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AN ACUTE DIETARY NITRATE SUPPLEMENT HAS NO EFFECT ON ENDOTHELIAL FUNCTION BUT HAS MODEST EFFECTS ON SYSTEMIC HEMODYNAMICS IN HEALTHY YOUNGER MEN

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by

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Abstract

Epidemiological studies suggest that diets rich in fruits and vegetables are protective against cardiovascular disease. Recent pre-clinical (rodent) and human studies suggest that inorganic nitrate supplementation in the form nitrate salts, concentrated beetroot juice, or other nitrate-rich vegetables, may be a key mediator of this cardioprotection via the serial (L-arginine and O_2-independent) reduction of ingested nitrate to nitrite and subsequently nitric oxide (NO). While nitrate supplementation has become established as a natural means of increasing NO, its influence on *in vivo* endothelial function in humans remains equivocal. The effects of nitrate supplementation on cardiovascular/mental stress reactivity, another *in vivo*/laboratory based prognostic indicator of cardiovascular disease risk (i.e., future risk of hypertension), are also not known.

PURPOSE: The primary objective of the present investigation was to determine if acute dietary nitrate supplementation augments the dilation of the brachial artery during graded handgrip exercise, a test previously shown to be nitric oxide-mediated in healthy younger men (Wray et al *J Appl Physiol* H1101-H1107, 2011). A secondary objective was to determine if this supplement attenuates the rise in systemic blood pressures or favorably modifies conduit artery shear patterns during an acute mental stress challenge.

METHODS: In a randomized, double-blind, placebo-controlled crossover study, thirteen young (22 ± 2 yrs) healthy men consumed a concentrated beetroot juice supplement (140 mL Beet-It Sport, James White Drinks Ltd.) or placebo (nitrate-depleted Beet-It Sport) on two study visits at least five days apart. Resting blood pressure (brachial auscultation) and venous nitrate/nitrite concentrations (ENO-20) were measured before and 2.5 hours post-consumption. Three hours post consumption, arterial pulse wave velocity was measured (Colin tonometry) followed by resting and handgrip exercise-induced changes in brachial artery diameter, blood flow, and blood velocity (Doppler Ultrasound). A sub-set of subjects (n=7) performed a serial subtraction test (SST) during which measures of superficial femoral artery diameter, flow, and blood velocity were assessed.

RESULTS: Dietary nitrate supplementation raised plasma nitrate (17-fold) and nitrite (1.6-fold) concentrations, and lowered resting pulse wave velocity (Colin VP-2000) versus placebo (all $p<0.05$) indicating absorption, conversion, and a biological effect of this supplement. However, nitrate supplementation had no effect on brachial artery diameter, flow, or shear rates at rest (all $p>0.28$) or during any of the six exercise work rates (all $p>0.18$). The increase in brachial diameter per unit change in shear rate across exercise intensities also did not differ between visits ($p=0.15$). Nitrate supplementation did not affect resting or SST-induced increases in systolic BP, heart rate, or femoral hemodynamics, but it did attenuate the peak rise in diastolic BP ($p=0.041$ vs. placebo visit).

CONCLUSION: These findings suggest that acute dietary nitrate supplementation favorably modifies arterial pulse wave velocity, but does not augment endothelial nitric oxide-mediated vasodilation in healthy younger men. The SST sub-study further suggests that acute nitrate supplementation may influence blood pressure responses to acute mental stress in healthy young men. The mechanisms and clinical significance of functional improvements in arterial compliance in healthy

adults, independent of any measureable improvements in vascular function in the arm or leg conduit arteries, remains to be established.

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Chapter 1

AN ACUTE DIETARY NITRATE SUPPLEMENT HAS NO EFFECT ON ENDOTHELIAL FUNCTION BUT HAS MODEST EFFECTS ON SYSTEMIC HEMODYNAMICS IN HEALTHY YOUNGER MEN

Introduction

Endothelial function is an important determinant of cardiovascular health. Accordingly, interventions that improve endothelial function may prevent or slow the progression of cardiovascular disease in both healthy and higher risk populations.[1-3] Emerging evidence suggests that dietary nitrate, derived in the diet primarily from vegetables, could contribute to cardiovascular disease prevention via its effects on nitric oxide status.[4-6] Indeed, clinical trials with dietary nitrate supplementation have observed improvements in several prognostic markers of cardiovascular health including short term lowering of systolic/diastolic blood pressure, arterial stiffness, and inhibition of platelet aggregation, with concomitant improvements in nitric oxide status.[6-15]

Impaired endothelial production of nitric oxide often precedes the development of cardiovascular disease.[3, 16-20] While dietary nitrate supplementation is now well established as a natural means of increasing bioavailable nitric oxide, the influence of such supplementation on endothelial function per se (i.e., one of the key mechanisms proposed to underlie the cardioprotective benefits of dietary nitrates) remains equivocal in humans. Some studies report modest increases in endothelial function, while others report no change.[12, 21] Each of these nitrate supplementation studies assessed endothelium-dependent dilation as the peak (absolute or % above baseline) dilation of the brachial artery during ischemia-induced reperfusion of the forearm (flow-mediated vasodilation [FMD] test).[12, 21] However, none of these studies normalized the brachial artery dilator response to the dilatory stimulus (vascular shear rate), which may have contributed to their variable and/or null findings. We hypothesized that such normalization, coupled with evaluation of vasodilator responses across a broad range of shear stimuli, would provide a more robust method with which to assess the impact of dietary nitrates on endothelial function. Accordingly, the primary objective of the present investigation was to determine if acute dietary nitrate supplementation augments the progressive dilation of the brachial artery during graded stages of handgrip exercise, a test previously shown to be endothelial nitric oxide-mediated in healthy younger men.[22]

A secondary aim of this study was to assess the acute effects of beetroot juice on local and systemic hemodynamics at rest and in response to various forms of physiological stress. Acute nitrate supplementation studies which implement beetroot juice, green leafy vegetable diets, or nitrate salts, have typically demonstrated a 4 to 5 mmHg drop in resting systolic blood pressure, providing further (pre-clinical) support for the cardioprotective (anti-hypertensive) effects of diets rich in nitrates. Whether nitrate-rich diets or supplements can reduce the blood pressure responses to psychological stressors (i.e., rise in blood pressure during acute mental stress) is entirely unknown, but is a clinically relevant question. Normotensive younger adults who are high "responders" to such stimuli have increased risk of developing hypertension 10 to 15 years later.[23, 24] To gain preliminary insight into this question, blood pressure responses to a serial subtraction test (SST) were compared in a sub-group of the subjects in the present study. Shear patterns in the superficial femoral artery (SFA), a large conduit artery, were also examined during the SST. The general hypothesis of this sub-study was that consumption of nitrate-rich beetroot juice would result in more favorable systemic (smaller rise in systolic BP and/or diastolic BP) and local (improved shear pattern characteristics in the SFA) vascular responses.

Methods

Subjects

Healthy men (n=13, 22 ± 2 years) were recruited from the local university population. Subjects were recreationally active, normotensive, non-diabetic, normolipidemic, non-obese (BMI range - 18.5-29kg/m^2), free of cardiovascular, metabolic, pulmonary or neurological disorders, and non-smokers (Table 1). All subjects provided written consent to participate after all protocols and potential risks were explained. Subjects did not take any blood pressure altering medications or donate blood throughout the duration of the study. Subjects were asked to avoid brushing their teeth or using mouthwash on the morning of each study visit, as to not disturb integral nitrate reducing bacteria in the oral cavity. All procedures were performed at approximately the same time of day in each subject. All procedures were approved by the Office of Research Protection at Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki along with the ethical standards in physiological research.

Procedures

Subjects were asked to abstain from caffeine for 12 hours prior to each of the three study visits. Subjects also abstained from exercise for 24 hours and alcohol for 48 hours prior to the morning of study visits. On the familiarization visit, subjects were given the opportunity to practice maintaining the correct frequency of contraction for the handgrip protocol. Subjects also performed three repeated maximal handgrip contractions with a handgrip dynamometer to quantify peak forearm muscle strength (Jamar, Sammons Preston, Bolingbrook, IL). On both experimental days (placebo and nitrate supplement visits), subjects came into the lab after a 12-hour fast. The order in which the supplementation was distributed was randomized. Blood pressure was taken in the supine position with an automated cuff (Omron Healthcare, Lake Forest, IL). A venous blood sample was obtained to establish baseline plasma nitrate and nitrite concentrations.

