Articles in PresS. Am J Physiol Heart Circ Physiol (May 5, 2017). doi:10.1152/ajpheart.00163.2017

Acute beetroot juice supplementation on sympathetic nerve activity: A randomized, double-blind, placebo-controlled proof-of-concept study

Karambir Notay[1], Anthony V. Incognito[1], Philip J. Millar[1,2]

[1] Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

[2] Toronto General Research Institute, Toronto General Hospital, Toronto, Ontario, Canada

Running Title: Acute dietary nitrate on sympathetic activity

Word Count of Manuscript: 4995

Word Count of Abstract: 250

Address for Correspondence and requests for reprints:

Philip J. Millar, PhD

ANNU 348A, 50 Stone Road East, Guelph, Ontario, Canada, N1G2W1

Telephone: 519-824-4120 x54818

Email: pmillar@uoguelph.ca

Abstract

Acute dietary nitrate supplementation reduces resting blood pressure in healthy normotensives. This response is attributed to increased nitric oxide bioavailability and peripheral vasodilation, though nitric oxide also tonically inhibits central sympathetic outflow. We hypothesized that acute dietary nitrate (NO_3^-) supplementation using beetroot (BR) juice would reduce blood pressure and muscle sympathetic nerve activity (MSNA) at rest and during exercise. Fourteen participants (7 men, 25±10 years) underwent blood pressure and MSNA measurements before and after (165-180 minutes) ingestion of 70ml high-nitrate (~6.4mmol NO_3^-) BR or nitrate-depleted BR placebo (PL; ~0.0055mmol NO_3^-) in a double-blind, randomized, crossover design. Blood pressure and MSNA were also collected during 2 minutes of static handgrip (30% maximal voluntary contraction). The changes in resting MSNA burst frequency (-3±5 vs. 3±4 bursts/min, p=0.001) and burst incidence (-4±7 vs. 4±5 bursts/100 heartbeats, p=0.002) were lower following BR vs. PL; whereas systolic (-1±5 vs. 2±5 mmHg, p=0.30) and diastolic blood pressure (4±5 vs. 5±7 mmHg, p=0.68), and spontaneous arterial sympathetic baroreflex sensitivity (p=0.95) were not different. During static handgrip, the change in MSNA burst incidence (1±8 vs. 8±9 bursts/100 heartbeats, p=0.04) was lower following BR vs. PL, while MSNA burst frequency (6±6 vs. 11±10 bursts/min, p=0.11), and systolic (11±7 vs. 12±8 mmHg, p=0.94) and diastolic (11±4 vs. 11±4 mmHg, p=0.60) blood pressure were not different. Collectively, these data provide proof-of-principle that acute BR supplementation can decrease central sympathetic outflow at rest and during exercise. Dietary nitrate supplementation may represent a novel intervention to target exaggerated sympathetic outflow in clinical populations.

Key Words: Sympathetic nervous system; Dietary nitrate; Blood pressure; Muscle sympathetic nerve activity; Exercise

New and Noteworthy

The hemodynamic benefits of dietary nitrate supplementation are attributed to nitric oxide-mediated peripheral vasodilation. We provide proof-of-concept that acute dietary nitrate supplementation using beetroot juice can decrease muscle sympathetic outflow at rest and during exercise in a normotensive population. These results have applications for targeting central sympathetic over-activation in disease.

Introduction

Acute and chronic dietary nitrate (NO_3^-) supplementation has been demonstrated to elicit dose-dependent reductions in resting blood pressure in normotensive and hypertensive populations in prospective studies (e.g. 13, 21, 22, 25, 47) and meta-analyses (2, 44). These results highlight the potential therapeutic benefit of targeting the nitrate-nitrite-nitric oxide pathway to increase the bioavailability of the potent vasodilator, nitric oxide (NO) (27, 28, 47). In contrast to conventional NO synthesis through the oxidation of L-arginine by nitric oxide synthase (38), this recently discovered pathway involves conversion of nitrate and nitrite back to bioactive NO without the need for oxygen. Nitrate and nitrite derive from dietary nitrate ingestion and as metabolic byproducts of NO oxidation (27, 28, 47). Following consumption and absorption through the gastrointestinal tract, ~25% of ingested dietary nitrate is taken up by the enterosalivary circulation where it is reduced to nitrite by facultative anaerobic oral bacteria (27, 28, 47). Swallowed nitrite can be spontaneously decomposed to produce NO and other bioactive nitrogen oxides in the acidic environment of the stomach, however a large proportion is again absorbed through the gastrointestinal tract, entering the circulation where it can be reduced to NO by endogenous nitrite reductases (e.g. deoxyhemoglobin, deoxymyoglobin, molybdenum-containing enzymes) during physiological and pathological hypoxic conditions (27, 28, 47). As a result, nitrate and nitrite are now considered to represent important reservoirs for NO production (1, 14).

