RAPID REPORT | Mining Natural Products for Cardiovascular Benefits

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

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Notay K, Incognito AV, Millar PJ. Acute beetroot juice supplementation on sympathetic nerve activity: a randomized, double-blind, placebo-controlled proof-of-concept study. Am J Physiol Heart Circ Physiol 313: H59–H65, 2017. First published May 5, 2017; doi:10.1152/ajpheart.00163.2017.—Acute dietary nitrate (NO₃⁻) supplementation has been demonstrated to elicit dose-dependent reductions in resting blood pressure in normotensive and hypertensive populations in prospective studies (e.g., Refs. 13, 21, 22, 25, and 47) and meta-analyses (2, 44). These results highlight the potential therapeutic benefit of targeting the NO₃⁻-nitrite-nitric oxide (NO) pathway to increase the bioavailability of the potent vasodilator NO (27, 28, 47). In contrast to conventional NO synthesis through the oxidation of l-arginine by NO synthase (38), this recently discovered pathway involves the conversion of NO₃⁻ and nitrite back to bioactive NO without the need for oxygen. NO₃⁻ and nitrite derive from dietary NO₃ ingestion and as metabolic byproducts of NO oxidation (27, 28, 47). After consumption and absorption through the gastrointestinal tract, ~25% of ingested dietary NO₃⁻ 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, and molybdenum-containing enzymes) during physiological and pathological hypoxic conditions (27, 28, 47). As a result, NO₃⁻ and nitrite are now considered to represent important reservoirs for NO production (1, 14). To date, the most common method of administering dietary NO₃⁻ 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 (change of ~5 mmHg) (22), whereas supplementation with 500 ml (~22.5 mmol) of BR juice was reported to elicit larger reductions (change of ~10/8 mmHg) (47). Importantly, the peak hypotensive responses in both studies occurred ~3 h after ingestion, aligned with the time course of peak plasma nitrite concentration (22, 47). Similar findings have been reported after daily BR supplementation for 4 wk 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

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pressure and endothelium-dependent vasodilation (18, 19); however, whether the beneficial effects of dietary NO₃⁻ 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 NO₃⁻ supplementation using BR can modulate central sympathetic outflow. We hypothesized that compared with NO₃⁻-depleted BR placebo (PL), acute consumption of high-NO₃⁻ 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 NO₃⁻ 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 and 10 women, age: 27 ± 11 yr) were recruited to participate in the study after providing informed written consent. All participants were normotensive, nonsmoking, in sinus rhythm, free of known cardiovascular or metabolic disease, and not taking any acute (<3 mo) or chronic medications, including birth control or antibiotics. All participants self-reported not currently or previously engaging in formal NO₃⁻ supplementation. Due to the requirement of oral bacteria to convert dietary NO₃⁻ to nitrite (15, 47), participants were required to abstain from using antibacterial mouthwash 30 days before 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-NO₃⁻ (~6.4 mmol) BR or NO₃⁻-depleted (~0.0055 mmol) PL (James White Drinks, Suffolk, UK). Before testing visits, all participants completed a familiarization visit to the laboratory (~1 h), where they underwent detailed verbal and visual explanations of the study requirements and protocols (e.g., micronurography) 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-NO₃⁻ BR (~6.4 mmol) juice is considered to be equivalent to ~250 ml (~5.5–6.4 mmol) of regular BR juice (21, 22), whereas the PL version is manufactured specifically for research occurring during 13 of the 28 study visits (BR: n = 5 and PL: n = 8).

Continuous beat-to-beat blood pressure was recorded from the right brachial blood pressure was recorded using an automated sphygmomanometer (BPTru Medical Devices, Coquitlam, BC, Canada). Continuous beat-to-beat blood pressure was recorded from the right middle finger using photoelectric plethysmography (Finometer MIDI, Finapres).

Postganglionic multiunit MSNA was measured from the right bulbar nerve by microneurography, as previously described (35, 40). A low-impedance tungsten microelectrode (2 MΩ, Frederick Haer, Brunswick, ME) was inserted percutaneously into a motor fascicle and adjusted until spontaneous multiunit 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 multiunit neurogram (model 662C-4, Nerve Traffic Analyzer, 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 were digitized and stored with LabChart (PowerLab, ADInstruments). Heart rate, respiration, blood pressure, and the integrated multiunit multiunit MSNA signal were recorded at a sampling frequency of 1,000 Hz, whereas the raw MSNA signal was collected at 10,000 Hz.

