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Plasma nitrate and nitrite are increased by a high nitrate supplement, but not by high nitrate foods in older adults

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Abstract

Little is known about the effect of dietary nitrate on the nitrate/nitrite/NO (nitric oxide) cycle in older adults. We examined the effect of a 3-day control diet vs. high nitrate diet, with and without a high nitrate supplement (beetroot juice), on plasma nitrate and nitrite kinetics, and blood pressure using a randomized four period cross-over controlled design. We hypothesized that the high nitrate diet would show higher levels of plasma nitrate/nitrite and blood pressure compared to the control diet, which would be potentiated by the supplement. Participants were eight normotensive older men and women (5 female, 3 male, 72.5±4.7 yrs) with no overt disease or medications that affect NO metabolism. Plasma nitrate and nitrite levels and blood pressure were measured prior to and hourly for 3 hours after each meal. The mean daily changes in plasma nitrate and nitrite were significantly different from baseline for both control diet+supplement ($p<0.001$ and $=0.017$ for nitrate and nitrite, respectively) and high nitrate diet+supplement ($p=0.001$ and 0.002), but not for control diet ($p=0.713$ and 0.741) or high nitrate diet ($p=0.852$ and 0.500). Blood pressure decreased from the morning baseline measure to the three 2 hr post-meal follow-up time-points for all treatments, but there was no main effect for treatment. In healthy older adults, a high nitrate supplement consumed at breakfast elevated plasma nitrate and nitrite levels throughout the day. This observation may have practical utility for the timing of intake of a nitrate supplement with physical activity for older adults with vascular dysfunction.

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Conflicts of interest

Kim-Shapiro is listed as a co-author on a patent application entitled "USE OF NITRITE SALTS FOR THE TREATMENT OF CARDIOVASCULAR CONDITIONS".

Keywords

older adult; beetroot juice; nitric oxide; cross-over controlled

Introduction

According to the most recent projections from the United States Census Bureau the number of individuals over the age of 65 years will double from ~35 million in 2000 to 71 million in 2030[1]. Maintaining function in this population is a public health priority. In older adults, the degree of vascular disease correlates with good health and function, and improvements in vascular function are linked to improved quality of life[2]. A key aspect of vascular endothelial dysfunction is a reduced ability to make nitric oxide (NO) via L-arginine dependent pathways by endothelial nitric oxide synthase. It is well documented that NO enhances blood flow, decreases platelet aggregation, improves mitochondrial function, and diminishes cellular vascular adhesion[3,4]. Nitric oxide is also produced independently of the L-arginine-NOS (nitric oxide synthetase) pathway through the nitrate/nitrite/NO cycle. Until recently nitrite was thought to be biologically inert[5]. However, it has been shown that supraphysiological amounts of plasma nitrite increase forearm blood flow and this activity is enhanced under hypoxic conditions[6].

Substantial elevations in plasma nitrite can occur through increasing dietary nitrate intake[7]. Absorbed nitrate is concentrated in the salivary gland and subsequently reduced to nitrite by symbiotic, oral bacteria; a portion of the nitrite is then absorbed and found in plasma[7,8]. In the United States, dietary intake of nitrate is around 40-100 mg/day, with 85% coming from vegetables[9]. Dietary nitrate can be greatly enhanced, reaching an intake of over 1200 mg (nearly 20 mmoles) by making appropriate selections of high nitrate containing foods as part of the DASH diet (Dietary Approaches to Stop Hypertension). This is more than 5 fold above the World Health Organizations Acceptable Daily Intake of 3.7 mg nitrate/kg body weight /day and more than 2 fold above the United States Environmental Protection Agency of 7.0 mg nitrate/kg body weight/day for a 60 kg individual[10].

Little is known about the effect of dietary nitrate intake on the nitrate/nitrite/NO cycle in older adults. This is an important gap in the literature since the cycle is known to be affected by a number of factors that may be altered in older adults, including achlorhydria, medications that alter gastric pH, dry mouth, oral bacteria populations, and intestinal health. In addition, the potential health implications of increasing dietary nitrate to raise plasma nitrate and nitrite are critically important for health and function in older adults. For example, dietary supplementation of nitrate has been shown to reduce blood pressure[11,12], increase exercise performance[13], and alter cerebral perfusion[14].