To date, the most common method of administering dietary nitrate has involved supplementation with beetroot (BR) juice (2, 13, 32, 44). In young normotensives, acute ingestion of 250 ml (~5.5 mmol) of BR juice decreased resting systolic blood pressure (Δ~5 mmHg) (22), while supplementation with 500 ml (~22.5 mmol) of BR juice was reported to

elicit larger reductions (Δ~10/8 mmHg) (47). Importantly, the peak hypotensive responses in both studies occurred ~3 hours following ingestion, aligned with the time course of peak plasma nitrite concentration (22, 47). Similar findings have been reported following daily BR supplementation for 4 weeks in hypertensives without evidence of tachyphylaxis (21). The mechanisms responsible for these hypotensive effects have been attributed to the peripheral vasodilatory actions of increased NO bioavailability (7, 47), as highlighted by an increase in endothelium-dependent vasodilation (47). However, in addition to its role as a vasodilator, NO exerts a tonic inhibitory influence on the central regulation of sympathetic outflow (41, 49). The sympathetic nervous system is known similarly to impact blood pressure and endothelium-dependent vasodilation (18, 19), however, whether the beneficial effects of dietary nitrate supplementation are mediated, at least in part, via a neural mechanism has not been studied.

Therefore, the purpose of the present investigation was to provide proof-of-concept that acute dietary nitrate supplementation using BR can modulate central sympathetic outflow. We hypothesized that compared to a nitrate-depleted BR placebo (PL), acute consumption of high-nitrate BR juice would reduce resting blood pressure and muscle sympathetic nerve activity (MSNA). To determine whether changes in MSNA were mediated by peripheral or central mechanisms we also examined spontaneous arterial sympathetic baroreflex sensitivity. Additionally, as acute nitrate supplementation can improve human exercise performance (32), we sought to determine whether the neural effects of BR were present during sympathoexcitation elicited by static handgrip exercise.

Methods

Participants

Twenty healthy men and women (10 men, 27 ± 11 years) were recruited to participate in the study after providing informed written consent. All participants were normotensive, non-smoking, in sinus rhythm, free of known cardiovascular or metabolic disease, and not taking any acute (<3 months) or chronic medications, including birth control or antibiotics. All participants self-reported not currently or previously engaging in formal nitrate supplementation. Due to the requirement of oral bacteria to convert dietary nitrate to nitrite (15, 47), participants were required to abstain from using antibacterial mouthwash 30 days prior to the first visit and for the duration of the study. Participants were reminded verbally throughout the study to ensure compliance. All procedures were approved by the University of Guelph Research Ethics Board.

Study Design and Experimental Protocol

Participants completed a double-blind, randomized, placebo-controlled crossover trial comparing the effects of 70 ml of high-NO_3^- (~6.4 mmol) BR or NO_3^--depleted (~0.0055 mmol) PL (James White Drinks Ltd, Suffolk, UK). Prior to testing visits, all participants completed a familiarization visit to the laboratory (~1 hour) where they underwent detailed verbal and visual explanations of the study requirements and protocols (e.g. microneurography), and attempted a practice static handgrip contraction. After this, participants were randomized (1:1) to start (on a subsequent visit) either BR or PL using a random sequence generator (Random.org) followed by crossover after a >30 day washout. The 70 ml dose of high-nitrate BR (~6.4 mmol) juice is considered to be equivalent to ~250 ml (~5.5-6.4 mmol) of regular BR juice (21, 22), while the PL version is manufactured specifically for research purposes, as described (24), and is

indistinguishable in physical appearance and taste. The independent Human Nutraceutical Research Unit at the University of Guelph maintained study blinding and controlled the dispensing of BR and PL. The experimental protocol was completed during two identical study visits conducted at the same time of day (±2 hours) and separated by at least one month. Diet was not restricted during the course of the study, however, each participant was instructed to refrain from making any major changes during the course of the study. Prior to each testing visit participants were asked to abstain from caffeine, alcohol, and strenuous exercise for 24 hours.

During each testing visit participants entered the laboratory following voiding, underwent anthropometric measurements, and were positioned supine on a comfortable bed for the remainder of the study. Next, participants were asked to perform two handgrip contractions in their left (non-dominant) hand to establish maximal volitional contraction (MVC) (Lafayette Instrument, Lafayette, LA). Each contraction lasted ~3 seconds and was separated by 30 seconds of rest and the highest value was taken as MVC. Participants then underwent instrumentation (~1 hour) and a 10 minute acclimatization period before continuous heart rate, blood pressure, MSNA, as well as discrete minute-to-minute brachial blood pressure data were collected over a 10 minute baseline. Upon completion, participants consumed either BR or PL and remained in the supine position for three hours. Continuous heart rate, blood pressure, and MSNA, along with five discrete measurements of brachial blood pressure were sampled 165-180 minutes following supplementation. This time period was chosen to align with peak hypotensive and plasma nitrite responses reported previously (13, 22, 46, 47). Immediately following the three hour rest protocol, participants underwent continuous measurements of heart rate, blood pressure, and MSNA during a 3 minute resting baseline and 2 minute static handgrip contraction at 30% MVC.