Data analysis. All data were analyzed and tabled by investigators blinded to the intervention allocation. MSNA was analyzed using custom semi-automated LabView software (National Instruments, Austin, TX) (35, 40). MSNA was quantified as burst frequency (in bursts/min) and burst incidence (in bursts/100 heart beats). Total MSNA was not calculated as a result of a singular site change occurring during 13 of the 28 study visits (BR: n = 5 and PL: n = 8). However, MSNA burst occurrence exhibits excellent intraday 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 previously described (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 α of 0.05 and β of 0.2. Rounding baseline variables were compared between BR and PL (and visit 1 vs. visit 2) using two-tailed paired t-tests. Intraclass 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 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 posttreatment value as the dependent variable. The ANCOVA method compares the effects of BR and PL on each outcome after correcting for differences in resting (preingestion) or handgrip (postingestion) 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, NY) with significance defined as \( P < 0.05 \). All values are presented as means ± 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 14 participants, although only 11 participants completed the static handgrip exercise protocol (3 dropouts due to time restrictions). Participant characteristics are shown in Table 1. Resting baseline heart rate, diastolic blood pressure, MVC, and MSNA were consistent between BR and PL visits (all \( P > 0.05 \)), whereas systolic blood pressure tended to be lower during the PL visit (\( P = 0.04 \)). The intertest 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 heart beats, \( P = 0.002\)) were lower after BR compared with PL (Fig. 1). In contrast, the changes in resting systolic blood pressure (\(-1 ± 5 \) vs. \(2 ± 5 \) mmHg, \( P = 0.30\)) and diastolic blood pressure (\(4 ± 5 \) vs. \(5 ± 7 \) mmHg, \( P = 0.68\); Fig. 1), heart rate (\(0 ± 4 \) vs. \(-1 ± 4 \) beats/min, \( P = 0.70\)), and spontaneous arterial sympathetic baroreflex sensitivity (\(0.2 ± 1.4 \) vs. \(0.2 ± 1.3 \) bursts/100 heartbeats\(^{-1}\) mmHg\(^{-1}\), \( P = 0.95\)) 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).

Table 1. Baseline participant characteristics between placebo and beetroot supplementation visits

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo Supplementation</th>
<th>Beetroot Supplementation</th>
<th>Intraclass Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>25 ± 10</td>
<td>26 ± 10</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Sex, men/women</td>
<td>7/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height, cm</td>
<td>167 ± 10</td>
<td>167 ± 10</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>63 ± 9</td>
<td>63 ± 9</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>24 ± 6</td>
<td>24 ± 6</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Maximal voluntary contraction, kg</td>
<td>37 ± 15</td>
<td>36 ± 15</td>
<td>0.97</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>64 ± 10</td>
<td>64 ± 8</td>
<td>0.88</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>103 ± 6(^\star)</td>
<td>106 ± 6(^\star)</td>
<td>0.62</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>65 ± 7</td>
<td>64 ± 6</td>
<td>0.71</td>
</tr>
<tr>
<td>MSNA burst frequency, bursts/min</td>
<td>21 ± 8</td>
<td>23 ± 7</td>
<td>0.76</td>
</tr>
<tr>
<td>MSNA burst incidence, bursts/100 heartbeats</td>
<td>34 ± 13</td>
<td>36 ± 10</td>
<td>0.77</td>
</tr>
<tr>
<td>Sympathetic baroreflex sensitivity, bursts/100 heartbeats(^{-1}) mmHg(^{-1})</td>
<td>-3.9 ± 1.4</td>
<td>-3.9 ± 1.5</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Values are means ± SD; \( n = 14 \) participants. MSNA, muscle sympathetic nerve activity. \(^\star P < 0.05 \) vs. placebo.

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

DISCUSSION

Supplementation with dietary NO\(_3\) 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 NO\(_3\) 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, primarily 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 NO\(_3\) 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 (5) has examined the effects of dietary NO\(_3\) supplementation on the autonomic nervous system, demonstrating that acute BR consumption increased time-domain heart rate variability, a noninvasive marker of cardiac autonomic modulation. The effects of dietary NO\(_3\) 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 NO₃⁻/H₁¹₀₀₂. Prior research has demonstrated that exogenous NO₃⁻ 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 NO₃⁻ 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 resting MSNA burst incidence independent of changes in sympathetic baroreflex sensitivity or blood pressure) have been reported in clinical patients after 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 and primary regulator of barosensitive sympathetic outflow (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 an 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 previously reported 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)

Fig. 1. Changes (Δ) in resting systolic blood pressure (A), diastolic blood pressure (B), muscle sympathetic nerve activity (MSNA) burst frequency (C), and MSNA burst incidence (D) after placebo (PL) or beetroot (BR) supplementation. Data were obtained from 14 participants and are expressed as means ± SD. P values were adjusted for resting baseline values.
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 NO3/H11002 supplementation as a therapeutic strategy to target sympathetic overactivation 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). Furthermore, some current antihypertensive 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 overactivation 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 1 mo (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, primarily 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 NO3/H11002 and nitrite concentrations for both BR [NO3/H11002: 3.5 mmol (range: 2.6–4.4 mmol) and nitrite: 0.1 mmol (range: 0.05–0.16 mmol)] and PL [NO3/H11002: 0.34 mmol (range: 0.05–0.56 mmol) and nitrite: 0.006 mmol (range: 0.001–0.1 mmol)] (6). The results confirm the difference between BR and PL but suggest that BR NO3/H11002 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 the low normotensive status of our participants), although hypotensive responses are not universal across the literature, even at higher NO3/H11002 doses (2, 44). Importantly, the present results suggest that the NO3− 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 and 3 h after consumption) has been characterized in prior studies with consistent findings (13, 21, 22, 25), although interindividual 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 BR 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 NO3 supplementation can cause central sympathoinhibition in a healthy, primarily young, population. Reductions in sympathetic outflow are likely to contribute to the cardiovascular benefits of dietary NO3 supplementation, while offering a new autonomic restorative intervention to be tested in future clinical trials in patient groups associated with sympathetic overactivation (3, 9, 10, 35).

REFERENCES