Currently, the effects of single meals and repetitive days of diets with different nitrate levels on the kinetics of plasma nitrate and nitrite concentrations have not been described in detail in older adults. Pragmatically it would be useful to know if, and for how long, plasma nitrate/nitrite was elevated throughout the day following a high nitrate meal and/or supplement because it may have implications for the timing of physical activity for older adults with poor vascular function. Therefore, the primary objective of this study was to examine, in 8 sedentary older adults, the effect of a 3-day control vs. high nitrate diet, with and without a beverage supplement (beetroot juice) high in nitrate, on the kinetics of plasma nitrate and nitrite concentrations using a randomized four period cross-over controlled design. All participants received all four treatments. We also examined the effect of the four treatments on blood pressure. We hypothesized that the high nitrate diet alone would raise plasma nitrate/nitrite and lower blood pressure compared to the control diet. Furthermore, it

was hypothesized that plasma nitrate and nitrite would be elevated and blood pressure lowered from the high nitrate supplement vs. no supplement, and that the high nitrate diet would potentiate this effect as compared to the control diet.

Methods and materials

Subjects

Participants were older men and women over the age of 65 years, who scored 10 or below on the Short Physical Performance Battery (SPPB) and had a Mini-Mental State Exam (MMSE) score of ≥ 24 . The goal was to have a minimum of eight subjects complete all four interventions. We anticipated that our study protocol would be burdensome given the age of our target sample and we expected participant dropout. Two participants withdrew from the study before they completed all interventions. Individuals with systemic, uncontrolled diseases (ex. diabetes, recent (last 6-months) or current treatment for cancer, hypertension, clinical hypotension, resting blood pressure less than 100/60 mmHg, thyroid disorders, cardiovascular disease, chronic obstructive pulmonary disease, and inflammatory bowel diseases) were excluded. Participants were also excluded if they were taking medications that may be contraindicated when on a high nitrate diet, such as nitroglycerin or nitrate preparations used with angina, or phosphodiesterase type 5 (PDE5) inhibitors, including sildenafil (Viagra®), proton pump inhibitors, or antihypertensive medications. We are reasonably confident that these stringent criteria allowed us to screen out individuals with a disease state and/or medications that might have confounded the effect of the treatments on the nitrate/nitrite/NO cycle.

Meal preparation and feeding, assessment of blood pressure and blood draws occurred at the Clinical Research Unit (CRU) of Wake Forest University Health Sciences. Potential participants attended a screening visit with a dietitian to assess safety and palatability of the diets and beet juice supplement. In addition, known allergies to foods were determined and compared with the planned food and beverages in the study diets. The study protocol was approved by the Wake Forest University School of Medicine Institutional Review Board and written informed consent was obtained from each participant by the study coordinator or staff prior to any assessments.

Design and dietary interventions

This study was a four arm randomized crossover design consisting of a high nitrate diet, a high nitrate diet plus high nitrate beverage, a control diet, and a control diet plus high nitrate beverage (see diet sample in Appendix 1). Study participants ($n=8$ completers of all 4 interventions) were randomly assigned to one of eight possible unique orderings of the four treatments: (1,2,4,3), (2,3,1,4), (3,4,2,1), (4,1,3,2), (1,4,3,2), (2,3,4,1), (3,1,2,4), and (4,2,1,3), where each number represents a specific intervention (1=control diet; 2=control diet+supplement; 3=high nitrate diet; 4=high nitrate diet+supplement), and each position within the parentheses represents the chronological ordering for the study (Table 1). This ensured that all participants received each of the four interventions, no two sequences of orderings were used twice, and each period of the study had two observations of each intervention. Study participants were randomly assigned to treatments using a Williams design to ensure each participant received all four treatments, and that carryover effects and the possibility that the ordering of treatments may have affected study conclusions were minimized[15].

Participants reported to the CRU in the early morning following an overnight fast. A venous blood sample (~3-4 ml) and blood pressure were obtained at this time (baseline). They then consumed their assigned diet and resting blood pressure and venous blood (~3-4 ml,

indwelling catheter) were obtained throughout the day for all three days of each treatment. Venous blood for determining plasma nitrate and nitrite was obtained prior to consuming each meal (breakfast, lunch, dinner) and then at every hour following the meal for 3-hours. Each meal was consumed in a 30-minute time period. In the statistical analysis, we used blood pressure measures obtained 2-hours after each meal. This time was chosen based on previous work showing a hypotensive effect of the nitrate treatment occurring between 1 and 3 hours post consumption[16,17].

