Protocol Document

Dose-response Effect of Dietary Nitrate on Muscle Function in Older Individuals

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Dose-response effect of dietary nitrate on muscle function in older individuals

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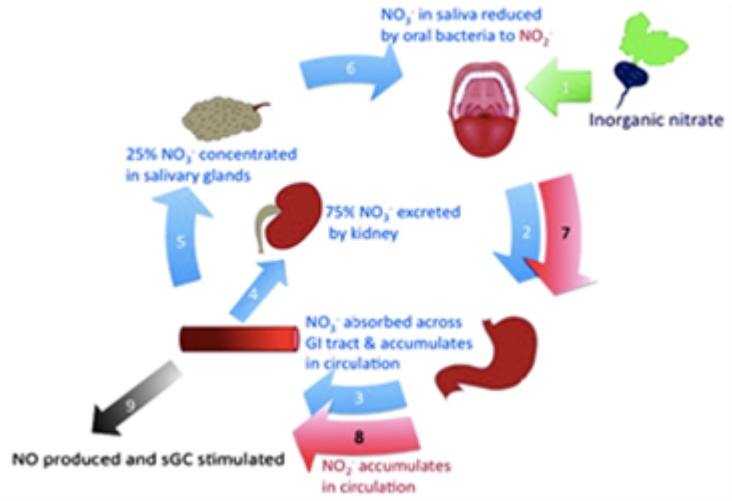
1.0 Background

By 2050, approximately 20% of the US population, or >80 million people, will be 65 y of age or older.[1] Unfortunately, aging is accompanied by a progressive reduction in the maximal strength, speed, and especially power of skeletal muscle. These age-related physiological changes often lead to functional limitations that are highly predictive of disability, institutionalization, and mortality in the elderly.[2,3] Thus, any intervention that significantly enhances muscle contractile function could potentially improve the health, quality of life, and possibly even the longevity of older individuals.

Numerous factors undoubtedly account for the decline in muscle function described above, with age-related changes in the size, properties, and neural control of muscle likely all playing a role.[4] Another important factor, however, may be a fall in NO bioavailability with aging. Although initially identified as a vasodilator, i.e., as "endothelium-derived relaxing factor", NO is in fact a key cellular signaling molecule with pleiotropic effects in many tissues. These include skeletal muscle, wherein among other effects NO helps modulate contractile function.[5-9] With aging, however, whole-body NO production decreases, as evidenced, e.g., by a progressive decline in the plasma concentrations of its downstream metabolites, NO_2^- and NO_3^-.[10,11] In skeletal muscle itself, there is a dramatic decrease in interstitial NO_2^- and NO_3^- concentrations,[12] as well as a decline in the activity of the neuronal form of NO synthase (nNOS, or NOS1),[13,14] the primary isoenzyme within muscle responsible for the synthesis of NO from L-arginine, O_2, and NADPH. These changes are accompanied by an age-related reduction in flow-mediated vasodilation,[15-18] perhaps the hallmark indicator of NO bioavailability. In turn, the latter (i.e., flow-mediated vasodilation) has been shown to correlate positively with muscular power and physical functioning in older men and women.[19] Taken together, these data suggest that decreased NO production may contribute to the age-associated decline in muscle contractile properties and hence in functional capacity.

In this context, the physiological effects of dietary NO_3^- are of considerable interest. It is now recognized that, rather than being metabolically inert or, worse, a potential carcinogen, NO_3^- in the diet is a significant source of NO in the body[20-24] (Fig. 1). In fact, this dietary pathway, which entails the reduction of NO_3^- to NO_2^- by facultative anaerobic bacteria in the mouth followed by further reduction of NO_2^- to NO by, e.g., deoxyhemoglobin, can account for as much as ~25% of basal whole-body NO production.[25,26] This dietary pathway serves as an important "backup" system to the more well-known NOS pathway. This is likely to be especially true in skeletal muscle, since unlike the NOS pathway the dietary pathway operates well at low pO_2 and is stimulated rather than inhibited by low pH,[20-24] conditions that regularly exist in muscle, both at rest and especially during contractile activity. Indeed, skeletal muscle has recently been shown to play a central role in whole-body $NO_3^-/NO_2^-/NO$ metabolism.[27,28]

Figure 1. Enterosalivary pathway of NO production. From Ref. 22.



