Effects of Chronic Dietary Nitrate Supplementation on the Hemodynamic Response to Dynamic Exercise

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ABSTRACT

While acute treatment with beetroot juice (BRJ) containing nitrate (NO$_3^-$) can lower systolic blood pressure (SBP), afterload and myocardial O$_2$ demand during submaximal exercise, effects of chronic supplementation with BRJ (containing a relatively low dose of NO$_3^-$, 400 mg) on cardiac output (CO), BP, total peripheral resistance (TPR) and the work of the heart in response to dynamic exercise are not known. Thus, in 14 healthy males (22±1 yr), we compared effects of 15 days of both BRJ and nitrate-depleted beetroot juice (NDBRJ) supplementation on plasma concentrations of NOx (NO$_3^-$/NO$_2^-$), SBP, diastolic blood pressure (DBP), mean arterial pressure (MAP), CO, TPR, and rate pressure product (RPP) at rest and during progressive cycling exercise. Endothelial function was also assessed via flow mediated dilation (FMD). BRJ supplementation increased plasma NOx from 83.8±13.8 to 167.6±13.2 uM. Compared to NDBRJ, BRJ reduced SBP, DBP, MAP and TPR at rest and during exercise ($p<0.05$). In addition, RPP was decreased during exercise, while CO was increased, but only at rest and the 30% workload ($p<0.05$). BRJ enhanced FMD-induced increases in brachial artery diameter (pre: 12.3±1.6%; post: 17.8±1.9%). We conclude that: 1) chronic supplementation with BRJ lowers BP and vascular resistance at rest and during exercise and attenuates RPP during exercise and 2) these effects may be due, in part, to enhanced endothelial-induced vasodilation in contracting skeletal muscle. Findings suggest that BRJ can act as a dietary nutraceutical capable of enhancing O$_2$ delivery and reducing work of the heart such that exercise can be performed at a given workload for a longer period of time before the onset of fatigue.

Key words: nitrate, nitrite, total peripheral resistance, afterload, rate x pressure product, endothelial function
Nitric oxide (NO) is a signaling molecule that has numerous functions in the human body, including blood flow regulation, neurotransmission, glucose and calcium homeostasis, muscle contractility and mitochondrial respiration (11, 12, 33). It is well documented that NO is produced by oxidation of L-arginine in a reaction catalyzed by the NO synthases (30). NO can also be produced from dietary nitrate (NO\textsubscript{3}\textsuperscript{−}) via the NO\textsubscript{3}−-nitrite (NO\textsubscript{2}\textsuperscript{−})-NO pathway (28). NO\textsubscript{3}\textsuperscript{−} is found in many vegetables such as, mustard leaf, lettuce, spinach, rucola and beetroot (BR) (20). Following dietary consumption, NO\textsubscript{3}\textsuperscript{−} can be reduced to NO\textsubscript{2}\textsuperscript{−} by commensal bacteria in the oral cavity and then further reduced to NO via numerous pathways that include xanthine oxidoreductase, deoxygenated myoglobin and deoxygenated hemoglobin (28). As such, NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} have been suggested to represent a storage reservoir for NO (5).

NO\textsubscript{3}\textsuperscript{−} appears to be an ergogenic nutraceutical that can improve exercise performance. In this regard, dietary supplementation with BR or sodium nitrate has been shown to reduce submaximal O\textsubscript{2} cost during cycling at a fixed power output (26) and improve exercise time trial performance (7). Since NO\textsubscript{3}\textsuperscript{−} can reduce resting blood pressure (25, 38), chronic dietary supplementation with BR juice (BRJ) may also have beneficial effects on the cardiovascular system during exercise. Acute administration of 500 ml of BRJ containing ~750 mg of NO\textsubscript{3}\textsuperscript{−} has been reported to have effects on the cardiovascular response to graded submaximal cycling exercise in young, overweight women (2). In this regard, BRJ reduced systolic blood pressure (SBP) and rate x pressure product (RPP) (i.e., heart rate [HR] x SBP; an index of myocardial oxygen demand) at rest and during all workloads. Total peripheral resistance (TPR) was also reduced at rest and during all but the highest work intensity. However, no effects on diastolic blood pressure (DBP), mean arterial pressure (MAP), cardiac output (CO) or HR were observed. These findings suggest that a single dose of NO\textsubscript{3}\textsuperscript{−} can reduce the afterload on the heart and lower myocardial oxygen demand at a given exercise intensity. However it is not known if chronic dietary supplementation can maintain these effects on exercise hemodynamics over time and/or modulate other important cardiovascular responses during exercise. For example, these cardiovascular effects of BRJ, particularly reductions in TPR, may be due, at least in part, to enhanced
vascular function, as a positive correlation between plasma levels of nitrite/nitrate and endothelial-induced vasodilation has been established (6). Therefore, we hypothesized that: 1) chronic dietary supplementation with BRJ attenuates SBP, DBP, MAP and RPP and enhances stroke volume (SV), CO and endothelial function responses to progressive elevations in exercise intensity and 2) these effects are partly due to endothelial-induced reductions in peripheral vasoconstriction.
Material and methods