Subjects were then given 140mL of a beet juice supplement containing 12.9 mmol of nitrate or placebo without nitrate (Beet It Sport, James White Drinks Ltd., Ipswich UK). Following 2.5 hours of quiet rest, a second venous sample was taken to determine post consumption [NO_3^-] and [NO_2^-]. Following this, subjects underwent several different testing protocols to assess nitrate consumption on vascular function.

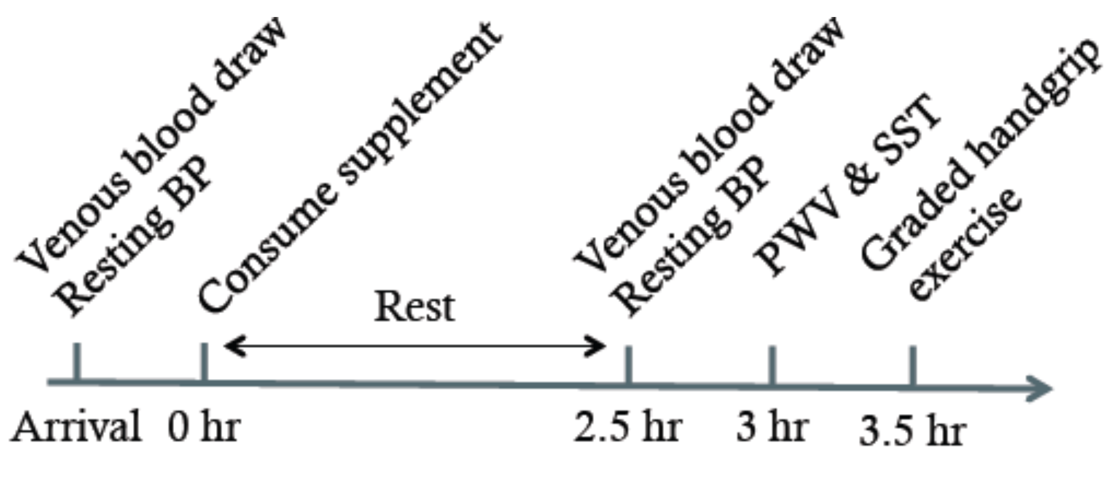


Table 1 Timeline of experimental visits

Methods

Blood pressure measurements during supplementation

Blood pressure measurements were taken by clinical research staff and the methods were in accordance with current AHA guidelines.[25] Of the thirteen subjects, eight subjects were seated for five minutes and five were supine for ten minutes before measurements were taken. The same methodology for measurement was employed, with the exception of posture. Briefly, a sphygmomanometer and manual cuff were used. The cuff was properly fitted around the subject's upper arm and proper cuff deflation techniques were used (2-3mmHg/second). Subjects were advised to remain silent through the duration of the test. Three measurements were taken with one minute of quiet rest between each measurement.

Pulse Wave Velocity

After 15 minutes of supine rest, resting pulse wave velocity was measured using an automated device (VP2000, Colin Medical). Cuffs are placed on the subject's upper arms and ankles and the distances from the heart are calculated automatically from subject height data and previously reported statistics. The machine then measures the amount of time a pressure pulse takes to travel the distance between the heart and brachial artery and between the heart and the ankle. These values (distance between the heart and each cuff, and the time value) are then used in an approved algorithm to calculate brachial to ankle PWV (Colin Medical).

Serial subtraction test (SST)

Doppler ultrasound (HDI 5000, ATL) was used to measure blood velocity and vessel diameter in the superficial femoral artery (SFA). A 4MHz probe was used to image the SFA at an insonation angle of 60 degrees. After resting SFA velocity, diameter and blood pressure were measured, subjects underwent a serial subtraction test for three minutes while blood velocity in the SFA was continuously monitored. Blood pressure was monitored at rest and at one-minute intervals during the test with an automated device (Omron Medical). A verbal Serial 13s and 17s test was employed. Briefly, subjects were given a random three digit number between 900-1000 and asked to verbally subtract 13 or 17 from that number as fast as possible. During the test, speed and accuracy were encouraged. The order in which the subjects subtracted 13 and 17 was random in relation to the supplement taken at the beginning of the visit.

Graded handgrip test

Subjects rested quietly for 5-10 minutes and then performed graded rhythmic handgrip exercise while Doppler Ultrasound (6MHz probe) was used to monitor changes in brachial artery diameter and velocity during the last 30 seconds of each workload. The model used for the handgrip test was adapted and modified from previous studies.[22] Subjects were asked to exercise at a rate of 30 contractions per minute moving the load on each contraction through a full range of motion (10cm). Each workload lasted three minutes, with one minute of quiet rest between workloads. Six exercise bouts were performed, and the workload progressively increased with each bout (200-1,200g). Heart rate and blood pressure measurements were taken from the contralateral arm while at rest and during the final minute of each exercise bout with an automated device (Omron Medical).

Plasma [Nitrate/Nitrite] analysis

Venous blood samples were drawn into EDTA tubes (10mL K_2 EDTA tubes, BD Vacutainer, Franklin Lakes, New Jersey) and immediately centrifuged at 3,200 rpm and 4°C for 10 minutes. Plasma was then extracted and stored in -80°C freezer for later analysis of [NO_3^-] and [NO_2^-]. ENO-20 (EICOM, San Diego, CA) was used to measure [NO_3^-] and [NO_2^-] in the plasma samples. Briefly, plasma was mixed with an equal volume of 100% methanol and centrifuged at 13,000rpm for 10 minutes. Samples were then loaded into a 96-well plate. Nitrate and nitrite were then separated via column chromatography and individually reacted with a Griess reagent, synthesizing a diazo compound. The absorbance of this red diazo compound were then read at an wavelength of 540nm using a visible light detector.

Calculated variables

Brachial and superficial femoral artery diameters were measured offline with automated edge detection software (Brachial Analyzer, MIA, Coralville, IA). Velocity measurements were taken from Doppler Ultrasound via Powerlab software (Chart 5, Colorado Springs, CO). Blood flow values were calculated from diameter and velocity (velocity*π((diameter(cm)/2)/4)*60). Anterograde and retrograde shear was calculated from their respective velocities (8*velocity/diameter). All analyses were conducted by a single investigator who was blinded to the subject and treatment conditions.

Statistical analysis

Data were analyzed using SPSS 20 (North Castle, NY). Resting parameters during the placebo and beetroot juice visits were compared using a paired t-test. ANOVA for repeated measures was used to assess the effects of beetroot juice supplementation on stress-induced (mental arithmetic and graded handgrip exercise) vascular and hemodynamic parameters. All data are presented as mean±SEM. A value of $p<0.05$ was considered statistically significant. Post hoc analyses of power were done using G*Power 3(Düsseldorf, Germany).

Results

Subject compliance

Ingestion of the placebo (PL) and nitrate rich beetroot juice (BRJ) was well tolerated by all subjects. Beeturia, a harmless side effect common with chronic beetroot juice supplementation, was not reported by any subjects.

Plasma nitrate and nitrite

Prior to any beverage ingestion, resting plasma nitrate and nitrite were not different between visits. Following consumption of BRJ, resting plasma [NO_3^-] and [NO_2^-] increased (29 µM ± 3 PL vs. 560 µM ± 36 BRJ: $p<0.001$) and (.36 µM ± .1 PL vs. .57 µM ± .1 BRJ: $p=0.02$).

Resting blood pressure

Resting supine blood pressures on the two experimental visits are presented in Figure 1. In the present study, resting blood pressures were taken three hours after consumption and were unchanged by nitrate consumption. (128/74 vs. 126/73 PL and 124/71 vs. 121/69, BRJ)

Pulse wave velocity

There was a significant decrease in PWV following the consumption of the BRJ compared to the placebo (1122 cm/s ± 31 PL vs. 1082 cm/s ± 34 BRJ; $p=0.02$).

Superficial femoral artery hemodynamics during a serial subtraction test

Resting SFA diameters after ingestion of supplement were not different with BRJ compared to PL (6.76 mm ± 0.12 PL vs. 6.85mm ± 0.15; $p=0.17$). Also, resting blood velocity and blood flow were not significantly different between treatments (6.16 ± 0.7cm/s PL vs. 5.59±0.4cm/s BRJ: 126 ± 12 mL/min PL vs. 120 ± 10 mL/min BRJ; respectively). Further, resting anterograde and retrograde shear were not different between treatments (194 ± 12, -115 ± 7 cm^{-1} PL and 199 ± 8, -122 ± 7 cm^{-1} BRJ).