The control diet and high nitrate diet were isoenergetic and contained a similar distribution of macronutrients. Our earlier work indicated that a diet of ~1900 calories was well accepted by older participants with health characteristics similar to the current study participants. All participants received the same quantity of food and caloric level across all diets. This ensured that all participants within each treatment received similar absolute levels of nitrate in their diet. Snacks were provided to consume in the evening at home after the participant left the CRU. The amount of food not consumed was determined by measuring plate waste and by asking individuals if they ate their snacks. We also asked participants if they ate any extra foods at home during the treatment days. In nearly all cases, participants did not report consuming extra food, and there was no significant plate waste. We used beetroot juice (Biotta®, Carmel, IN) as the high nitrate supplement. For the supplement treatment conditions, participants consumed 500 ml of the beverage in the morning with breakfast.

There was at least a 3-day and no more than a 12-day washout period between each testing period. This range was due to scheduling challenges when identifying three consecutive days when participants were available to come to the CRU. Prior publications on nitrate supplementation provide evidence that there is likely to be little or no carryover effect between periods due to the relatively short half-life of nitrate in the human body; it is expected that one day following supplementation, nitrate levels will have fallen to 3% of their post-supplementation maximum[18]. Therefore, we are confident that our three day minimum washout period removed any physiological effects of the previous period's treatment.

Outcome measures

Demographics and health status

Age, gender, medical history, the Mini Mental State Exam[19], blood pressure, and health status were obtained at the screening visit.

Body mass and height

Body mass and height were measured without shoes and with outerwear removed using standard techniques on a calibrated scale and stadiometer. These assessments were done at baseline (day 1) for each treatment.

Plasma nitrate and nitrite

All blood draws were obtained by CRU nursing staff. Plasma was separated from red blood cells by sedimentation and frozen on dry ice for subsequent analysis as previously described (Presley et al, 2011). Measurements were conducted on the Eicom ENO-20. The ENO-20 instrument is designed specifically for measurements of nitrite and nitrate and is used routinely for plasma samples. It is based on the colorimetric Griess assay.

Blood pressure

Systolic and diastolic blood pressure was measured at rest in a seated position prior to and each hour for three hours after each meal by the nursing staff at the CRU. We used an

automated blood pressure cuff (Dinamap Pro100V2 and Dinamap Procare, General Electric) using standard techniques and the instruments were serviced and calibrated regularly. As noted above, for the statistical analyses, we used the blood pressure assessed 2-hr after each meal.

Acceptance of beetroot juice supplement

To determine the tolerance for the taste and any after-effects from the consumption of the high nitrate supplement, a 5 question Likert scale survey was administered to participants after breakfast on day 3 when they consumed the high nitrate beverage. The survey was anchored at 1 rating by “strongly agree”, 3 rating of “neutral”, and 5 rating of “strongly disagree”.

Statistical analyses

Demographic and baseline characteristics were summarized using means and standard deviations for continuous measures and frequencies and relative frequencies for categorical measures. The fixed diet and supplement effects on plasma nitrate, plasma nitrite, systolic blood pressure, and diastolic blood pressure were analyzed using mixed models. The day and hour effects were treated as fixed to allow for testing of daily and hourly time trends, while within-individual variability was assumed a random source of variation. Nitrate and nitrite means are summed over all days, time points, and individuals, while blood pressure measures are summarized for only the 2-hour post-meal time points. The models assume a compound symmetric covariance structure to accommodate the small sample size. All figures present the model-adjusted least square mean of nitrate and nitrite values over time. All statistical analyses and graphs were executed using SAS v9.2. All statistical comparisons were performed assuming a Type I error rate of 0.05, and pairwise comparisons maintained this error rate using Tukey's method for multiple comparisons.

Results

Ten individuals were recruited for the study and signed a consent to participate. Eight participants (5 women, 3 men) completed all testing visits and all analyses are based on these data. There were two dropouts due to recurring scheduling conflicts and time burden. The mean age of the participants was 72.5 ± 4.7 years. The MMSE ranged from 27 to 30 (28.5 ± 0.9). Mean body mass was 81.4 ± 15.1 kg and mean body mass index was 28.5 ± 3.9 kg/m². The SPPB ranged from 6 to 11 (9.6 ± 1.6) indicating that all participants were experiencing some lower extremity mobility dysfunction. Study participants reported no hypertension, diabetes, or chronic kidney disease and were not on any medications known to influence the study outcomes.

In general, the beetroot juice supplement was well accepted. The mean score for enjoying the taste of the beverage was 2.5 with only one participant rating it below neutral. Side effects included gastrointestinal upset ($n=1$) and headache ($n=1$). Beeturia was reported by all but one individual.