Fourteen healthy male subjects (age 22±1) participated in this study. Prior to testing, all subjects gave written informed consent. All procedures were reviewed and approved by the Kyung Hee University Institutional Review Board (KHU 2014-G08). Subjects were sedentary, non-smokers in good health who were not taking any cardiovascular medications. They were instructed to adhere to their normal living and dietary routines throughout the study. Subjects completed one maximal exercise test and two submaximal tests, and were instructed to avoid strenuous exercise for 48 h prior to each testing session. All exercise tests were performed at the same time of day for each subject. They were asked to refrain from consuming alcohol for 48 h and caffeine for 3 h prior to each test.

Exercise protocols

Resting blood pressure was measured while the subject was in a seated position. At least two measurements were obtained, 2 min apart, using a sphygmomanometer and pressure cuff. To determine the relative exercise intensities for the three workloads used in this study, a maximal exercise test was conducted using a cycle ergometer (Monark 828, Sweden). The protocol began with 2 min of unloaded baseline cycling followed by increases in workload of 30 watts/min until the subject could no longer maintain a pedal cadence of 60 rpm. Pulmonary gases were measured on a breath-by-breath basis using an Ultima CPX Metabolic Measurement Cart (Medgraphic, USA). VO\textsubscript{2peak} obtained from this test was used as an index of functional capacity. Subjects then completed 3 bouts of cycling exercise at constant submaximal workloads corresponding to 30%, 60%, and 80% of their predetermined VO\textsubscript{2peak} values. The duration of each workload was 5 min. To avoid muscle fatigue, subjects performed the 30% and 60% VO\textsubscript{2peak} workloads on the same day, and the 80% VO\textsubscript{2peak} test 48 h later. Subjects exercised at the same absolute workloads before and after BRJ supplementation.

Supplementation

Following completion of the submaximal tests, subjects were randomly assigned via a
double-blind, crossover design to receive 15 days of dietary supplementation with either NO$_3^-$ (400 mg administrated as 70 ml of BRJ/day for a total dose of 6.4 mmol/day) or 70 ml of organic nitrate-depleted beetroot juice/day (NDBRJ) (Beet It™, James White Drink, Ipswich, UK). The BRJ contained protein (2.6g), sugar (9.3g), carbohydrates (15g), fat (0.2g), saturates (<0.1g), fiber (<0.5g), and sodium (<0.1g). Beetroot was chosen because it is condensed compared to other vegetables containing NO$_3^-$ and because it can be consumed as a juice.

Subjects were instructed to drink the juice around the same time each morning. Neither NDBRJ nor BRJ supplements were taken for 24 h prior to exercise testing. NDBRJ was similar to the experimental beverage in appearance, odor, taste, and texture. A two week washout period separated the supplementation periods. The experimental order between the BRJ and NDBRJ supplementation was balanced such that 7 subjects took BRJ first, while the other 7 initially received NDBRJ. The investigators administering the exercise tests were not aware of the type of beverage being consumed by the subjects.