Measurements

Electrocardiography (Lead II) was used to acquire beat-to-beat heart rate (ADInstruments Pty Ltd, Australia). Respiratory movements were tracked to ensure spontaneous breathing patterns using a piezoelectric transducer placed around the abdomen (Pneumotrace II, UFA, Morro Bay, CA). To obtain accurate recordings of blood pressure, discrete left brachial blood pressure was recorded using an automated sphygmomanometer (BPTru Medical Devices, Coquitlam, Canada). Continuous beat-to-beat blood pressure was recorded from the right middle finger using photoelectric plethysmography (Finometer MIDI, Finapres Inc, Netherlands).

Postganglionic multi-unit MSNA was measured from the right fibular nerve by microneurography, as described previously (35, 40). A low-impedance tungsten microelectrode (2mΩ; Frederick Haer, Brunswick, ME) was inserted percutaneously into a motor fascicle and adjusted until spontaneous multi-unit bursts of sympathetic activity were observed. Muscle sympathetic activity was confirmed by reflexive increases in response to a breath hold and the absence of responsiveness to unexpected clapping. The MSNA signal was amplified, band-pass filtered (0.7-2.0 kHz), rectified, and integrated to obtain the mean voltage multi-unit neurogram (Nerve Traffic Analyzer, Model 662C-4; Absolute Design and Manufacturing Services, Salon, IA). The neural signal was monitored both audibly and visually to identify changes in the recording site throughout the study protocol.

All continuous data was digitized and stored with LabChart (PowerLab, ADInstruments, Colorado Springs, CO). Heart rate, respiration, blood pressure, and the integrated multi-unit MSNA signal were recorded at a sampling frequency of 1000 Hz, while the raw MSNA signal was collected at 10,000 Hz.

Data Analysis

All data was analyzed and tabled by investigators blinded to intervention allocation. MSNA was analyzed using custom semi-automated LabView software (National Instruments, Austin, Texas, USA) (35, 40). MSNA was quantified as burst frequency (bursts/min) and burst incidence (bursts/100 heartbeats). Total MSNA was not calculated as a result of a singular site change occurring during 13 of the 28 study visits (BR, n=5; PL, n=8). However, MSNA burst occurrence exhibits excellent intra-day reproducibility within two or more time points on the same day (16), justifying the use of MSNA burst frequency and incidence. Time-domain calculation of spontaneous arterial sympathetic baroreflex sensitivity was completed by assessing the relationship between diastolic blood pressure (input) and MSNA burst occurrence (output), as described previously (17, 23). A weighted linear regression line was fit between the likelihood of a MSNA burst (incidence) within 2 mmHg bins of diastolic blood pressure for each participant. If the regression line possessed an r value ≥ 0.5, the slope of the line was taken as sympathetic baroreflex gain.

Statistical Analysis

This study was powered to detect a change in MSNA burst frequency (primary variable). An *a priori* sample size calculation estimated a required sample of 12 participants assuming a 20% reduction in MSNA burst frequency in a crossover trial with an assigned alpha of 0.05 and beta of 0.2. Resting baseline variables were compared between BR and PL (and visit 1 vs. visit 2) using two-tailed paired t-tests. Intra-class correlation coefficients were used to evaluate reliability of resting measures between BR and PL study visits. As recommended to improve precision and reduce bias in crossover (33) and randomized control trials (4), the effects of BR

on neural and hemodynamic variables were examined using an analysis of covariance (ANCOVA) with the change from baseline (Δ) as the dependent variable and the absolute baseline value as the covariate. This provides the same statistical result as using the absolute post-treatment value as the dependent variable. The ANCOVA method compares the effects of BR and PL on each outcome after correcting for differences in resting (pre-ingestion) or handgrip (post-ingestion) baseline values. As this method may be limited for testing our hypothesis during exercise, due to the fact that neural and hemodynamic responses could be influenced by changes at rest, we also compared the change from the handgrip baseline (Δ) during exercise for each variable using a two-tailed paired t-test. All analyses were performed using IBM SPSS Statistics 24 (Armonk, New York, USA) with significance defined as $P<0.05$. All values are presented as mean ± SD, unless otherwise stated.

Results

We recruited 20 participants between July 2015 and April 2016. Three participants were excluded due to the inability to secure a MSNA recording site during visit 1 and three participants withdrew from the study before completing visit 2. Complete high-quality microneurographic recordings were obtained in fourteen participants, though only 11 participants completed the static handgrip exercise protocol (3 dropouts due to time restrictions). Participant characteristics are reported in Table 1. Resting baseline heart rate, diastolic blood pressure, MVC, and MSNA were consistent between BR and PL visits (All $p>0.05$), while systolic blood pressure tended to be lower during the PL visit ($p=0.04$). The inter-test reliability was good-to-excellent ($r > 0.6$) for all resting baseline variables.

The changes in resting MSNA burst frequency (-3 ± 5 vs. 3 ± 4 bursts/min, $p=0.001$) and

burst incidence (-4 ± 7 vs. 4 ± 5 bursts/100 heartbeats, p=0.002) were lower following BR compared to PL (Figure 1). In contrast, the changes in resting systolic (-1 ± 5 vs. 2 ± 5 mmHg, p=0.30) and diastolic (4 ± 5 vs. 5 ± 7 mmHg, p=0.68) blood pressure (Figure 1), heart rate (0 ± 4 vs. -1 ± 4 bpm, p=0.70), and spontaneous arterial sympathetic baroreflex sensitivity (0.2 ± 1.4 vs. 0.2 ± 1.3 bursts/100 heartbeats/mmHg, p=0.95) were not different between BR and PL. No differences were detected (All p>0.05) for any of the parameters when measured during visit 1 or visit 2 (i.e. no order effects).