Dietary nitrate and nitrite

The daily intake of nitrate and nitrite from the control and high nitrate diet treatments, presented per day and broken out by meal is shown in Table 1. The beetroot juice supplement had 8.5 mmoles (527 mg; 6.5 mg/kg body weight) of nitrate and 345 μ moles (15.9 mg) of nitrite. Total daily nitrate in the control diet was 0.7 mmoles (43 mg; 0.5 mg nitrate/kg body weight) and in the high nitrate diet it was 2.5 mmoles (155 mg; 1.9 mg/kg body weight), nearly a 4 fold difference. Daily nitrite intake was 0.6 μ moles (28 μ g) from the control diet and 5.3 μ moles (246 μ g) from the high nitrate diet. The dinner meal had the

highest nitrate content for the high nitrate diet (1.2 mmol). Adding the nitrate level from the beetroot juice supplement raised the total daily nitrate intake for the two supplement treatments to 9.2 mmol nitrate for the control+supplement diet and 11.0 mmol for the high nitrate+supplement diet. This also increased the nitrate content of the breakfast meal in the high nitrate+supplement treatment to 9.0 mmol and to 8.5 mmol in the control nitrate+supplement treatment.

Plasma nitrate and nitrite

There were no differences between treatments in the baseline (i.e., prior to breakfast) plasma nitrate or plasma nitrite concentrations on day one of each treatment (Table 2). Values for nitrate ranged between treatments from 34.1 to 54.5 μM and from 0.24–0.30 μM for nitrite. The mean change from baseline for plasma nitrate concentrations across the day for each treatment is shown in Figure 1. Meal times were 30 minutes long and occurred at -0.5 hours, 3.5 hours, and 7.5 hours (as indicated in Figure 1 and Figure 2). There were no significant differences between the days within the same treatment, thus we collapsed the data for day for each treatment. Plasma nitrate increased when participants consumed the high nitrate supplement independent of the diet. Maximal mean plasma nitrate levels were achieved at approximately 2 hours post-supplement consumption. Plasma nitrate was elevated over the course of the day in the supplement treatments. The mean daily change in plasma nitrate from baseline was statistically significant for both control diet+supplement ($p<0.001$) and high nitrate diet+supplement ($p<0.001$), while control diet ($p=0.713$) and high nitrate diet ($p=0.852$) were not significantly different from baseline (Table 2). The adjusted multiple comparison of means indicated that the control diet+supplement vs. high nitrate diet +supplement were not significantly different ($p=0.990$) and control diet vs. high nitrate diet were not significantly different ($p=1.00$). All other pairwise comparisons were significantly different ($p<0.001$).

Consistent with the plasma nitrate analysis, treatments with the high nitrate supplement achieved the largest elevations in plasma nitrite, although the plasma nitrite following the high nitrate diet+supplement diet appears to be markedly greater than the control diet +supplement between hours 1 to 5 post-consumption (Figure 2). Plasma nitrite levels were maximized at approximately 3 hours following supplement consumption, and remained elevated throughout the day. The mean daily change in plasma nitrite from baseline was statistically significant for both control diet+supplement ($p=0.017$) and high nitrate diet +supplement ($p=0.002$), while control diet ($p=0.742$) and high nitrate diet ($p=0.500$) were not significantly changed from baseline (Table 1). The adjusted multiple comparison of intervention means indicated that the mean daily nitrite levels for control diet+supplement and high nitrate diet+supplement were significantly different ($p=0.021$) from each other with plasma nitrite being higher for the high nitrate diet+supplement treatment; control diet and high nitrate diet were not significantly different ($p=0.700$) from each other. All other pairwise comparisons were significantly different ($p<0.001$) from each other.

Blood Pressure

Systolic and diastolic blood pressure, determined before breakfast on day one of each treatment and then at two hours after each meal (averaged across the three days of treatment), are shown in Table 3. Data are means with 95% confidence intervals. There were significant reductions in systolic and diastolic blood pressure between the pre-breakfast measure and the post-meal assessments for all four treatments. However, there were no significant differences between treatments for any of the measures indicating no diet effect ($p=0.272$ for systolic, $p=0.111$ for diastolic). The model controlled for baseline values, treatment day, and the three days' time points.