No change in diameter was observed during the SST. Blood velocity and flow peaked during the PL day at 30 seconds (14.5 ± 3.1cm/s and 303.3 ± 67.6mL/min, respectively). The peak velocity and flow responses occurred at 45 seconds on the BRJ day (15.3 ± 3.3cm/s and 311.8 ± 70.0mL/min, respectively). Figure 4 illustrates this delayed hyperemic response to a SST during BRJ supplementation compared to PL. Although there was a significant time effect on blood flow during the SST ($p<0.001$), the peak response was not different between the two interventions ($p=0.40$). As indicated in figure 5, no differences were seen in the shear patterns during the SST.

The serial subtraction test resulted in substantial increases in heart rate (23 bpm above resting baseline values), systolic (27 mmHg), and diastolic (19 mmHg) blood pressures in all subjects during both study visits. The magnitudes of these responses are consistent with published reports involving healthy young adults.[26,27] Blood pressure and heart rate responses to the SST are represented in figure 6. The peak change in heart rate from rest during the SST occurred at variable time points and was not significantly different between treatment days (23.9 ± 2.7bpm PL vs. 22 ± 3.7bpm BRJ; p=0.29). Peak change in systolic pressure was unchanged between treatments, but peak change in diastolic pressure was significantly reduced on BRJ compared to PL visit (21.1 ± 2.4mmHg PL vs. 17.4 ± 2.4mmHg BRJ; p=0.041). Peak change in MAP was not different on the BRJ visit. (22.7 ± 2.6mmHg PL vs. 20.0 ± 2.0mmHg BRJ; p=0.053). All values are presented as delta change from baseline rest.

Forearm hemodynamics during graded handgrip exercise

Resting brachial artery diameters were taken before the handgrip exercise and were not different between the treatment days (4.18 ± 0.1 mm PL vs. 4.17 ± 0.1 mm BRJ). Resting velocity and flow were not different between treatment days. As expected, there was a workload effect on both velocity and flow (p<0.001). Resting anterograde and retrograde shear were also not different (376.0 ± 29.2, -112.1 ± 8.7 cm^{-1} PL and 382.7 ± 29.8, -116.7 ± 17.1 cm^{-1} BRJ, respectively). Brachial artery responses across workloads during the graded handgrip exercise are illustrated in figure 6. This demonstrates a workload dependent dilation of the brachial artery. Velocity, flow, and % dilation in the brachial artery during the graded handgrip test did not differ between treatments (all p>0.05).

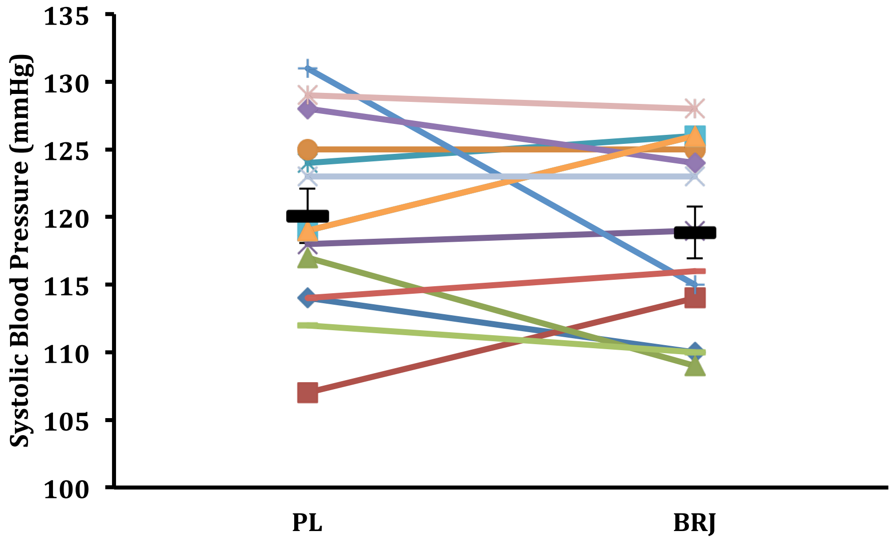
Heart rate, systolic and diastolic blood pressure values increased modestly during graded rhythmic handgrip, but were not different between treatments. (11 ± 3 bpm, 7 ± 3mmHg, 11 ± 3 mmHg PL vs. 10 ± 2 bpm, 8 ± 2 mmHg, 8 ±3 mmHg BRJ)

Subject Characteristics	
Height, cm	179.2±5.4
Weight, kg	78.8±8.4
Age, years	22.3±2.1
BMI, kg/m2	24.5±2.3
Glucose, mg/dL	90.5±4.8
Cholesterol, mg/dL	145.1±19.9
HDL, mg/dL	52±6.9
LDL, mg/dL	77.7±15.9
Triglycerides, mg/dL	76.5±27.2
Systolic Blood Pressure, mmHg	120.1±7.3
Diastolic Blood Pressure, mmHg	63.6±7.1
HR, bpm	57.6±10.5
Handgrip MVC, kg	50±7.4

Table 2 Subject Characteristics. Values are mean ± SEM. BMI: Body Mass Index, HDL: High density Lipoprotein, LDL: Low density Lipoprotein, HR: Heart Rate, MVC: Maximal Voluntary Contraction

Resting Parameters	N	Pre (PL)	Post (PL)	Pre (BR)	Post (BR)	p-value	(1-β)
SBP (mmHg)	13	128 ± 2	126 ± 2	124 ± 1	121 ± 1	0.10	0.172
DBP (mmHg)	13	74 ± 2	73 ± 2	71 ± 2	69 ± 2	0.11	0.301
PWV (cm/s)	13	--	1123 ± 31	--	1082 ± 34*	0.02	0.922
BA Diameter (mm)	12	--	4.18 ± 0.1	--	4.17 ± 0.1	0.29	0.066
BA Mean Shear (dynes)	12	--	108 ± 15	--	118 ± 15	0.30	0.475
BA Flow (mL/min)	12	--	48 ± 7	--	53 ± 9	0.28	0.119
SFA Diameter (mm)	12	--	6.76 ± .1	--	6.85 ± .2	0.17	0.241
SFA Mean Shear (dynes)	12	--	60 ± 6	--	57 ± 5	0.19	0.107
SFA Anterograde (dynes)	12	--	210 ± 12	--	211 ±8	0.48	0.134
SFA Retrograde (dynes)	12	--	-115 ± 7	--	-122 ± 7	0.21	0.201
SFA Flow (mL/min)	12	--	112 ± 9	--	117 ± 12	0.28	0.195

Table 3 Resting hemodynamics. SBP: Systolic blood pressure, DBP: Diastolic blood pressure, PWV: Pulse wave velocity, BA: Brachial artery, SFA: Superficial Femoral Artery. Values are mean ± SEM.



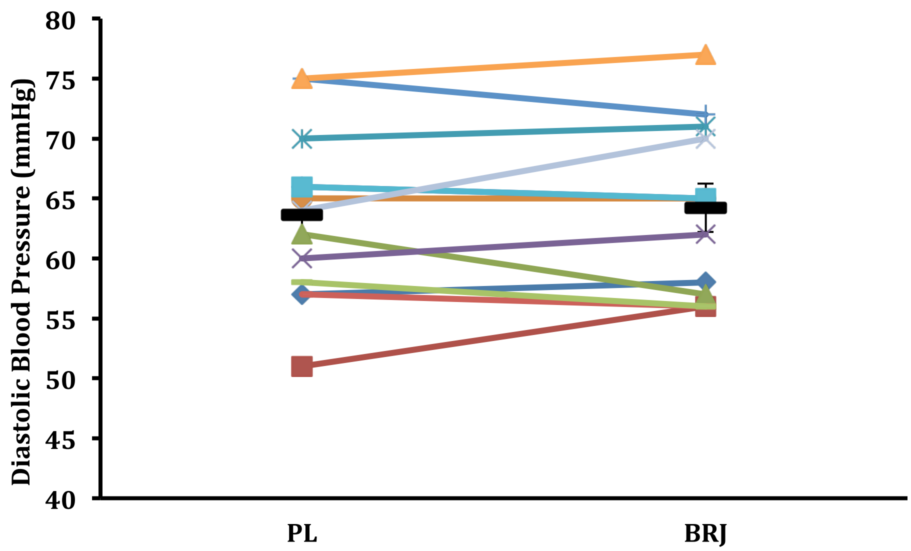


Figure 1 Individual subjects' systolic and diastolic blood pressure three hours post-consumption of either nitrate removed placebo or nitrate rich beetroot juice. Black bar represents mean values.