Measurement of hemodynamic variables

SV and HR were measured continuously via impedance cardiography (Physioflow, Manatec Biomedical, France). This device provides real-time CO data and measures cardiac parameters in healthy subjects (21, 31, 36). The bioimpedance device consists of two impedance cardiography electrodes, placed above the supraclavicular fossa at the base of the left side of the neck, two electrocardiography electrodes used for recording the ECG, and two electrodes placed at the xiphoid process. Impedance cardiography measures changes in thorax impedance during the cardiac cycle to calculate SV (3). The Physioflow is an impedance technique that emits a high-frequency (75 kHz) and low magnitude (1.8 mA) alternating electrical current via skin electrodes during the cardiac cycle. This results in a waveform from which SV is calculated (9). Initially, the stroke volume index (SVi) is calculated at rest over 24 heartbeats during an auto calibration procedure and then resting SBP and DBP are entered. The auto calibration measures the largest impedance variation during systole, the largest rate of variation of the impedance signal (i.e., the contractility index), the thoracic flow
inversion time and HR.

CO was calculated according to the following formula: CO = HR x SVi x BSA, where HR is obtained from the R-R interval determined from the first derivative of the ECG. SVi is the SV index (i.e., SV/BSA). BSA (body surface area) (m²) was determined according to the Haycock formula: BSA = 0.024265 x BM^{0.5378} x H^{0.3964}, where BM is the body mass in kg and H is the height in cm. The Physioflow technique has been validated against the direct Fick method at rest and during exercise (9). CO assessed by the direct Fick method is not significantly different from that obtained by the Physioflow at rest and during submaximal and maximal workloads (9, 31).

Brachial artery blood pressure was measured by a sphygmomanometer during the 30%, 60%, and 80% workloads under steady-state conditions. Blood pressure was measured by the same investigator in each subject throughout the experiment. MAP was calculated using the formula: MAP = [(SBP-DBP) x 1/3] + DBP. Total peripheral resistance (TPR) was calculated as MAP/CO and RPP as HR x SBP.

Flow mediated dilation (FMD)

The FMD technique was used to determine endothelial function in the brachial artery before and after BRJ supplementation. This non-invasive technique is a well-known bioassay of peripheral endothelial function that involves the release of a temporary occlusion of the arm vasculature to induce an acute increase in shear stress (8). The subsequent FMD is dependent, in part, on endothelial release of NO (13). Brachial artery diameter and velocity were measured by an ultrasound probe 12 MHz linear-array vascular probe (ClearVue 550, USA). The probe was placed 3-5 cm proximal to the bifurcation of the antecubital fossa. When images were obtained, the width of the artery was insonated at an angle of 60°. Blood velocity was acquired simultaneously using pulsed wave Doppler. Measurements were made by the same examiner in a blinded manner. Ten cardiac cycles were evaluated to calculate baseline arterial diameter. To elicit reactive hyperemia and increase shear stress, a pressure cuff was placed on the upper arm and inflated to 200 mmHg for 5 min, followed by a rapid deflation. Brachial artery images were recorded for 2 min. Peak diameter was determined as the
average of 5 cardiac cycles. Brachial artery images were selected when they occurred near the end of diastole. The absolute change in diameter was determined and FMD was expressed as percent change in diameter from baseline (%Δ FMD).

Measurement of plasma NO\textsubscript{3}⁻ and NO\textsubscript{2}⁻ (NOx)

To obtain blood samples during the NDBRJ and BRJ conditions for measurement of plasma NOx, which was used as an index of NO\textsubscript{3}⁻ levels, a catheter with a 12-gauge needle was inserted into a brachial vein. Five ml of venous blood were obtained at rest before and after both NDBRJ and BRJ supplementation. Samples were then centrifuged at 3,000 rpm for 10 min. Subsequently, the supernate was collected and immediately frozen at -80°C until analysis for NOx.

Plasma concentrations of NOx were assessed by a colorimetric assay using the Griess Reagent in a microtiter format (Cayman, Ann Arbor, Michigan, USA) (37). Spectrophotometric quantitation of nitrite, based on the Griess Reagent, was straightforward and the NADH-dependent enzyme nitrate reductase was used to convert nitrate to nitrite prior to quantitation by the Griess Reagent. The intraassay and interassay coefficients of variation for NOx measurement with this method were 6.6% and 9.2%, respectively.