Discussion

This is the first trial to examine the kinetics of plasma nitrate and nitrite across multiple days following dietary manipulation of nitrate using foods and a beverage supplement in older adults. A surprising result of this study was that there was no difference in plasma nitrate or nitrite between the high nitrate diet compared to the control diet, contrary to our original hypothesis. Also, the high nitrate diet alone did not appear to potentiate the effect of the supplement on plasma nitrate or nitrite levels. Therefore our data did not support our research hypothesis. However, we demonstrated that a high nitrate supplement consumed at breakfast substantially elevated both plasma nitrate and nitrite levels over the course of the day regardless of the diet. We did not observe any difference in systolic or diastolic blood pressure between the four treatments. Therefore our hypothesis that the high nitrate diet alone or when combined with the supplement would lower blood pressure compared to the control condition was not supported. We suggest the reader uses caution in the interpretation of these blood pressure data given the small sample size.

The level of nitrate contained in the beetroot juice supplement was 6.5 mg/kg body weight vs. 1.9 mg/kg body weight in the high nitrate diet and 0.5 mg/kg body weight in the control diet. In comparison, the World Health Organizations Acceptable Daily Intake is 3.7 mg nitrate/kg body weight /day and the United States Environmental Protection Agency is 7.0 mg nitrate/kg body weight/day. The addition of the beetroot juice supplement to the high nitrate diet increased total nitrate intake to 8.4 mg/kg body weight, about 20% above the United States Environmental Protection Agency acceptable intake, and to 7.0 mg nitrate/kg body weight for the control+supplement treatment.

From a practical standpoint, our data suggest that in the diets provided to our participants, the level of nitrate in the diet has minimal impact on plasma nitrate and nitrite, but that a high nitrate source (beetroot juice) consumed with the breakfast meal can significantly increase plasma nitrate and nitrite throughout the day. Our findings have real-world significance for older adults who seek to use beetroot juice with conditions such as peripheral arterial disease, or more generally, in those with impaired vascular function or vascular disease who wishes to engage in physical activity. Previous work by Kenjale and colleagues showed that individuals with peripheral arterial disease who consumed beetroot juice three hours prior to an exercise session had improved physical function and increased time to onset of claudication pain during an exercise test as compared to consuming a low nitrate containing beverage[20]. Our data show that plasma nitrate and nitrite are significantly increased throughout the day with a single dose of beetroot juice. Thus older adults may not need to concern themselves with timing the intake of a high nitrate supplement prior to an exercise session or a bout of moderate-to-high intensity physical activity. It is likely that this would lead to better adherence to a treatment regimen.

We showed that plasma nitrate peaked 1-2 hours after the morning meal following the consumption of the high nitrate supplement. These peak values and changes following the supplement approximated the levels reported earlier by us[14] and Webb and colleagues[17]. Interestingly, the level of nitrate in the beetroot juice product used by Webb et al., was about 2.5 times higher than the beetroot juice in our two studies. Furthermore, there was about a 2 fold higher plasma peak change in nitrite for our current work vs. that of Webb et al. The change in plasma nitrite concentrations reported by Webb et al., were about 0.2 μ M occurring 3-5 hours following the beetroot juice supplement as compared to the nearly 1.0 μ M change in our work for the control diet+supplement treatment. The difference between studies in plasma nitrite may be related to the conditions under which the supplement was consumed; we had participants consume it with a meal in both of our studies, whereas it is not stated this was the case in the work by Webb and colleagues. Also,

the amount of nitrite in the supplements (and diet) differed, which may have contributed to the difference; Webb et al., reported $< 0.05 \mu\text{M}$ nitrite in their beetroot juice, whereas our product had $0.3 \mu\text{M}$ of nitrite and our diets contributed an additional 0.06 and $3.4 \mu\text{M}$ for the breakfast meals of the control and high nitrate diets, respectively. Finally, differences in the results may also be due to the differences in demographics and potential health conditions between the two studies. Webb et al. recruited a younger and leaner cohort (mean age = 25 years; BMI = 22 kg/m^2) compared to our sample.

There was a progressive decline in plasma nitrate throughout the day, although it remained substantially elevated above baseline levels. In the supplement conditions, the rise in plasma nitrite tracked the rise in plasma nitrate during the initial part of the day with the exception that in the high nitrate diet+supplement treatment there was a substantial rise in plasma nitrite concentration during the first several hours before they matched the control diet +supplement levels. Furthermore, in the supplement conditions, plasma nitrite showed a secondary rise following the evening meal beginning at the 7.5 hour time point. We believe this may be due to an increase in saliva production from the evening meal, subsequent conversion of nitrate to nitrite, and then the swallowing of the saliva while eating. The two supplemented treatments had higher plasma nitrate at these times which may have been due to a higher concentration of nitrate in the salivary glands. If so, when saliva production increased at a meal, this would have led to conversion of nitrate to nitrite by oral bacteria and a subsequent rise in plasma nitrite. Changes in plasma nitrite from baseline were higher for high nitrate diet+supplement compared to the other conditions. The observation of a larger change in plasma nitrite for high nitrate diet+supplement vs. control diet+supplement is surprising considering there was no difference in the levels of substrate for plasma nitrate between the two treatments. This might indicate a more efficient conversion of nitrate to nitrite in the high nitrate diet+supplement condition; however pursuing this possibility is beyond the scope of the study.