During static handgrip exercise (Figure 2), the changes in systolic (11 ± 7 vs. 12 ± 8 mmHg, p=0.94) and diastolic (11 ± 4 vs. 11 ± 4 mmHg, p=0.60) blood pressure, heart rate (13 ± 10 vs. 12 ± 12 bpm; p=0.75), and MSNA burst frequency (6 ± 6 vs. 11 ± 10 bursts/min, p=0.12) were similar following BR and PL. The change in MSNA burst incidence (1 ± 8 vs. 8 ± 9 bursts/100 heartbeats, p=0.04) was smaller following BR vs. PL. Secondary analyses without adjusting for baseline as a covariate also found no differences during static handgrip in the changes in systolic (p=0.77) and diastolic (p=0.58) blood pressure, and heart rate (p=0.72), while the changes in MSNA burst frequency (p=0.04) and burst incidence (p=0.01) were smaller following BR vs. PL.

Discussion

Supplementation with dietary nitrate has been shown to reduce resting blood pressure (2, 44), a response attributed to the vasodilator actions of increased NO bioavailability (7, 47). The present study is the first to investigate the effects of acute dietary nitrate supplementation on peripheral sympathetic outflow. In support of our hypothesis, BR resulted in lower resting MSNA and attenuated sympathetic responses during static handgrip in healthy young

normotensives. The changes in resting MSNA occurred independent of alterations in spontaneous arterial sympathetic baroreflex sensitivity suggesting a central mechanism of action. Surprisingly, no differences in blood pressure were detected at rest or during exercise. These results provide proof-of-concept that dietary nitrate supplementation can modulate central sympathetic outflow and suggest that the established cardiovascular benefits are likely to involve a neural contribution.

To our knowledge, only one study has examined the effects of dietary nitrate supplementation on the autonomic nervous system, demonstrating that acute BR consumption increased time-domain heart rate variability (5), a non-invasive marker of cardiac autonomic modulation. The effects of dietary nitrate supplementation on direct measures of peripheral sympathetic activity have not been studied. Our hypothesis for reductions in MSNA was based on evidence that pharmacological blockade of NO synthase (i.e. decreasing NO bioavailability) raises central sympathetic outflow independent of changes in arterial pressure (41, 49). Alternatively, increased NO may also influence sympathetic outflow by modulating peripheral artery compliance and arterial baroreflex transduction (36, 48). Given that the present study observed a ~17% difference in post-BR and PL resting MSNA independent of a change in spontaneous arterial sympathetic baroreflex sensitivity, our findings support a central sympathoinhibitory action of dietary nitrate. Prior research has demonstrated that exogenous nitrate or nitrite supplementation has the capacity to cross the blood-brain barrier and increase NO bioavailability in the central nervous system (20, 42), providing a potential mechanism for the observed reduction in MSNA. The capacity for BR to exert a central neural effect is important as many of the benefits of dietary nitrate supplementation on blood pressure (2, 44),

endothelium-dependent vasodilation (47), and exercise capacity (32), may be caused similarly by reductions in neurogenic vasoconstriction (18, 19, 39).

Supporting the biological plausibility that BR can exert central sympathoinhibition, analogous results (i.e. 12-30% reductions in MSNA burst incidence independent of changes in sympathetic baroreflex sensitivity or blood pressure) have been reported in clinical patients following lipophilic statin therapy (31, 34). A known pleiotropic benefit of statin therapy is the upregulation of endothelial NO synthase and increase in endothelium-dependent vasodilation (26). Work in animal models has established that the autonomic effects of statins are secondary to NO-mediated reductions in oxidative stress in the rostral ventrolateral medulla (12), the brainstem region considered the final central relay station to integrate sympathetic outflow which provides the major excitatory input to sympathetic pre-ganglionic neurons (8).

During exercise, acute BR supplementation led to smaller MSNA burst incidence responses during static handgrip than PL, while secondary analyses found significantly smaller responses in both MSNA burst frequency and burst incidence. The latter results suggest that a portion of the benefit BR supplementation has on sympathetic responses during exercise involves modulation of resting baseline values. Given that BR has been shown to produce ergogenic effects (32), lower MSNA could be the result of increased static handgrip time-to-failure. However, an increase in handgrip endurance would be expected to elicit parallel responses in blood pressure. Similar to our findings at rest, no differences in blood pressure responses were found during static handgrip between BR and PL. In young healthy individuals MSNA does not correlate with blood pressure at rest (19), and highlighting the complexity of integrative blood pressure regulation, discordance in muscle sympathetic outflow and blood pressure responses during static handgrip exercise have been reported previously in the literature (29, 37). It is

important to remember that microneurographic assessments of sympathetic outflow reflect central discharge, not the quantity of neurovascular transduction. It is also possible that reduced MSNA occurred in parallel with increased sympathetic outflow to other tissues (e.g. renal) to maintain systemic blood pressure responses. Nevertheless, reducing sympathetic vasoconstrictor responses directed at skeletal muscle may permit greater blood flow during exercise and improve exercise capacity (39).