Based on the above, it is logical to hypothesize that increased dietary NO_3^- intake might enhance NO bioavailability in older men and women, leading to an improvement in

muscle function. Indeed, we have observed significant increases in muscle speed and power following dietary NO_3^- intake in healthy younger subjects,[29] athletes,[30] and especially middle-aged heart failure patients.[31] (See Preliminary Data.) Along the same lines, Haider and Folland[32] and Whitfield et al.[33] have reported that dietary NO_3^- intake enhances the rate of force development and peak force output of the quadriceps muscle of healthy young men during electrically-stimulated contractions. To date, however, no study has specifically examined the impact of dietary NO_3^- on muscle contractile function in older persons. Furthermore, it cannot be automatically assumed that dietary NO_3^- will be equally efficacious in older subjects. For example, in rodents the effects of dietary NO_3^- are more prominent in fast- vs. slow-twitch muscle,[34,35] and some,[36-39] but not all,[40-42] studies of humans have found that aging results in a reduction in the percentage of fast-twitch muscle fibers. Alternatively and/or in addition, more rapid destruction of NO due to increased production of reactive oxygen species by aging muscle[43] could limit the beneficial effects of dietary NO_3^- in older persons.

Even if the *efficacy* of a particular dose of NO_3^- could be established in older subjects, this would not necessarily mean that it is the *optimal* dose. There is limited data in the literature regarding the dose-response relationship for dietary NO_3^- for various outcomes, and only one study has examined the response to exercise over a wide range of doses.[44] These authors found that acute ingestion of either 8.4 or 16.8, but not 4.2, mmol of NO_3^- improved performance equally during high intensity *aerobic* exercise in healthy young men. However, it is not known whether a similar plateau exists with respect to improvements in muscle contractile function, nor is it known whether aging influences this relationship. Such information is obviously critical to any attempt to pursue funding for larger (e.g., multicenter) trials evaluating dietary NO_3^- as a potential treatment for reduced muscle function in older persons, especially since the incidence of negative side effects (e.g., GI distress) may increase at higher doses.

Our Preliminary Data (see below) suggest that older subjects may require a greater dose of NO_3^- than younger individuals to yield a significant improvement in muscle speed and/or power.

Preliminary data

Using methods essentially identical to those to be employed in the proposed experiments (see below), we previously demonstrated that acute dietary NO_3^- intake increased maximal knee extensor speed (Vmax) and power (Pmax) in 12 healthy, younger men and women by 11±5% ($P<0.05$) and 6±3% ($P<0.05$), respectively.[29] We observed similar dietary NO_3^--induced improvements in muscle speed and power in 13 athletes performing multi-joint, multi-muscle, i.e., sprint cycling, exercise.[30] Lastly, we found even greater increases in Vmax and Pmax (of 12±5%, $P=0.09$, and 13±4%, $P<0.05$, y) in nine middle-aged patients with systolic heart failure (HF).[31]

Using the same methods, we have also recently determined the effects of dietary NO_3^- in six healthy men and women 64-79 y of age. As shown in Table 1, plasma NO_3^- and NO_2^- and breath NO increased ($P<0.05$) by 1309±188%, 268±91%, and 142±19%, respectively, by 2 h after ingestion, and all three remained elevated for the remainder of the experiment. These findings are consistent with our previous research.[29-31] This increase in NO bioavailability resulted in a 9±5% increase in maximal muscle speed, i.e., Vmax (Fig. 2, *left panel*), with five out of the six subjects demonstrating an improvement. This increase, however, was not statistically significant (i.e., $P=0.13$), and maximal muscle power, i.e., Pmax, improved on average by only 2±3%