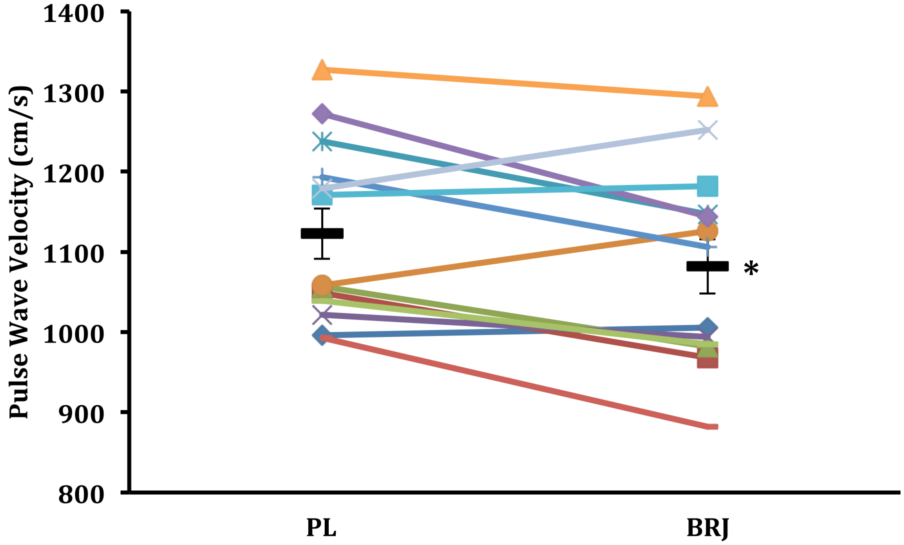


Figure 2 Global pulse wave velocity for n=13. Black bar represents mean values. *p<0.05

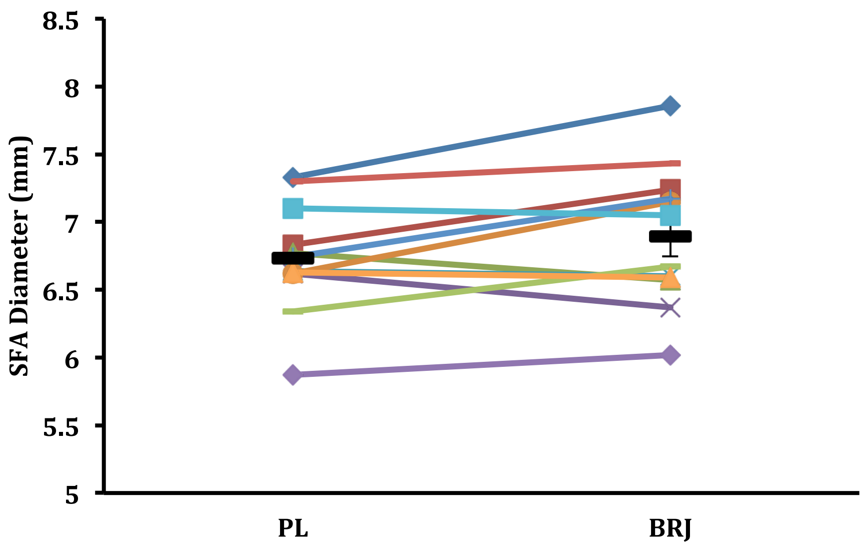


Figure 3 Resting SFA diameters following both placebo and beetroot juice supplementation. Black bar represents mean values.

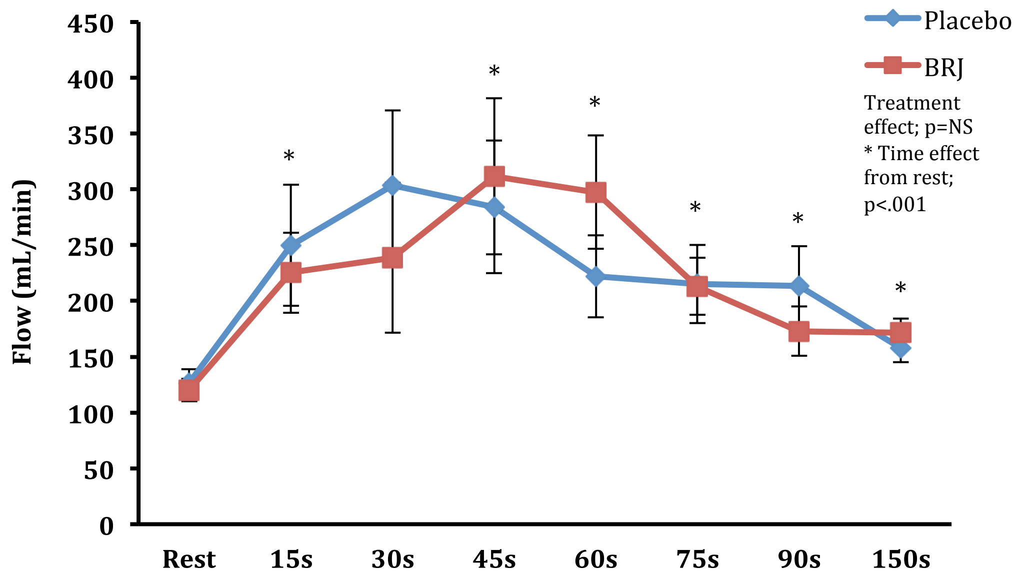


Figure 4 Blood flow through the SFA during a three-minute serial subtraction test. Values are presented as mean ± SEM.

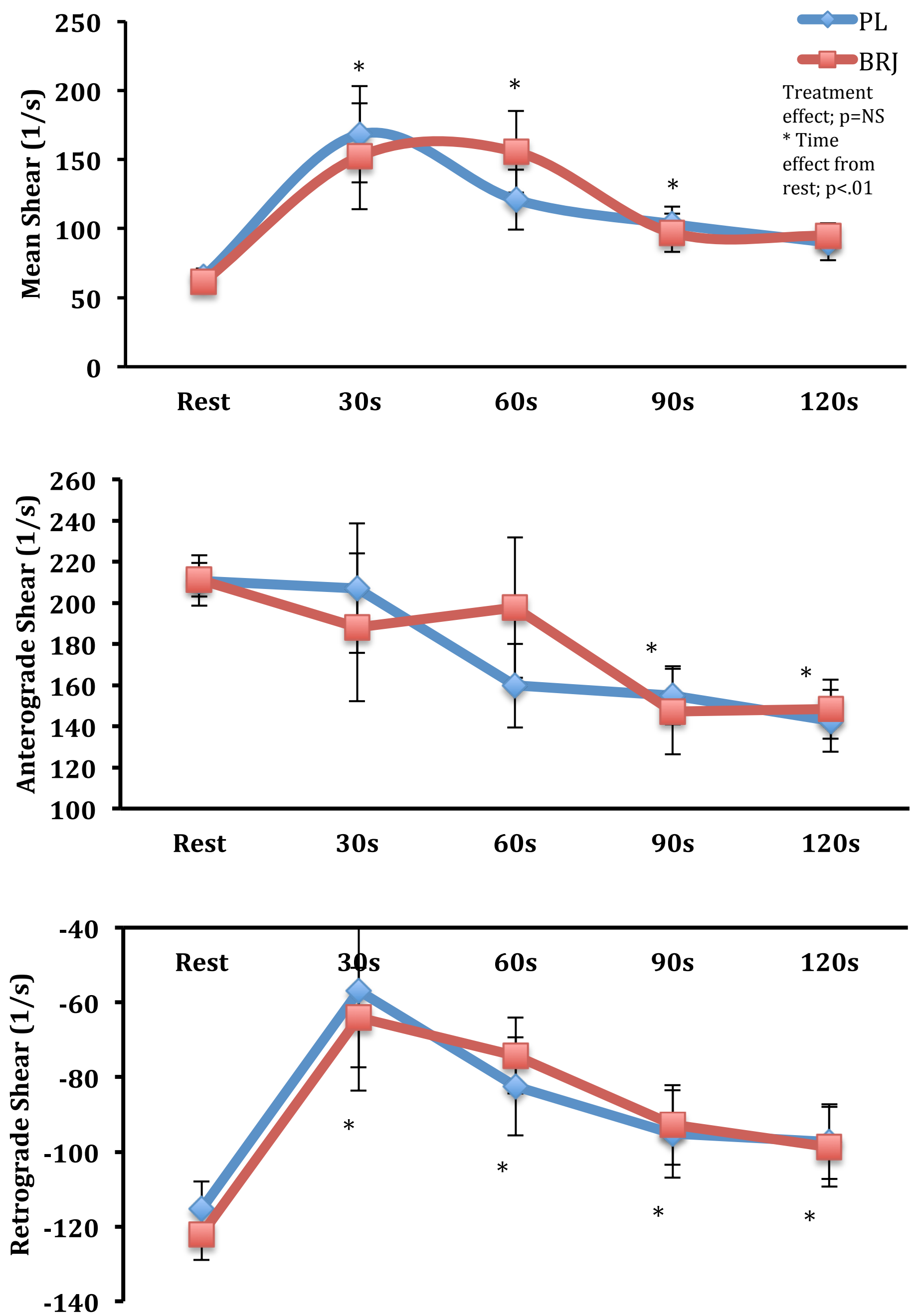


Figure 5 Mean, anterograde and retrograde shear during the first 2 minutes of a serial subtraction test. Values are presented as mean ± SEM