Data analysis

SV assessed by impedance cardiography was averaged over 30 s periods during steady-state conditions (21, 31, 36). This technique accurately measures absolute values of SV at rest and during submaximal exercise. Effects of dietary NO\textsubscript{3}⁻ on cardiovascular responses during exercise were expressed as absolute values. Mean values of HR, SV, CO TPR and RPP for each 30 sec interval at rest and during exercise were used for comparison purposes. Blood pressure was measured between the 4th and 5th min of each workload, just before blood collection. To compare the effects of BRJ over workloads and between groups, two way repeated-measures ANOVA and Tukey’s post hoc test were used. Mean values of all variables were compared between groups via an independent t test. The Student’s paired t test was used to compare mean differences between NDBRJ and BRJ
supplementation. Statistical significance was accepted at $p < 0.05$. 
Results

Physical characteristics of the subjects are shown in Table 1.

Table 2 presents effects of BRJ supplementation on plasma NOx and brachial artery FMD. BRJ supplementation caused significant increases in resting plasma NOx concentrations and in FMD. NDBRJ supplementation had no effect on NOx concentrations or FMD. FMD was positively correlated to NOx concentrations ($r = 0.4; P < 0.05$).

Absolute values of all hemodynamic variables at rest and during the 30%, 60%, and 80% of VO$_{2\text{peak}}$ workloads were not altered by NDBRJ supplementation.

When compared to pre-treatment conditions, BRJ supplementation reduced SAP, DBP, MAP and TPR at rest and during all workloads, while RPP was attenuated at every workload ($p<0.05$) (Figs. 1 and 2). No effects of BRJ on HR were seen (Fig. 1). SV was higher at rest and at the 30% and 60% of VO$_{2\text{peak}}$ workloads after BRJ supplementation while CO was elevated at rest and during exercise at the 30% of VO$_{2\text{peak}}$ workload ($p<0.05$) (Fig. 2).

Absolute values of all hemodynamic variables at rest and during all workloads were similar when compared between pre NDBRJ and pre BRJ supplementation conditions.

When compared to NDBRJ supplementation, BRJ attenuated SBP, DBP, MAP and RPP responses at rest and during exercise ($p<0.05$) (Figs 3 and 4). In addition, BRJ augmented SV and attenuated TPR at rest and during the 30% and 60% VO$_{2\text{peak}}$ workloads and augmented CO at the workloads of 30% and 60% of VO$_{2\text{peak}}$ ($p<0.05$) (Fig. 4). No differences in HR were found between the two conditions at rest or across any workload (Fig 3).
Discussion

An important new finding of this investigation is that chronic supplementation with dietary BRJ (i.e., 15 days) in healthy males increased baseline concentrations of plasma NOx and decreased SBP, DBP, and MAP and at rest and during exercise. It appears that these NO3- induced attenuations in blood pressure were associated with concomitant attenuations of TPR, suggesting that the afterload on the heart was reduced; a contention that is supported by the fact that our index of myocardial oxygen consumption, RPP, was also attenuated. In the rat model, BRJ supplementation has been reported to increase blood flow and conductance in the kidney in resting conditions. During exercise, this effect dissipates but appears to be compensated for by augmentations in blood flow and conductance in exercising skeletal muscle compared to control animals (15, 16). Such responses to BRJ supplementation in the current study might also account for the subsequent reductions in TPR.

In our subjects, it was unclear how NO3- supplementation caused attenuations in TPR during exercise. This effect may have been related to an increase in NO bioavailability associated with a greater storage of NO3- for conversion to NO (5). Supporting this possibility is the observation that acute administration of NO3- increases plasma concentrations of NO2- available for conversion to NO (2). Data from both human and animal studies have found that endothelial-based, NO-dependent vasodilation contributes to exercise-induced vasodilation in contracting skeletal muscle (19, 44). Coupled with our findings that BRJ supplementation markedly enhanced endothelial function (i.e. increased FMD), it seems reasonable to speculate that NO-induced vasodilation in contracting skeletal muscle was enhanced to the point where it affected subsequent reductions in TPR.

Another explanation for the potential influence of NO3- induced increases in NO on skeletal muscle TPR relates to effects on sympathetic nerve activity. During exercise, increases in sympathetic nerve activity occur that are capable of causing vasoconstriction in contracting skeletal muscle (4, 40). However, this vasoconstriction can be reduced by NO-evoked functional sympatholysis that is associated with a direct inhibitory effect on α1-receptor responsiveness to norepinephrine (4, 34). This phenomenon could lead to a scenario where BRJ supplementation causes increases in NO bioavailability that, in turn, enhance the release of NO from the vascular endothelium of contracting
skeletal muscle and subsequently reduce local vasoconstriction and TPR.