The present results suggest a novel clinical application of dietary nitrate supplementation as a therapeutic strategy to target sympathetic over-activation characteristic of most cardiovascular disease states, including primary hypertension and heart failure (3, 9, 10, 35). Increased MSNA is known to be a significant predictor of mortality in patients with heart failure (3). Other pathological sequelae of increased norepinephrine include arrhythmogenesis, cardiac and vascular remodeling, insulin resistance, and sudden cardiac death (3, 30, 43, 50). Further, some current anti-hypertensive medications used frequently in these populations may actually increase central sympathetic outflow despite blood pressure reductions due to baroreflex unloading (11). Without addressing the cause of sympathetic over-activation at the source (i.e. central) the systemic clinical consequences are not abated. The observation that BR supplementation is not associated with tachyphylaxis, at least after one month (21), and can reduce central sympathetic outflow supports future autonomic studies in clinical populations.

We acknowledge several limitations in the present study. First, our study recruited a convenience sample composed of young healthy participants for determining proof-of-concept. Based on the knowledge that MSNA increases both with age (45) and many cardiovascular disease states (3, 9, 10, 35), we would hypothesize that BR supplementation would elicit a greater drop in sympathetic outflow in these populations. Second, prior batch analysis of the

commercial BR and PL products has reported mean nitrate and nitrite concentrations [range] for both BR (Nitrate: 3.5 mmol [2.6-4.4 mmol]; Nitrite: 0.1 mmol [0.05-0.16 mmol]) and PL (Nitrate: 0.34 mmol [0.05-0.56 mmol]; Nitrite: 0.006 mmol [0.001-0.1 mmol]) (6). The results confirm the difference between BR and PL but suggest that BR nitrate concentrations may be lower than reported by the manufacturer. This discrepancy could have contributed to the lack of change in resting blood pressure (in addition to low normotensive status of our participants), though hypotensive responses are not universal across the literature, even at higher nitrate doses (2, 44). Importantly, the present results suggest that the nitrate dose required to modulate blood pressure and MSNA may not be equivalent. Finally, we did not determine plasma nitrite levels in our study. The plateau in peak plasma nitrite (between 2-3 hours following consumption) has been characterized in prior studies with consistent findings (13, 21, 22, 25), though inter-individual differences in peak nitrite concentration may contribute to between-participant variability. Future studies are required to examine the dose-response relationships between changes in plasma nitrite and MSNA.

In summary, acute supplementation with beetroot juice decreased resting MSNA and attenuated muscle sympathetic activation during handgrip exercise. In concert with the findings of unaltered arterial sympathetic baroreflex sensitivity, these results provide proof-of-concept that dietary nitrate supplementation can cause central sympathoinhibition in a young healthy population. Reductions in sympathetic outflow are likely to contribute to the cardiovascular benefits of dietary nitrate supplementation, while offering a new autonomic restorative intervention to be tested in future clinical trials in patient groups associated with sympathetic over-activation (3, 9, 10, 35).

Sources of Funding

This research was supported by a Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant (P.J.M. #1256447-2015), the Canada Foundation for Innovation (P.J.M. #34379), and the Ontario Ministry of Research, Innovation, and Science (P.J.M.). A.V.I. was supported by a CIHR Fredrick Banting and Charles Best Canada Graduate Scholarship.

Disclosures

The authors declare no conflicts of interest relevant to the content of this study.

Author Contributions

P.J.M. conceived and designed research; K.N., A.V.I., and P.J.M. performed experiments; K.N., and A.V.I. analyzed data; K.N., A.V.I., and P.J.M. interpreted results; K.N. prepared figures and drafted manuscript; K.N., A.V.I., and P.J.M. edited and revised manuscript; K.N., A.V.I., and P.J.M. approved final version of manuscript.

References

1. Ahluwalia A, Gladwin M, Coleman GD, Hord N, Howard G, Kim□Shapiro DB, Lajous M, Larsen FJ, Lefer DJ, McClure LA, Nolan BT, Pluta R, Schechter A, Wang C-Y, Ward MH, Harman JL. Dietary Nitrate and the Epidemiology of Cardiovascular Disease: Report From a National Heart, Lung, and Blood Institute Workshop. *J Am Heart Assoc* 5: e003402, 2016.

2. Ashor AW, Lara J, Siervo M. Medium-term effects of dietary nitrate supplementation on systolic and diastolic blood pressure in adults: a systematic review and meta-analysis. *J. Hypertens.* (March 17, 2017). doi: 10.1097/HJH.0000000000001305.

3. Barretto ACP, Santos AC, Munhoz R, Rondon MUPB, Franco FG, Trombetta IC, Roveda F, de Matos LNJ, Braga AMW, Middlekauff HR, Negrão CE. Increased muscle sympathetic nerve activity predicts mortality in heart failure patients. *Int J Cardiol* 135: 302–307, 2009.