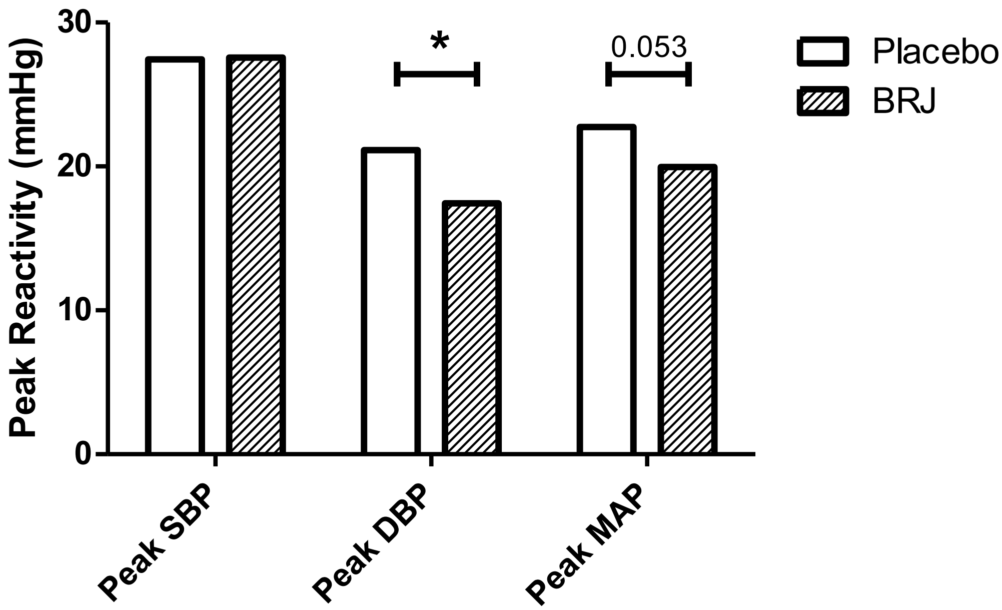


Figure 6 Peak increase in blood pressures above baseline during a three-minute serial subtraction test. *p<0.05

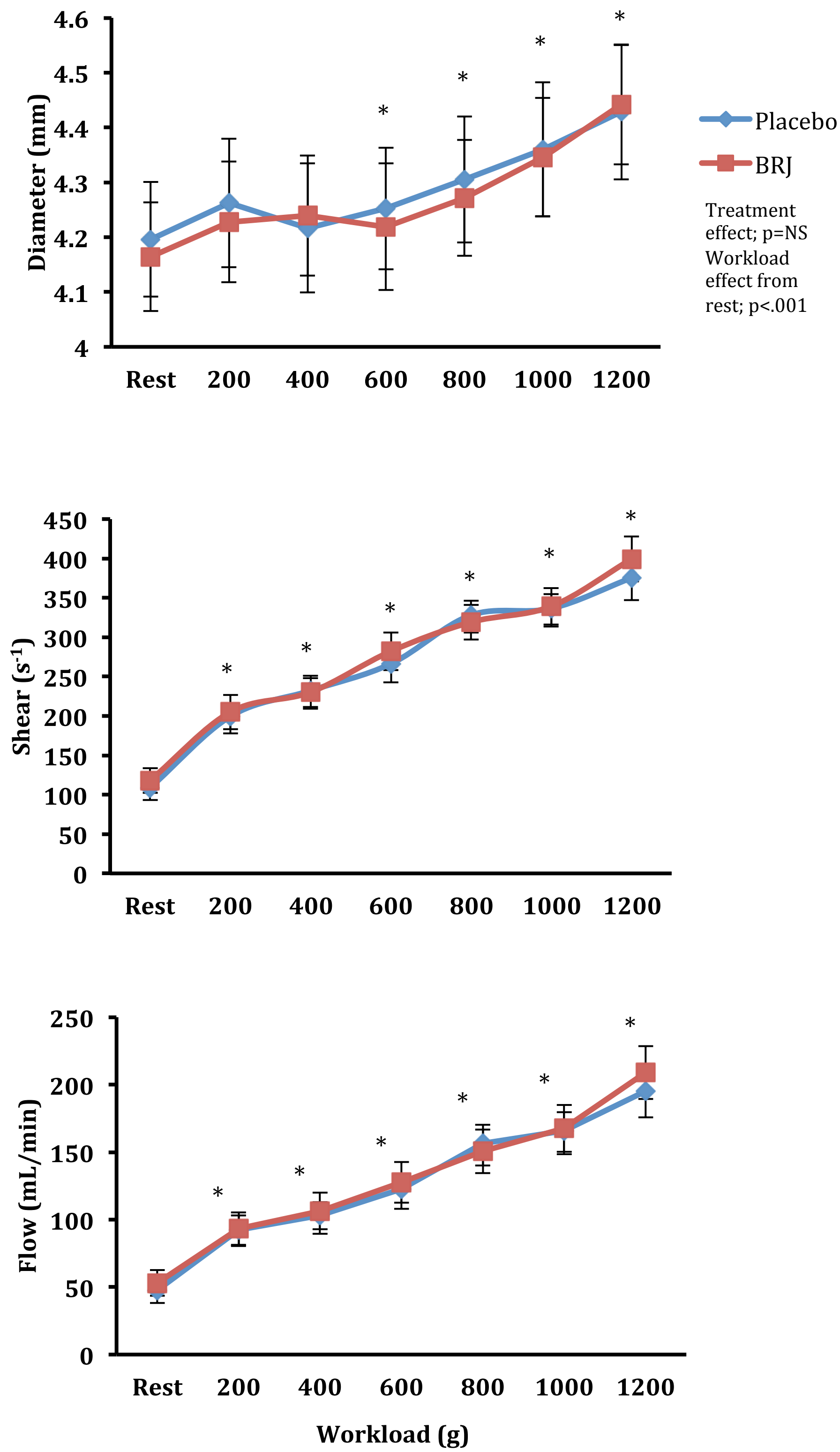


Figure 7 Diameter, shear and flow responses in the brachial artery to graded handgrip exercise. Values are presented as mean ± SEM.

Discussion

The cardiovascular health benefits of diets rich in fruits and vegetables are well established.[28, 29] The influence of nitrate-rich/nitrate-supplemented foods and beverages on cardiovascular health has received particularly close attention lately. Inorganic nitrate found in high quantities in certain vegetables and fruits can provide the physiologic substrate for reduction to nitrite and nitric oxide, which in turn can produce vasodilation and other vasoprotective actions. The primary aim of the present investigation was to determine if a nitrate-rich beetroot juice supplement enhances endothelium-dependent vasodilation in the brachial artery of healthy young adults. Vasodilatory responses to graded handgrip exercise were examined in an attempt to examine endothelium-dependent responses across a broad range of shear stimuli. A secondary aim of this investigation was to gain preliminary insight into the effects of this supplement on systemic (blood pressure and pulse wave velocity) and local (femoral artery vasodilation and shear patterns) responses to a mental stress task (serial subtraction).