A recent study investigated the effect of acute dietary nitrates (500 ml, 1,500 mg/L) on physiological responses in healthy women during graded cycling exercise (40%, 60%, 80% VO\textsubscript{2peak}) (1). They reported that BRJ treatment did not change cardiac output (CO) at rest or across workloads. This is an important consideration, since it is not known whether there are similar effects following chronic supplementation. Our study found that BRJ supplementation augmented exercise-induced increases in SV and CO at the two lower workloads compared to NDBRJ treatment. These increases in SV were probably due to a reduction in the inhibitory effects of afterload associated with concomitant reductions in MAP and TPR. It is not clear why increases in SV and CO reductions in TPR were not augmented at the highest workload. However, the blood pressure response was greatest during this workload, while the absolute reduction in MAP evoked by NO\textsubscript{3} supplementation was not different from that seen at the lower workloads. Thus, the corresponding reduction in afterload at the highest workload may not have been sufficient to allow SV to increase. Additional findings from the present study also differ from those reported by Bond et al (1, 2), who assessed acute effects of a single dose of BRJ on the cardiovascular response to cycling. Chronic supplementation with our dose of NO\textsubscript{3}, which was approximately half of the acute dose used by Bond et al (1, 2), caused modifications in the cardiovascular responses to cycling at similar work intensities that were not seen following acute administration of BRJ. They included reductions in DBP and MAP at all workloads compared to NDBRJ and increases in CO at the two lower workloads. The pattern of effects on SBP and TPR were similar. Thus, our results indicate that effects of an acute dose of NO\textsubscript{3} on cardiovascular function during exercise can not only be maintained via dietary supplementation with a smaller dose but also can be extended such that cardiac function (i.e., SV and CO) is enhanced and afterload on the heart is reduced further (i.e., reductions in DBP and MAP). However, since the subjects in the Bond et al studies (1, 2) were females, we cannot rule out possible intervening effects of gender. BRJ contains vasoactive factors other than nitrates (i.e., polyphenols, flavonoids and quercetin) (22) that may have contributed to the attenuations in blood pressure, resistance and work of the heart that we observed following our supplementation period. However, since our alternative to a
placebo (i.e., NDBRJ) consisted of BRJ that had been selectively removed of only NO₃⁻; any effects of other vasoactive factors would have been present in equal amounts in both conditions (BRJ and NDBRJ). Consequently, we feel confident that differences observed between the two conditions were due to specific effects of NO₃⁻.

We selected a dose of NO₃⁻ of 400 mg/day for dietary supplementation because it is within the lower range of acute and chronic doses that have been shown to lower resting MAP (1, 2, 38, 45). In addition, this dose was chosen because there is evidence suggesting that high intakes of dietary NO₃⁻ may have harmful effects on health (e.g., cancer), which has led to some regulation of nitrate concentrations in food and drinking water (43). Consequently, we opted to use a lower dose to limit exposure of NO₃⁻ beyond that received from routine dietary sources and to minimize safety issues that might arise compared to supplementation with higher doses of this ion. That being said, it should be noted that the traditional diet of the Japanese is high in nitrates (1300 mg/day for a 73 kg individual) (32), yet they have the highest longevity in the world and a low occurrence of heart disease and cancer (46). This observation, and other emerging evidence suggesting that dietary nitrates have beneficial cardiovascular effects, suggests that the risk-to-benefit ratio associated with dietary intake of nitrates and nitrates should be reevaluated.

**Limitations of the Study**

A limitation to our study was the fact that we measured NOx, which represents values of both NO₂⁻ and NO₃⁻. As a result, specific effects of BJR supplementation on plasma concentrations of NO₂⁻ were not quantified. This is an important distinction because conversion of NO₃⁻ to NO₂⁻ is necessary for biological effects to occur (42).

**Perspectives and significance**

Compared to acute effects of BRJ, the relevance of our results relates to the ability of NO₃⁻ to act chronically as a dietary neutraceutical that is capable of maintaining or enhancing its acute effects on oxygen delivery at a given level of exercise, while also causing reductions in blood pressure and
work of the heart. Consequently, the onset of fatigue may be delayed in healthy individuals and athletes, allowing for exercise to be performed for longer periods of time.