4. Bland JM, Altman DG. Best (but oft forgotten) practices: testing for treatment effects in randomized trials by separate analyses of changes from baseline in each group is a misleading approach. *Am J Clin Nutr* 102: 991–994, 2015.

5. Bond V, Curry BH, Adams RG, Asadi MS, Stancil KA, Millis RM, Haddad GE. Effects of Nitrate Supplementation on Cardiovascular and Autonomic Reactivity in African-American Females. *ISRN Physiology* 2014: e676235, 2014.

6. Bondonno CP, Liu AH, Croft KD, Ward NC, Shinde S, Moodley Y, Lundberg JO, Puddey IB, Woodman RJ, Hodgson JM. Absence of an effect of high nitrate intake from beetroot juice on blood pressure in treated hypertensive individuals: a randomized controlled trial. *Am J Clin Nutr* 102: 368–375, 2015.

7. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498–1505, 2003.

8. Dampney RA. The subretrofacial vasomotor nucleus: anatomical, chemical and pharmacological properties and role in cardiovascular regulation. *Prog Neurobiol* 42: 197–227, 1994.

9. Fisher JP, Young CN, Fadel PJ. Central Sympathetic Overactivity: Maladies and Mechanisms. *Auton Neurosci Basic Clin* 148: 5–15, 2009.

10. Floras JS, Hara K. Sympathoneural and haemodynamic characteristics of young subjects with mild essential hypertension. *J Hypertens* 11: 647–655, 1993.

11. Fu Q, Zhang R, Witkowski S, Arbab-Zadeh A, Prasad A, Okazaki K, Levine BD. Persistent Sympathetic Activation During Chronic Antihypertensive Therapy. *Hypertension* 45: 513–521, 2005.

12. Gao L, Wang W, Zucker IH. Simvastatin inhibits central sympathetic outflow in heart failure by a NOS mechanism. *J Pharmacol Exp Ther* 326: 278–285, 2008.

13. Gee LC, Ahluwalia A. Dietary Nitrate Lowers Blood Pressure: Epidemiological, Pre-clinical Experimental and Clinical Trial Evidence. *Curr Hypertens Rep* 18: 17, 2016.

14. Gladwin MT, Raat NJH, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol* 291: H2026-2035, 2006.

15. Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide Biol Chem* 19: 333–337, 2008.

16. Grassi G, Bolla G, Seravalle G, Turri C, Lanfranchi A, Mancia G. Comparison between reproducibility and sensitivity of muscle sympathetic nerve traffic and plasma noradrenaline in man. *Clin Sci* 92: 285–289, 1997.

17. Hart EC, Joyner MJ, Wallin BG, Karlsson T, Curry TB, Charkoudian N. Baroreflex control of muscle sympathetic nerve activity: a nonpharmacological measure of baroreflex sensitivity. *Am J Physiol Heart Circ Physiol* 298: H816–H822, 2010.

18. Hijmering ML, Stroes ESG, Olijhoek J, Hutten BA, Blankestijn PJ, Rabelink TJ. Sympathetic activation markedly reduces endothelium-dependent, flow-mediated vasodilation. *J Am Coll Cardiol* 39: 683–688, 2002.

19. Joyner MJ, Charkoudian N, Wallin BG. Sympathetic Nervous System and Blood Pressure in Humans. *Hypertension* 56: 10–16, 2010.

20. Jung K-H, Chu K, Ko S-Y, Lee S-T, Sinn D-I, Park D-K, Kim J-M, Song E-C, Kim M, Roh J-K. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke* 37: 2744–2750, 2006.

21. Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension* 65: 320–327, 2015.

22. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* 56: 274–281, 2010.

23. Kienbaum P, Karlsson T, Sverrisdottir YB, Elam M, Wallin BG. Two sites for modulation of human sympathetic activity by arterial baroreceptors? *J Physiol* 531: 861–869, 2001.

24. Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *J Appl Physiol* 110: 591–600, 2011.

25. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* 355: 2792–2793, 2006.

26. Liao JK, Laufs U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* 45: 89–118, 2005.

27. Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37: 395–400, 2004.

28. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 7: 156–167, 2008.

29. Mark AL, Victor RG, Nerhed C, Wallin BG. Microneurographic studies of the mechanisms of sympathetic nerve responses to static exercise in humans. *Circ Res* 57: 461–469, 1985.

30. Masuo K, Mikami H, Ogihara T, Tuck ML. Sympathetic nerve hyperactivity precedes hyperinsulinemia and blood pressure elevation in a young, nonobese Japanese population. *Am J Hypertens* 10: 77–83, 1997.

31. McGowan CL, Murai H, Millar PJ, Notarius CF, Morris BL, Floras JS. Simvastatin reduces sympathetic outflow and augments endothelium-independent dilation in non-hyperlipidaemic primary hypertension. *Heart* 99: 240–246, 2013.

32. McMahon NF, Leveritt MD, Pavey TG. The Effect of Dietary Nitrate Supplementation on Endurance Exercise Performance in Healthy Adults: A Systematic Review and Meta-Analysis. *Sports Med* 47: 735–756, 2017.