Nitrate Supplementation and Endothelial Function

The primary finding of the present study was that oral consumption of nitrate-rich beetroot juice had no effect on brachial artery hemodynamics at rest or in response to graded handgrip exercise in healthy young men, despite significantly elevated plasma nitrate (17-fold) and nitrite (1.6-fold) concentrations relative to the placebo study visit. The similarity of the brachial artery diameter response (absolute or relative to the vessel shear stimulus) during graded handgrip exercise on the two study visits suggests that endothelium-dependent dilation was not enhanced by a single dose of inorganic nitrate in these healthy young volunteers. This is consistent with the findings of Bahra et al who reported no effect of potassium nitrate ingestion (capsules) on brachial artery flow mediated vasodilation (FMD, the conventional test of endothelium-dependent vasodilation) in healthy 18 to 45 year old men and women.[12] Both studies measured endothelial function at 2.5 to 3 hours post nitrate consumption, a time frame repeatedly shown to result in peak plasma nitrite concentrations.[9, 11, 14, 30-32]

The absence of an acute effect of dietary nitrate supplementation on endothelium-dependent dilator responses in the subjects from our study and that of Bahra et al was not simply due to the fact that FMD cannot be enhanced in healthy subjects with presumably normal endothelial function. Very small, but statistically significant improvements in brachial FMD have been reported in healthy volunteers following ingestion of 200 mg of spinach (+0.5%), flavonoid-rich apples + spinach (+0.9%), and sodium nitrate.[21, 33] Non-significant alterations in endothelium-dependent responses in the present study are also not likely due to a lack of biological effect of the beetroot juice supplement we used, as we observed a significantly reduced pulse wave velocity on the nitrate study visit (vs. placebo visit) in our subjects.

Nitrate Supplementation and Limb Blood Flow

Gladwin & colleagues have observed significant increases in blood flow to the resting forearm of healthy humans in response to intra-arterial infusions of supraphysiological (+175% increase in blood flow) and physiological (+30% increase in blood flow) doses of sodium nitrite.[32, 34] This group also reported enhanced forearm blood flow during handgrip exercise during a single handgrip exercise workload (unknown) after sodium nitrite infusions.[34] To our knowledge, the present study is the first to determine if dietary nitrate supplementation impacts limb blood flow at rest or during exercise in humans. We hypothesized, based on the intra-arterial nitrite infusion studies of Gladwin and colleagues, that dietary nitrate supplementation would augment forearm (brachial artery) blood flow at rest and during graded handgrip exercise. To the contrary, we observed no differences in resting or exercising forearm blood flow with this intervention. The reasons for this are unclear. Acute nitrate supplementation via beetroot juice has also been linked to improvements in muscle tissue oxygenation during exercise in a hypoxic environment.[35, 36] Moreover, Ferguson et al reported that chronic nitrate supplementation via beetroot juice augmented blood flow and vascular conductance in rodent hind limb muscles during submaximal treadmill exercise that were composed predominantly of type IIb+d/x fibers (fast-twitch glycolytic). Previous non-exercise studies have also demonstrated the importance of inorganic nitrate during periods of hypoxia.[34, 37-41] While highly speculative, the light to moderate workloads utilized in the present study may not have caused sufficient metabolic perturbation to induce the conversion of NO from tissue nitrite stores in our subjects.

Nitrate Supplementation and Resting Blood Pressure

Multiple studies have reported a decrease in resting systolic blood pressure (and in some cases diastolic pressure) in healthy volunteers following consumption of nitrite salt (capsules) or nitrate enriched juice.[9, 10, 12, 14, 15, 31, 42-45] We did not observe a nitrate treatment effect on resting blood pressure, with mean values on the two study visits nearly identical (within 1-2 mmHg) for both SBP and DBP. Moreover, we could not attribute the substantial inter-subject variability in blood pressures between placebo and nitrate supplement visits (figure 1) to any of our other physiological outcome variables (e.g., baseline nitrate/nitrite concentrations, etc). The absence of any consistent effect on resting blood pressure in our study was not likely due to a low sample size *per se*, as our subject number (n=13) meets or exceeds that of 8 studies in younger healthy participants included in a recent meta-analysis of dietary nitrate supplementation and resting blood pressure.[46] Our exclusive use of male subjects would also tend to weight the study in favor of a blood pressure lowering effect, given a previous report showing more marked effects in men compared to women.[10] Given the above, we speculate that variability in our procedures for measuring resting blood pressure (i.e., seated rest for subjects 1 through 8: supine rest for subjects 9-13), coupled with a relatively modest effect of acute dietary nitrate supplementation on resting [systolic] blood pressure in normotensive adults, (~4 mmHg reduction) may explain our null findings with respect to resting blood pressure.[46] It is important to point out, however, that there are several published studies that have not observed a nitrate supplementation treatment effect on resting blood pressure.[33, 42, 43, 47, 48]

Nitrate Supplementation and Cardiovascular Responses to Acute Mental Stress

Normotensive individuals who exhibit large increases in blood pressure during psychological/mental stress are at higher risk of developing future hypertension.[23] The prognostic power of stress-induced BP responses varies by type of stress challenge and sex, with heightened responses to challenging mental achievement tasks (video games, mental arithmetic) being more predictive of future hypertension in younger men than traditional cold pressor testing.[23, 49] The present study provides preliminary evidence for a blunted peak diastolic BP response to mental arithmetic following consumption of nitrate-rich beetroot juice in young men (figure 7). While modest in magnitude (~4 mmHg smaller rise during supplement vs. placebo visit), the lower peak diastolic BP responses observed during the nitrate-supplemented visit in these young men may be clinically relevant given the association of heightened BP reactivity with future risk of hypertension and with ambulatory BP elevations in response to everyday stressful circumstances.[23, 50-54] Nitrate supplementation had no effect on systolic BP or heart rate reactivity during serial subtraction testing in our subjects, despite substantial increases in these variables (+27 mmHg and +23 bpm above baseline values).

In addition to raising systemic blood pressure, mental stress evokes transient vasodilation/hyperemia in the forearm muscles.[55-57] This local response is mediated by beta-2 (circulating epinephrine) and shear-induced (nitric oxide) stimuli, and is attenuated in patients at risk of hypertension.[50, 51, 58-60] Whether leg arterial vessels respond in a similar fashion to mental stress has not been extensively studied, and variability exists in the limited available studies that have included these measurements.[27, 61-63] However, the 165% increase in femoral blood flow we observed is consistent with a nearly 200% increase in femoral blood flow previously reported in healthy young men and women.[55] Augmented retrograde flow patterns, which have previously been observed during sympathetic "vasoconstrictor" maneuvers (e.g., cold pressor testing), were not observed in our subjects during mental stress testing under either supplemental condition. Such findings, if replicated in larger trials, would be suggestive of a systemic (circulating epinephrine) effect of mental stress on the limb arterial vasculature in healthy adults.

Leg vasodilation and femoral artery shear patterns during (the first 1-2 minutes of) mental stress did not appear to be impacted by nitrate supplementation, although our limited sample size precludes any definitive conclusions.

Experimental Considerations

Strengths of the current investigation include our use of a double-blind, randomized cross-over study design in a carefully screened sample of healthy younger men. This was also one of only two identified studies examining vascular outcomes of dietary nitrate supplementation to use a "true placebo" (i.e., nitrate-depleted Beetroot juice; e.g., Gilchrist et al 2013) and one of very few acute supplementation studies that did not "artificially" deplete subjects' diets of nitrate containing foods prior to supplementation (i.e., nitrate-free run in diet; e.g., Coles & Clifton 2012). We view the use of a true placebo plus standardized pre-visit instructions to avoid caffeine, exercise, and oral anti-bacterial hygiene (teeth brushing, mouthwash) as the most rigorous means of testing the effects of nitrate supplementation *per se* (in the context of supplementing a typical American diet) on vascular function.

Despite these rigorous study design features/experimental controls, the increases in plasma nitrite concentration seen in the present study were lower than previously reported. We utilized standardized blood collection methods and the plasma samples were analyzed with the ENO-20 absorbance technique. Increases in plasma [nitrite] of 2.5-4 fold are typical in the literature and elicit a blood pressure lowering effect.[30, 47] We observed a 1.6 fold increase in plasma [nitrite], and although it was statistically significant, there may not have been enough nitrate-nitrite-NO conversion to cause a significant attenuation of resting blood pressures in these normotensive subjects.

Although the sample size used in this study was consistent with previous studies using healthy, younger males, post hoc power analyses suggest that many of the outcome variables were underpowered (see table 3).

Beetroot juice contains several components in addition to nitrate, which may facilitate the reduction of nitrate to nitrite and NO (anti-oxidants, polyphenols) and/or have vascular (e.g., blood pressure lowering) effects. As such, the use of a true placebo (with only the nitrate extracted) rather than water or other juice product could obscure the effects of the active treatment.   However, dose-response studies by Kapil et al (2010) and Lansley et al (2011) suggest this is unlikely, at least with respect to the outcomes they studied (resting blood pressure and exercise tolerance).