These effects of chronic dietary supplementation with NO3⁻ also have clinical implications. It is well known that functional capacity and exercise tolerance are reduced in pathological conditions such as hypertension, heart failure, coronary heart disease and diabetes, which can limit the ability to perform work and participate in activities of daily life (10, 23, 27). Since such limitations are related to endothelial dysfunction, increases in vascular resistance and reductions in skeletal muscle blood flow (10, 14, 27, 39), regular treatment with dietary NO3⁻ may at least partially offset these debilitating effects.

It is also of note that cardiac events such as myocardial infarction can be precipitated by physical exertion (29, 35) and that high levels of blood pressure may play a role (17, 24). Thus, dietary BRJ may provide a non-pharmacological intervention that reduces the risk of cardiac events during exercise; especially in conditions where cardiovascular function is compromised (e.g., hypertension, heart failure, ischemic heart disease).

Conclusions

The results of our study demonstrate that chronic dietary supplementation with BRJ (containing a daily dose of 400 mg of NO3⁻) increases NOx in the plasma, lowers blood pressure and vascular resistance at rest and during exercise. Reduces RPP during exercise. It also profoundly augments endothelial function, particularly when compared to effects of other neutraceuticals such as polyunsaturated fatty acids and flavanols (18, 41).

Supplementation additionally enhanced cardiac function via augmented exercise-evoked increases in SV and CO at our lower workloads. The cardiovascular effects seen during exercise were most likely due to reductions in the afterload on the heart and to diminished peripheral vasoconstriction (most likely in the exercising skeletal muscle).

These findings suggest that BRJ can act as a dietary ergogenic supplement capable of enhancing oxygen delivery and reducing work of the heart, allowing exercise to be performed at a given
workload for a longer period of time before the onset of fatigue. BRJ supplementation may also represent an alternative, more natural intervention for individuals that suffer from reductions in functional capacity and exercise tolerance related to cardiovascular diseases such as hypertension, heart failure and cardiovascular ischemia.
Figure Legends

Fig. 1. SBP, DBP, MAP and HR at rest and during exercise before (Pre) and after (Post) supplementation with beetroot juice (BRJ). *p<0.05, vs. pre-supplementation

Fig. 2. CO, SV, TPR and RPP at rest and during exercise before (Pre) and after (Post) supplementation with beetroot juice (BRJ). *p<0.05, vs. pre-supplementation

Fig. 3 SBP, DBP, MAP and HR at rest and during exercise following supplementation with NO3− depleted beetroot juice (NDBRJ) or beetroot juice (BRJ). *p<0.05, vs. NDBRJ.

Fig. 4 CO, SV, TPR and RPP at rest and during exercise following supplementation with NO3− depleted beetroot juice (NDBRJ) or beetroot juice (BRJ). *p<0.05, vs. NDBRJ.
Reference List


Figure 1
Figure 2
Figure 3
Figure 4
Table 1. Physical characteristics of the subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subjects (n=14)</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
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<td>Height (cm)</td>
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<td>Body weight (kg)</td>
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<td>BMI (kg/m²)</td>
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<td>SBP (mmHg)</td>
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<td>VO₂ peak (ml/kg/min)</td>
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Values are expressed as means ± standard error; BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, HR: heart rate, VO₂ peak: peak oxygen uptake.
Table 2. Effects of beetroot juice with or without dietary nitrates on plasma NOx and brachial artery FMD at rest.

<table>
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<th>NDBRJ</th>
<th>BRJ</th>
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<td>Pre</td>
<td>Post</td>
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<tr>
<td>NOx (uM)</td>
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<td>Baseline diameter (mm)</td>
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<td>Maximal diameter (mm)</td>
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<td>Δ Absolute diameter (mm)</td>
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<td>0.54±0.09</td>
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<tr>
<td>FMD (%)</td>
<td>14.5±1.9</td>
<td>14.4±2.5</td>
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</table>

NDBRJ: nitrate depleted beetroot juice, BRJ: beetroot juice; NOx: nitrate/nitrite, FMD: flow mediated dilation. Values are expressed as mean ± standard error. *p<0.05 vs. Pre-supplementation.