Previous work with dietary nitrates has shown that consumption of an acute dose of nitrate isolated in a supplement or as a whole food product, such as beetroot juice, increases plasma nitrate, and subsequently plasma nitrite[11-13,17,21-25]. These previous studies were conducted primarily to investigate the acute effect of ingestion of nitrate on blood pressure and exercise performance following a single acute dose of nitrate supplement, or up to 15-days of a supplement. We manipulated nitrate intake through changes across the whole diet for three consecutive days. Sobko and colleagues also manipulated nitrate via the diet, but their study was conducted in healthy participants who consumed a traditional Japanese diet with foods high in nitrates[26] (see reference for list of their specific foods). They observed elevated plasma and saliva levels of nitrate and nitrite on day 10 following a 10-hour overnight fast; however, they did not perform serial measures of plasma nitrate and nitrite to study the intra- and inter-day kinetics of the dietary manipulations. Our study is the first to look at the changes in plasma nitrate and nitrite at multiple time points across several consecutive days of treatment. Our study adds unique information to the literature in that we examined older adults and manipulated the nitrate intake through both the diet and supplement over several days.

Surprisingly, the high nitrate diet alone did not elevate either plasma nitrate or nitrite and high nitrate diet+supplement did not elevate plasma nitrate/nitrite above that of control diet +supplement. We hypothesized that the high nitrate diet alone would result in a significant rise in plasma nitrate compared to the control diet alone. This was based on our calculations of the nearly 4 fold higher nitrate load in the high nitrate diet compared to the control diet. In comparison to other work[16], the 2.5 mmol in the high nitrate diet is lower than the single dose administration of 4 mmol of nitrate that has been found to cause physiological effects and increases in plasma nitrate and nitrite. Apparently our diet dose was not

sufficient to significantly raise plasma nitrate and nitrite above the control diet. Furthermore, the amount was spread across the three meals and snacks throughout the day and may have not provided a large enough bolus dose of nitrate at any meal or snack to raise plasma levels of either nitrate or nitrite. The meal with the largest nitrate dose from foods alone (not including the beetroot juice supplement) was at dinner with about 1.25 mmoles of nitrate or one-half of the daily nitrate dose. This was not sufficient to significantly increase plasma nitrate/nitrite compared to the control diet. A second possibility is that consumption of nitrate in whole foods (both cooked and raw) may provide a lower bioavailability of nitrate vs. from a beverage. However, we do not believe this is a satisfactory explanation because van Velzen and colleagues demonstrated an absolute bioavailability of nitrate of 100% from several high nitrate containing vegetables (cooked spinach, raw lettuce, and cooked beetroot) when participants consumed a single dose of the product[27].

Based on earlier work, we hypothesized that the high nitrate diet and supplement, both together and separately, would lower resting blood pressure compared to the control diet and no supplement interventions[11,16,17]. Previously, Kapil and colleagues showed that a nitrate dose of 5.5 mmoles delivered in beetroot juice lowered diastolic blood pressure by over 4 mm Hg 2.5-3 hours after drinking the beverage as compared to water[16]. Systolic and diastolic blood pressures were also reduced by up to 10 mm Hg at nitrate daily doses ranging from 5.2 to 22.5 mmoles[13,16,17,20,21,28-30]. These were primarily in young, healthy adults. We did not observe a nitrate treatment effect on blood pressure which may have been related to variability in blood pressure measures and the small sample size of our study. We used a single measure of the blood pressure at each of the time points whereas others have obtained 3 or more measurements and used the mean of these as the data point. Additionally, our sample of older adults was quite healthy and we excluded participants with hypertension. This may have blunted the effect of the nitrate treatments (both diet and supplement).

The hypotensive effect found in DASH studies may be as much attributable to inorganic nitrate found in certain vegetables and fruits as to the high potassium, polyphenols, and calcium content of the DASH diet. Based on analysis by Hord et al., dietary nitrate levels could easily reach over 16 mmoles (1000 mg; 12 mg/kg body weight)[10] in a DASH dietary pattern by astutely choosing high nitrate containing vegetables and fruit. In comparison, our high nitrate diet (without the supplement) was only 2.5 mmoles. With the documented health benefits from the DASH diet and diets abundant in vegetables and fruits, it has been suggested that the current World Health Organization and United States Environmental Protection Agency Acceptable Daily Intake for nitrate needs to be reevaluated[10,31].

