitric oxide metabolism and breakdown. Biochim Biophys Acta. 1999;1411(2–3):273–89.
16. Lykkesfeldt J, Tveden-Nyborg P. The pharmacokinetics of vitamin C. Nutrients. 2019;11(10):2412.
17. Rowlands AV, Olds TS, Hillsdon M, Pulsford R, Hurst TL, Eston RG, et al. Assessing sedentary behavior with the GENEActiv: introducing the sedentary sphere. Med Sci Sports Exerc. 2014;46(6):1235–47.
18. Craig CL, Marshall AL, Sjöström M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity questionnaire: 12-country reliability and validity. Med Sci Sports Exerc. 2003;35(8):1381–95.
19. 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(3):e004503.
20. 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. J Appl Physiol. 2010;109(1):135–48.
21. 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. Am J Physiol Regul Integr Comp Physiol. 2010;299(4):R1121–31.
22. Whitehead AL, Julious SA, Cooper CL, Campbell MJ. Estimating the sample size for a pilot randomised trial to minimise the overall trial sample size for the external pilot and main trial for a continuous outcome variable. Stat Methods Med Res. 2016;25(3):1057–73.
23. Ho E, Karimi Galougahi K, Liu CC, Bhindi R, Figtree GA. Biological markers of oxidative stress: applications to cardiovascular research and practice. Redox Biol. 2013;1(1):483–91.
24. Van't Erve TJ, Lih FB, Jelsema C, Deterding LJ, Eling TE, Mason RP, et al. Reinterpreting the best biomarker of oxidative stress: the 8-iso-prostaglandin F2α/prostaglandin F2α ratio shows complex origins of lipid peroxidation biomarkers in animal models. Free Radic Biol Med. 2016;95: 65–73.
25. Gee LC, Ahluwalia A. Dietary nitrate lowers blood pressure: epidemiological, pre-clinical experimental and clinical trial evidence. Curr Hypertens Rep. 2016;18(2):17.
26. Carlström M, Persson AE, Larsson E, Hezel M, Scheffer PG, Teerlink T, et al. Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension. Cardiovasc Res. 2011;89(3):574–85.
27. Kemmner S, Lorenz G, Wobst J, Kessler T, Wen M, Günthner R, et al. Dietary nitrate load lowers blood pressure and renal resistive index in patients with chronic kidney disease: a pilot study. Nitric Oxide. 2017;64:7–15.
28. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993;329(27):2002–12.
29. Anderson OK, Martinez-Ferran M, Lorenzo-Calvo J, Jiménez SL, Pareja-Galeano H. Effects of nitrate supplementation on muscle strength and mass: a systematic review. J Strength Cond Res. 2022;36(12):3562–70.
30. de Oliveira GV, do Nascimento LAD, Volino-Souza M, do Couto Vellozo O, Alvares TS. A single oral dose of beetroot-based gel does not improve muscle oxygenation parameters, but speeds up handgrip isometric strength recovery in recreational combat sports athletes. Biol Sport. 2020;37(1):93–9.
31. Sim M, Lewis JR, Blekkenhorst LC, Bondonno CP, Devine A, Zhu K, et al. Dietary nitrate intake is associated with muscle function in older women. J Cachexia Sarcopenia Muscle. 2019;10(3):601–10.
32. Papadopoulos S, Dipla K, Triantafyllou A, Nikolaidis MG, Kyparos A, Touplikioti P, et al. Beetroot increases muscle performance and oxygenation during sustained isometric exercise, but does not alter muscle oxidative efficiency and microvascular reactivity at rest. J Am Coll Nutr. 2018;37(5):361–72.

33. de Oliveira GV, Morgado M, Conte-Junior CA, Alvares TS, Acute effect of dietary nitrate on forearm muscle oxygenation, blood volume and strength in older adults: A randomized clinical trial. PLoS One. 2017;12(11):e0188893.
34. Kim-Shapiro DB, Gladwin MT. Mechanisms of nitrite bioactivation. Nitric Oxide. 2014;38:58–68.
35. North BJ, Sinclair DA. The intersection between aging and cardiovascular disease. Circ Res. 2012;110(8):1097–108.

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Conflict of interest
The authors declare that there are no conflicts of interest.

Authors' contribution
MS and EL designed the study. EL and MS and drafted the manuscript, performed the laboratory analysis and analysed the data. MS and II supervised the project. EL, AM and JR conducted the study. All authors contributed to discussion and reviewed/edited the manuscript. The corresponding author (MS) is the guarantor for the manuscript and 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. All authors read and approved the final version of the paper.

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