Chapter 2

CONCLUSIONS AND FUTURE DIRECTIONS

1. The present results and those of Bahra et al suggest that acute nitrate supplementation does not alter conduit artery endothelial function in healthy young adults.[12] The graded handgrip exercise model employed in the present study (including normalization of dilator responses to the local shear stimulus) should be used to examine similar questions in populations with established endothelial dysfunction such as older adults, hypertensives, and diabetics. It would also be informative to examine the ability of dietary nitrate supplementation to restore endothelial function in a healthy young cohort following disturbed blood flow-induced or ischemia reperfusion-induced injury.[9, 64] Such studies would expand our understanding of the therapeutic (and cytoprotective) potential of dietary nitrate supplementation beyond its potential blood pressure lowering effects.

2. The present study is the first to directly assess the effects of acute dietary nitrate supplementation on blood flow to exercising skeletal muscle in humans. While no supplementation effects were observed during these lower intensity exercise workloads, further studies of similar design should be conducted to determine the effects on muscle blood flow during higher intensity exercise; conditions under which the nitrite reduction to NO would more likely be induced.[13, 65] Such investigations could have important implications for exercise tolerance in clinical populations with ischemic muscles (e.g. peripheral artery disease).

3. We did not observe a significant blood pressure lowering effect at rest in these healthy young men, which is not without precedent i.e., references [4, 33, 42, 43, 47, 48], but which could have been due to the variation in measurement posture (upright in first 8 subjects, supine in last 5 subjects), a limited overall sample size, and/or our use of a true placebo (see discussion above). The findings of the present study suggest that each of these respective factors should be carefully controlled/considered in future studies on this topic.

4. The results of the SST sub-study (n=7) suggest that acute dietary nitrate supplementation may reduce diastolic blood pressure responses to mental stress in healthy adults. Given the small sample size and potential clinical relevance of this preliminary finding, further investigation of systemic and local hemodynamics in a larger group of subjects may be warranted. Peripheral venous pressure, which rises instantly and stays elevated during mental arithmetic[66], could also be measured in follow-up studies, given the well-established veno-dilator effects of nitrates.[34]

Appendix

Informed Consent Form

Informed Consent Form for Biomedical Research
The Pennsylvania State University

Title of Project: Acute vascular effects of nitrate-rich beet juice

Principal Investigator: *David N. Proctor, Ph.D.*
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1. Purpose of the study:

This research study will examine the effects of a single dose of beet juice on the stiffness of the arteries and the ability of blood vessels in the arms and legs to dilate (i.e., widen). The knowledge gained from this study will be useful in evaluating the growing use of dietary nitrate supplements in the prevention and treatment of cardiovascular diseases such as high blood pressure. This study is being paid for by the Social Science Research Institute at Penn State University.

2. Procedures to be followed:

This research is being performed by David Proctor, Professor of Kinesiology, trained graduate and undergraduate students working in his laboratory, and Dr. Proctor's colleagues in the College of Health and Human Development and the Penn State College of Medicine. A member of Dr. Proctor's research team will fully explain each procedure that applies to your participation. The study and its procedures are outlined below:

Visit 1 – Screening/Familiarization Visit. *Prior to this visit, you should be fasted (no food, water only for 12 hours prior to arrival) and should not consume any alcohol or dietary supplements (48 hours prior) or participate in any exercise workouts (24 hours prior: no weight lifting or sustained whole body exercise lasting longer than 15 minutes).* Upon arrival, your height, weight, resting heart rate and blood pressure will be measured at the Clinical Research Center (CRC). Medical staff at the CRC will also give you a medical review and provide you with questionnaires to evaluate your medical history, healthy history, and physical activity/dietary habits. Also, a blood sample from a vein in your arm will be taken (approximately 15 mL or about 1 tablespoon). This blood sample will be analyzed to ensure that various markers of the function and health of your kidney, liver, and heart/blood (e.g., cholesterol, fat, and oxygen carrying proteins) are within normal limits for your age. A CRC medical staff member experienced in performing blood draws will take this blood sample. If the initial blood draw is unsuccessful it will need to be repeated, possibly from a vein in your hand, with your permission.

You will then be escorted down the hall to the Vascular Aging and Exercise Laboratory (room 201 Noll Laboratory) where you will become familiarized with our blood vessel measurement devices and forearm exercise testing machine. Finally, you will be asked to squeeze a handgrip device as hard as you can. The highest force you develop during three such maximal efforts will determine your maximal grip strength.

Visit 2 – Experimental Visit. You will be asked to arrive at the CRC in the morning, after a 12 hour overnight fast (no food, only water). *Prior to this visit, you should not consume any alcohol or dietary supplements (48 hours prior) or participate in any exercise workouts (24 hours prior: no weight lifting or sustained whole body exercise lasting longer than 15 minutes).* After a seated resting blood pressure measurement, a blood sample from a vein in your arm will be taken by a CRC staff member. This sample will be used to determine the amount of nitrate (a naturally occurring substance in your body that relaxes blood vessels) and its by-products in your blood. If the initial blood draw is unsuccessful it will need to be repeated, possibly from a vein in your hand, with your permission. Following the blood draw, you will be given a small snack (e.g., granola bar or other carbohydrate food, with water) to help reduce feelings of hunger. Shortly after, you will be asked to consume 140 mL (9.5 tablespoons) of either nitrate-rich beet juice (known as the "active" drink) or beet juice with nitrates removed (known as the "placebo" drink). You will not be able to tell which drink you are consuming. You will then be asked to wait in the CRC or Noll lab for approximately 2.5 hours to allow time for the active (or placebo) drink to be fully digested and absorbed. During these 2.5 hours, you will be able to use a personal laptop computer or other portable electronic device. We will also have a CRC staff member take your seated blood pressure one hour, and two hours, after consuming the drink. A second blood sample will then be collected in the CRC after 2.5 hours, to determine if there is an increase in nitrate or its by-products in your blood after consuming the beet juice. If the initial blood draw is unsuccessful it will need to be repeated, possibly from a vein in your hand, with your permission. After this blood sample is collected, you will be escorted to the Vascular Aging and Exercise Laboratory (room 201 Noll Laboratory) to have the following procedures:

1. Measurement of blood pressures and vessel stiffness in your arms and legs
2. Measurements of resting blood flow through an artery in your mid-thigh.
3. Measurements of blood flow through an artery in your mid-thigh and blood pressure during a serial subtraction test
4. Measurements of the size of an artery in your arm before (rest), during, and after rhythmic exercise of your forearm muscles.

*Each of the above procedures is described in more detail below.

Visit 3 – Experimental Visit. At least 4 days after visit 2, you will return to the CRC to repeat the same procedures as you did during visit 2. Procedures and timeline will be identical to those during visit 2 except that you will consume whichever drink supplement you did not receive during visit 2, i.e., the active (nitrate-containing) drink or the placebo (nitrate-removed) drink. The order of these two drink supplements will be randomly determined for each participant.

The details of the measurements and procedures conducted during visits **2** and **3** are as follows:

Consumption of beet juice (nitrate-rich form). Shortly after finishing your snack, you will be asked to consume a 140 mL (9.5 tablespoons) dose of a commercially available nitrate rich ("active") beet juice supplement. Beets are naturally high in nitrates; the nitrate content in this beet juice supplement is 0.8 grams of nitrate, which is about the amount of nitrate found in 5 servings of spinach (American Heart Association recommends 5 servings of vegetables a day). Nitr**a**tes obtained from beets or other food/drink sources are broken down in the body to nitr**i**tes and eventually to nitric oxide, a substance that can dilate (widen) blood vessels and mildly reduce blood pressure.

Consumption of beet juice (without nitrates): On either visit 2 or visit 3 the placebo drink you consume will contain all of the same components as the "active" drink except for the dietary nitrates.

Experimental visit (visits 2 and 3) blood samples. Approximately 10 mL (two thirds of a tablespoon) of blood will be taken from a vein in your forearm (or hand vein if necessary) before and approximately 2.5 hours after you consume the beet juice supplement. A CRC medical staff member experienced in performing blood draws will take these samples.

Measurement of blood pressures and vessel stiffness in your arms and legs: After your second blood draw, you will be escorted from the CRC to the Vascular Aging and Exercise Laboratory where we will ask you to lay comfortably on a hospital stretcher flat on your back. Dr. Proctor's research assistants will place blood pressure cuffs around both of your upper arms and ankles, and a small sensor over your shirt (mid-chest level). You will lie quietly on the stretcher for about 15 minutes to allow your blood pressure and heart rate to stabilize. The cuffs allow us to measure the blood pressure in your arms and legs and to determine how fast heart pulses travel through your blood vessels (an indicator of the stiffness of your arteries).