There are limitations to this study. The small sample of older adults in this study was ostensibly healthy and individuals were screened for disorders or drugs that may have interfered with the metabolism of nitrate and nitrite. This information was obtained from self-report but it is possible that participants had some underlying disease and may have misreported medications. The low inter-subject variability suggests that this was not a major concern. Participants were also asked to refrain from using antibacterial mouthwash for the duration of the study but may not have done so. It seems highly unlikely that participants, who were blind to the study purpose, could have manipulated the data via medications/ mouthwash, to suppress the effect of the high nitrate diet. The level of nitrate in the high nitrate diet treatment may have led to the lack of response from this treatment alone. Developing a higher nitrate diet menu may provide differences in dietary treatments. Finally, since we excluded individuals with conditions common in the older adult population, caution should be exercised when generalizing these data.

This study confirms that the nitrate-nitrite cycle is intact in ostensibly healthy older adults when a high nitrate supplement is consumed in a single dose over a 30-minute period. In summary, in healthy older adults, a high nitrate supplement consumed at breakfast increased plasma nitrate and nitrite levels throughout the day. This observation may have practical utility for the timing of physical activity and a high nitrate supplement for older adults with vascular dysfunction.

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APPENDIX 1

Sample One-Day Menu of High Nitrate (with high nitrate supplement) and Control Diet

Each provides ~1900 calories and are 26-28% of energy from fat, 52-55% from carbohydrates, and 17-18% from protein

High Nitrate Diet

Breakfast

1 egg Omelet, Canadian bacon (1 ounce), tomatoes (1/4 cup) Whole Wheat Toast - 1 slice
Jelly – 2 T Banana High nitrate beverage (Beetroot juice)

Lunch

Tuna salad sandwich with American cheese on Whole Wheat toast

- 1 T mayo

- 3 oz tuna in water, drained

- 1 slice cheese

Pear – 1 medium

Pretzels – 0.5 ounce

Dinner

Spinach tossed salad with beets and spinach – 1 Tbsp Salad Dressing Veggie burger on whole wheat bun with cheese

- catsup – 1.5 T

Steamed mixed vegetables - ½ cup Cantaloupe – 1.5 cups

Snacks

Mixed nuts – 0.5 oz

Carrots – 1 cup

Control Diet

Breakfast

Nonfat Milk – 1 cup

Breakfast cereal (Raisin Bran) – 1 cup

Whole wheat toast – 1 slice

Margarine – 1 tsp

Jelly – 1 T

Lunch

Egg Salad (1/2 cup dice egg and 1T mayo) sandwich on 2 slices whole wheat bread Pretzels – 1 oz Grapes – ¾ cup Cottage cheese (1% milk fat) – ¼ cup

Dinner

Baked Chicken breast – 4 oz Macaroni salad (mayo, cooked macaroni, mustard, celery seed, boiled egg, salt) – ½ cup

- ¼ cup macaroni

- 1 T mayo

- 1 tsp mustard

- 1/4 diced egg

Roll and margarine – 1 with 1 tsp

Snacks

Graham Crackers – two large crackers

Apple – 1 medium

Use distilled water for drinking and coffee and tea

Abbreviations

NO	nitric oxide
NOS	nitric oxide synthetase
DASH	Dietary Approaches to Stop Hypertension
CRU	Clinical Research Unit
MMSE	Mini Mental State Examination
SPPB	Short Physical Performance Battery

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Figure 1. Plasma Nitrate (hourly mean change from baseline). Data points are least squares means. Mealtimes were 30 min long starting at time -0.5, 3.5, and 7.5 hr.

Figure 2. Plasma Nitrate (hourly mean change from baseline). Data points are least squares means. Mealtimes were 30 min long starting at time -0.5, 3.5, and 7.5 hr.

Table 1

Nitrate and nitrite in the diet (not including beetroot juice) for the control diet and high nitrate diet treatments. The beetroot juice contained 8.5 mmol (527 mg; 6.5 mg/kg body weight) of nitrate and 0.3 μ mol (16 μ g) of nitrite.