33. Metcalfe C. The analysis of cross-over trials with baseline measurements. *Stat Med* 29: 3211–3218, 2010.

34. Millar PJ, Floras JS. Statins and the autonomic nervous system. *Clin Sci* 126: 401–415, 2014.

35. Millar PJ, Murai H, Floras JS. Paradoxical Muscle Sympathetic Reflex Activation in Human Heart Failure. *Circulation* 131: 459–468, 2015.

36. Monahan KD, Dinenno FA, Seals DR, Clevenger CM, Desouza CA, Tanaka H. Age-associated changes in cardiovagal baroreflex sensitivity are related to central arterial compliance. *Am J Physiol Heart Circ Physiol* 281: H284-289, 2001.

37. Monahan KD, Wilson TE, Ray CA. Omega-3 Fatty Acid Supplementation Augments Sympathetic Nerve Activity Responses to Physiological Stressors in Humans. *Hypertension* 44: 732–738, 2004.

38. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002–2012, 1993.

39. Notarius CF, Millar PJ, Murai H, Morris BL, Marzolini S, Oh P, Floras JS. Divergent muscle sympathetic responses to dynamic leg exercise in heart failure and age-matched healthy subjects. *J Physiol* 593: 715–722, 2015.

40. Notay K, Seed JD, Incognito AV, Doherty CJ, Nardone M, Burns MJ, Millar PJ. Validity and reliability of measuring resting muscle sympathetic nerve activity using short sampling durations in healthy humans. *J Appl Physiol* 121: 1065–1073, 2016.

41. Owlya R, Vollenweider L, Trueb L, Sartori C, Lepori M, Nicod P, Scherrer U. Cardiovascular and Sympathetic Effects of Nitric Oxide Inhibition at Rest and During Static Exercise in Humans. *Circulation* 96: 3897–3903, 1997.

42. Pereira C, Ferreira NR, Rocha BS, Barbosa RM, Laranjinha J. The redox interplay between nitrite and nitric oxide: From the gut to the brain. *Redox Biol* 1: 276–284, 2013.

43. Schlaich MP, Kaye DM, Lambert E, Sommerville M, Socratous F, Esler MD. Relation between cardiac sympathetic activity and hypertensive left ventricular hypertrophy. *Circulation* 108: 560–565, 2003.

44. 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* 143: 818–826, 2013.

45. Vallbo ÅB, Hagbarth K-E, Wallin BG. Microneurography: how the technique developed and its role in the investigation of the sympathetic nervous system. *J Appl Physiol* 96: 1262–1269, 2004.

46. van Velzen AG, Sips AJAM, Schothorst RC, Lambers AC, Meulenbelt J. The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol Lett* 181: 177–181, 2008.

47. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 51: 784–790, 2008.

48. Wilkinson IB, Qasem A, McEniery CM, Webb DJ, Avolio AP, Cockcroft JR. Nitric oxide regulates local arterial distensibility in vivo. *Circulation* 105: 213–217, 2002.

49. Young CN, Fisher JP, Gallagher KM, Whaley-Connell A, Chaudhary K, Victor RG, Thomas GD, Fadel PJ. Inhibition of nitric oxide synthase evokes central sympatho-excitation in healthy humans. *J Physiol* 587: 4977–4986, 2009.

50. Zipes DP, Rubart M. Neural modulation of cardiac arrhythmias and sudden cardiac death. *Heart Rhythm* 3: 108–113, 2006.

Table 1. Baseline participant characteristics between placebo (PL) and beetroot (BR) supplementation visits (n=14).

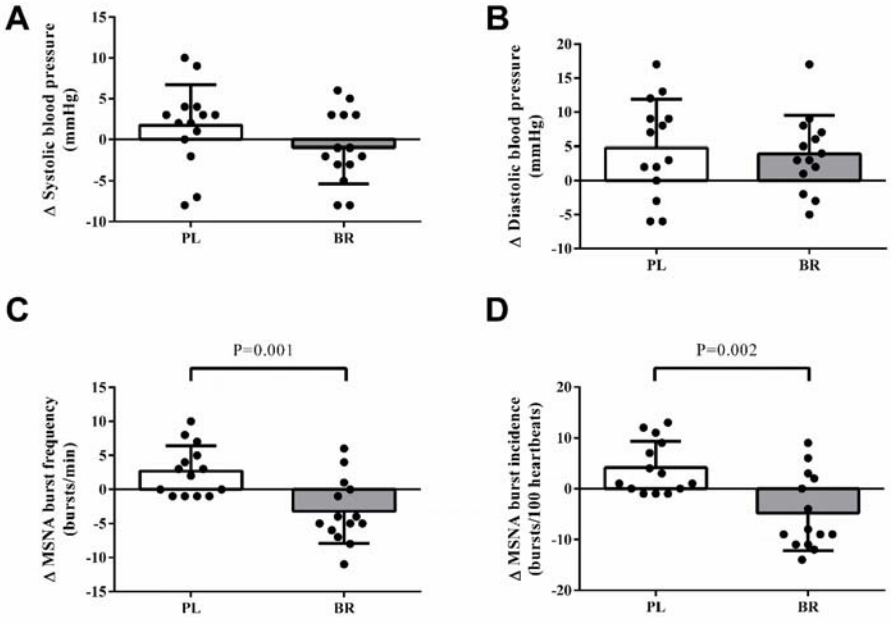
Characteristic	PL	BR	ICC
Age (years)	25 ± 10	--	--
Sex (Male/Female)	7/7	--	--
Height (cm)	167 ± 10	167 ± 10	>0.99
Weight (kg)	63 ± 9	63 ± 9	>0.99
Body mass index (kg/m^2)	24 ± 6	24 ± 6	>0.99
Maximal volitional contraction (kg)	37 ± 15	36 ± 15	0.97
Heart rate (bpm)	64 ± 10	64 ± 8	0.88
Systolic blood pressure (mmHg)	103 ± 6	106 ± 6*	0.62
Diastolic blood pressure (mmHg)	65 ± 7	64 ± 6	0.71
MSNA burst frequency (bursts/min)	21 ± 8	23 ± 7	0.76
MSNA burst incidence (bursts/100 heartbeats)	34 ± 13	36 ± 10	0.77
Sympathetic BRS (bursts/100 heartbeats/mmHg)	-3.9 ± 1.4	-3.9 ± 1.5	0.63