During each of these resting measurements, the cuffs will inflate to a level that temporarily stops blood flow to both hands and feet, followed by a slow deflation (just like a typical blood pressure measurement in your arm). Three to six measurements of blood pressure/artery stiffness will be collected, depending on the quality of these recordings.

Measurements of blood flow through an artery in your mid-thigh: After removing the blood pressure cuffs from your wrists and ankles, you will remain on the stretcher for detailed measurements of blood flow patterns in your leg. For this measurement, you will need to wear shorts, which we can provide for you if necessary. One of our research assistants will place an ultrasound probe (device which sends/receives sound waves) over a large artery in your mid-thigh. This ultrasound device allows us to measure the size (width) of this artery and the speed/patterns of blood flow through your leg. Blood pressure will also be monitored periodically during these measurements using an arm and/or finger cuff.

Measurements of blood flow and blood pressure during a serial subtraction test: After the resting measures of blood flow patterns are taken in the leg, a research assistant will instruct you to count backwards by a set number starting from a random number between 900-1000. The same ultrasound device as used above will measure the speed/patterns of blood flow through your leg during this subtraction test. Blood pressure will also be monitored continuously using a finger cuff.

Measurements of artery size during forearm exercise: For the final part of this visit, you will be asked to perform repeated hand squeeze exercise (as practiced during visit 1) in which the contraction speed and/or muscle force your produce is gradually increased every 2 to 3 minutes until you cannot maintain the required work rate. The size (width) of your brachial artery (located on the inside surface of your upper arm just above your elbow) will be measured using an ultrasound sensor before, during, and immediately following this graded exercise test to determine how much this vessel in your arm can dilate (widen). Blood pressure will also be monitored during these measurements using an arm and/or finger cuff.

Future use of stored blood samples: During visits 2 and 3, we will collect approximately 3 mL (or 0.2 tablespoons per blood draw) of blood above the minimum we need to complete the measurements we have planned for this study (i.e., measurement of blood nitrates and it's by products). The 3 extra mL of blood we collect provides additional sample in case an error occurs (equipment and/or human error) during our laboratory analysis procedures and we need to re-run a measurement or entire test. It is also possible that some time after we have completed your study visits, we might decide that additional blood measures not described in this consent form (for example, a newly discovered protein that influences blood vessel function) may be useful in helping us learn even more about the effects of beet juice consumption on blood vessel health. Permission is needed in order for us to use your extra stored blood samples for these additional/new measurements. **Please choose one of the following two options below:**

I **DO** give permission to use my stored blood in future studies on vascular health

____ your initials

I **DO NOT** give permission to use my stored blood in future studies on vascular health.

____ your initials

3. Discomforts and risks:

It is not possible to identify all potential risks associated with these research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known or potential risks.

Blood draw: For each of the blood draws (one during visit #1, two during visit #2, and two during visit #3; total of 5 for the entire study), you will feel a small pinch or discomfort while the needle is inserted in your arm. If the initial blood draw is unsuccessful it may need to be repeated, with your permission. Blood draws can also cause mild swelling, bruising (blood under the surface of the skin), and/or continued bleeding at the insertion site; the risk of these happening will be minimized by pressing on the site until any visible bleeding stops. There is also a slight chance of infection developing at the insertion site in the hours following a blood draw. This risk will be minimized and most likely eliminated by having a trained CRC medical staff member draw the blood in the CRC using sterile supplies. Dizziness or fainting during or following a blood draw is also a possible risk. If these symptoms occur, we will have you lie down and help you raise your feet until symptoms improve. If this does occur, we may ask that you remain at the CRC until we have checked your blood pressure, and we are sure that you feel okay.

Consumption of beet juice: There are no known health risks or toxicities associated with daily consumption of nitrate-rich beet juice, a commonly sold health drink/supplement in Europe. The most common side-effect of beet juice consumption is pinkish-colored urine (known as "beeturia"). This can occur after consuming either the nitrate-rich or placebo version of the drink. If this does occur, the pinkish urine usually disappears in 24 hours or less. Because nitrates dilate (widen) blood vessels, there is also a possibility that consuming this supplement will cause your blood pressure to decrease slightly (i.e., 3 to 5 millimeters). Most people will not have any sensations of this reduction in blood pressure. However, in the unlikely event that blood pressure drops enough to cause lightheadedness at any point during this study visit, we will have you remain in the building under the supervision of a CRC staff member until the lightheadedness goes away completely.

Measurement of blood pressures and vessel stiffness in your arms and legs: You may feel a tightness and/or tingling sensation in your hands and feet while the upper arm and ankle cuffs are inflated. These sensations are very temporary since each cuff inflation measurement only lasts for about 1 minute.

Measurements of blood flow through an artery in your mid-thigh: You may feel minor discomfort (pressure) when the research assistant is pressing the ultrasound probe against your mid-thigh to locate a good image of the underlying artery. This discomfort, if it occurs, is very mild and stops immediately after the measurement is completed (measurement takes about 5 minutes). There is also a small risk that the ultrasound gel will irritate your skin. This irritation/redness, if it occurs, should go away soon after the study is completed.

Measurement of artery size during forearm exercise: Minor discomfort and skin irritation associated with ultrasound imaging (described immediately above) may also be felt in your arm before and during the forearm exercise testing. These sensations, if they occur, will go away soon after the study is completed. Forearm muscle fatigue (tiredness), particularly during the higher workloads near the end of the test, is likely. This discomfort goes away quickly after the completion of the exercise. Mild soreness in the muscles of the forearm/hand could develop sometime after the exercise test is completed (usually the day following). This soreness, if it occurs, usually goes away in about 2 days.

4. Benefits:

There are no direct benefits to you for participating in this study other than receiving information about your cardiovascular risk factors (i.e., blood pressure, blood cholesterol, etc.). However, this study may benefit society by providing information about the health effects of nitrate-rich beet juice.

5. Duration/time of the procedures and study:

You will be involved in the research study for no longer than 6 weeks, which will consist of 3 separate study visits. The first study visit will take approximately 3 hours. The following two experimental visits (visits 2 and 3) will each take approximately 4.5 hours. Thus, the total duration of all the research sessions will be approximately 12 hours.

6. Alternative procedures that could be utilized:

There are alternative dietary modifications that could be used to reduce the stiffness of blood vessels (example: salt restriction) or to test the ability of blood vessels to widen (example: temporarily blocking blood flow to the arm). There are also alternative methods of measuring these vascular responses, some non-invasive, others invasive. The measurements we have selected, however, should be sensitive enough to determine if this particular food supplement (i.e., nitrate-rich beet juice) can de-stiffen blood vessels.

7. Statement of confidentiality:

Your participation in this research is confidential. Moreover, data will be stored and secured in the Vascular Aging and Exercise Laboratory (201 Noll Laboratory) in password protected computer files. Any hard copies of data will be stored in locked filing cabinets. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

The Pennsylvania State University's Office for Research Protections, the Institutional Review Board, and the Office for Human Research Protections in the Department of Health and Human Services may review records related to this research study.

8. Right to ask questions:

Please contact Dr. David Proctor at 814-863-0724 (office phone) or 814-571-5234 (cell phone) with questions, complaints or concerns about the research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

9. Payment for participation:

You will be paid a total of $150 for your participation in the entire study ($0 for visit 1, $75 each for study visits 2 and 3). If for some reason you do not complete the study, you will be paid for the visits you did complete with the exception of the screening/familiarization visit.

10. Voluntary participation:

Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise.

Additionally, if you do not comply with the study protocol (e.g., you skip/miss an excessive number of study visits or fail to follow pre-visit instructions) we may not seek your continued participation in this study.

11. Injury Clause:

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

12. Abnormal Test Results:

In the event that abnormal lab test results are obtained during initial screening or subsequently throughout this study, the Clinical Research Center will inform you as quickly as possible of these results and instruct you to contact your private physician for further assessment. The lab test results will be made available to your private physician at your request.

If you have read the information in this form and agree to and give your permission for your participation as a volunteer in the study entitled **"Acute vascular effects of nitrate-rich beet juice"** please sign below. You must be 18 years of age or older. You will receive a signed copy of this consent form.

Participant Signature ___________________________________ Date___________

Person Obtaining Consent _______________________________ Date___________

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