	Control Diet	High Nitrate Diet
Diet - Total		
Nitrate		
mmol	0.70	2.50
mg	43.4	155.0
mg/kg BW	0.5	1.9
Nitrite		
μ mol	0.60	5.35
μ g	27.7	246.0
μ g/kg BW	0.3	3.0
Breakfast		
Nitrate		
mmol	0.03	0.55
mg	1.9	33.8
Nitrite		
μ mol	0.06	3.4
μ g	2.7	156.4
Lunch		
Nitrate		
mmol	0.47	0.55
mg	29.0	34.1
Nitrite		
μ mol	0.19	0.31
μ g	8.56	14.40
Dinner		
Nitrate		
mmol	0.08	1.24
mg	4.80	76.9
Nitrite		
μ mol	0.23	1.17
μ g	10.58	53.82
Snacks/Coffee		
Nitrate		
mmol	0.08	0.11
mg	4.7	7.0
Nitrite		
μ mol	0.13	0.46
μ g	5.9	21.3

Table 2

Mean baseline values for plasma nitrate and nitrite on day 1 and mean daily changes in plasma nitrate and nitrite by treatment

Diet	Plasma Nitrate (μM) Mean (SE)	Plasma Nitrite (μM) Mean (SE)
Control Diet		
Baseline	54.4 (31.9)	0.25 (0.19)
Change	-4.3 (11.4) ^a	0.09 (0.27) ^a
Control Diet+Supplement		
Baseline	34.1 (15.5)	0.30 (0.15)
Change	275.4 (11.4) ^{b, †}	0.73 (0.27) ^{b, †}
High Nitrate Diet		
Baseline	54.5 (35.8)	0.29 (0.12)
Change	-2.2 (11.3) ^a	0.19 (0.27) ^a
High Nitrate Diet+Supplement		
Baseline	40.7 (23.0)	0.24 (0.11)
Change	275.3 (11.4) ^{b, †}	1.03 (0.27) ^{c, †}

Change values are the diet-specific daily mean (SE) change from pre-prandial baseline values, averaged over the 12 post-baseline time points and 3 days.

Presented statistical tests are partial F-tests from mixed effects linear models. Means within each column with the same alphabetical letter are not statistically significantly different. All pairwise comparisons are adjusted for Type I error inflation using Tukey's method.

Presented baseline values are pre-breakfast for day 1 of each treatment.

[†] indicates treatment mean is significantly changed from daily baseline.

Table 3

Systolic and diastolic blood pressure by treatment prior to breakfast on day 1 and 2 hours after each meal across days 1, 2 and 3. Values are presented as means with 95% confidence intervals.

Treatment	Systolic Blood Pressure (mm Hg)	Diastolic Blood Pressure (mm Hg)
Control Diet		
Pre Breakfast Day 1	132.4 (111.6, 153.1)	64.0 (55.6, 72.4)
2 Hour Post Breakfast	116.7 (107.9, 125.6)	60.3 (56.8, 63.7)
2 Hour Post Lunch	116.5 (107.8, 125.3)	61.2 (57.9, 64.5)
2 Hour Post Dinner	116.3 (107.6, 125.1)	58.1 (54.7, 61.4)
Control Diet +Supplement		
Pre Breakfast Day 1	128.0 (107.5, 148.5)	69.5 (61.8, 77.2)
2 Hour Post Breakfast	118.5 (109.7, 127.2)	59.2 (55.9, 62.6)
2 Hour Post Lunch	114.1 (105.3, 122.9)	60.2 (56.8, 63.5)
2 Hour Post Dinner	118.6 (109.9, 127.4)	57.6 (54.3, 61.0)
High Nitrate Diet		
Pre Breakfast Day 1	130.1 (117.6, 142.6)	67.7 (60.9, 74.4)
2 Hour Post Breakfast	121.1 (112.6, 129.7)	62.2 (59.0, 65.4)
2 Hour Post Lunch	121.1 (112.6, 129.7)	63.0 (59.8, 66.2)
2 Hour Post Dinner	116.1 (107.6, 124.7)	59.9 (56.7, 63.1)
High Nitrate Diet +Supplement		
Pre Breakfast Day 1	130.6 (115.3, 145.8)	64.6 (60.4, 68.7)
2 Hour Post Breakfast	116.4 (107.4, 125.4)	59.8 (56.3, 63.3)
2 Hour Post Lunch	114.9 (105.9, 123.8)	60.1 (56.7, 63.6)
2 Hour Post Dinner	115.1 (106.2, 124.0)	60.5 (57.0, 64.0)

Presented baseline estimates are pre-breakfast for day 1 of each treatment.

Presented post-meal estimates are the diet-specific mean (95% CI) systolic and diastolic blood pressure, averaged at 2-hour post-meal time points over the 3 days.