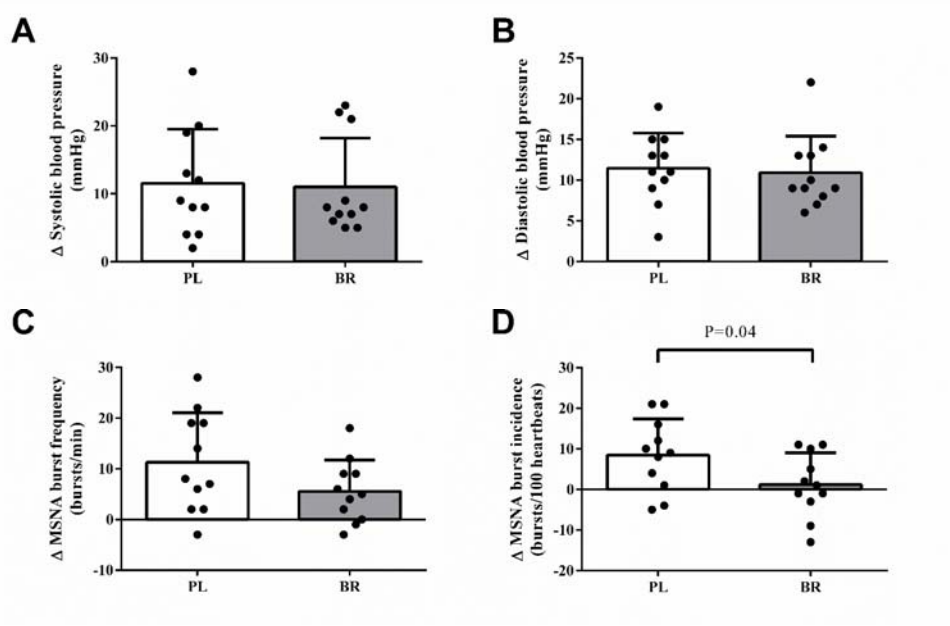
Mean ± SD. *, $p<0.05$ versus placebo. PL, placebo; BR, beetroot; BRS, baroreflex sensitivity; ICC, intra-class correlation coefficient.

Figure Legends

Figure 1. The change (Δ) in resting systolic blood pressure (A), diastolic blood pressure (B), MSNA burst frequency (C), and MSNA burst incidence (D) following placebo (PL) or beetroot (BR) supplementation. Data obtained from 14 participants and expressed as means ± SD. P-values adjusted for resting baseline values.

Figure 2. The change (Δ) in systolic blood pressure (A), diastolic blood pressure (B), MSNA burst frequency (C), and MSNA burst incidence (D) during 30% MVC static handgrip following placebo (PL) or beetroot (BR) supplementation. Data obtained from 11 participants and expressed as means ± SD. P-values adjusted for handgrip baseline values.


A
Δ Systolic blood pressure (mmHg)
15
10
5
0
-5
-10
PL
BR
B
Δ Diastolic blood pressure (mmHg)
20
15
10
5
0
-5
-10
PL
BR
C
P=0.001
Δ MSNA burst frequency (bursts/min)
15
10
5
0
-5
-10
-15
PL
BR
D
P=0.002
Δ MSNA burst incidence (bursts/100 heartbeats)
20
10
0
-10
-20
PL
BR


A
Δ Systolic blood pressure (mmHg)
30
20
10
0
PL
BR
B
Δ Diastolic blood pressure (mmHg)
25
20
15
10
5
0
PL
BR
C
Δ MSNA burst frequency (bursts/min)
30
20
10
0
-10
PL
BR
D
P=0.04
Δ MSNA burst incidence (bursts/100 heartbeats)
30
20
10
0
-10
-20
PL
BR