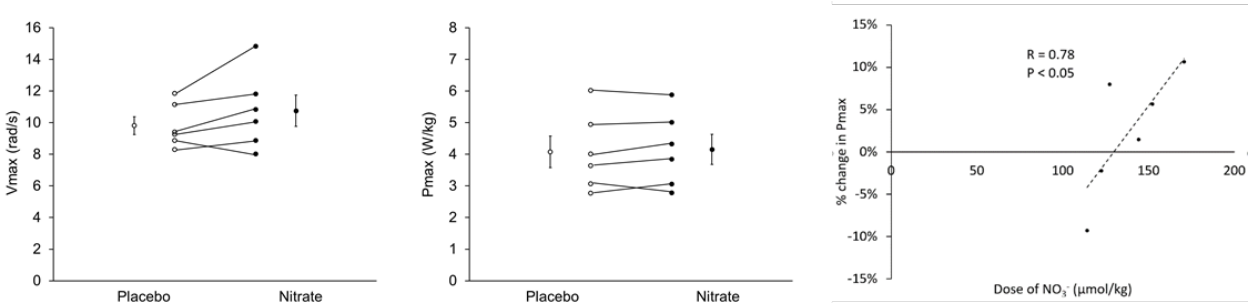
Table 1. Changes in plasma NO_3^- and NO_2^- and breath NO in response to dietary NO_3^- intake in older study participant.

	Trial	Pre	1 h	2 h	10 min post exercise
Plasma NO_3^-	Placebo	26±4	24±3	24±3	22±4
(μmol/L)	Nitrate	31±7	285±27*	295±27*	282±33*
Plasma NO_2^-	Placebo	0.21±0.03	0.26±0.06	0.26±0.06	0.20±0.03
(μmol/L)	Nitrate	0.27±0.04	0.43±0.13	0.49±0.06*	0.56±0.10*
Breath NO	Placebo	23±5	26±4	27±4	23±5
(ppb)	Nitrate	27±4	49±7*	38±4*	52±10*

Values are mean±S.E. for n=7. *Nitrate trial significantly higher than Placebo trial at same time point (P<0.05).

(P=0.47) (Fig. 2, *center panel*). This is only 1/3rd as much as we previously found in younger

Figure 2. Responses of Vmax (left panel) and Pmax (center and right panels) to dietary NO_3^- in older subjects.



men and women.[29,30] Closer examination of the data suggested that this diminished effect was due to an inadequate dose of NO_3^-, as there was a significant positive relationship between NO_3^- intake relative to body mass and the relative improvement in Pmax (Fig. 2, *right panel*). These preliminary results therefore suggest that older individuals remain responsive to NO_3^-, but that a higher dose may be required to elicit improvements in muscle contractile function.

2.0 Rationale and Specific Aims

In light of the above, we will use a double-blind, placebo-controlled, crossover design to address the following Specific Aim:

Specific Aim #1: To determine the dose-response relationship between acute dietary NO_3^- supplementation and muscle contractile function in healthy, older (i.e., 65-79 y old) men and women.

3.0 Inclusion/Exclusion Criteria

Inclusion:

- Men and women age 65-79
- In good health, as determined by the physician's review of history, physical examination, resting EKG, and routine blood tests

Exclusion:

- Men and women <65 or >79 years of age
- Unable to provide informed consent
- Currently pregnant or lactating

- Current smokers
- Significant orthopedic limitations or other contraindications to strenuous exercise
- Those taking phosphodiesterase inhibitors (e.g., Viagra)
- Those taking proton pump inhibitors, antacids, xanthine oxidase inhibitors, or on hormone replacement therapy
- History of major metabolic disease (e.g., type I and type II diabetes, thyroid disorders), history of neuromuscular disease (e.g., cervical spondylotic radiculomyelpathy, lumbar

Figure 3. Study design.

spondylosis, amyotrophic lateral sclerosis, Guillain-Barré syndrome, and acquired demyelinating polyneuropathies), cardiovascular disease (e.g., > stage II hypertension, heart failure, myocardial infarction/ischemia, significant myocardial or pericardial diseases (e.g. amyloidosis, constriction), moderate or severe valvular disease, renal disease, liver disease, or anemia

Twenty study participants will be enrolled.

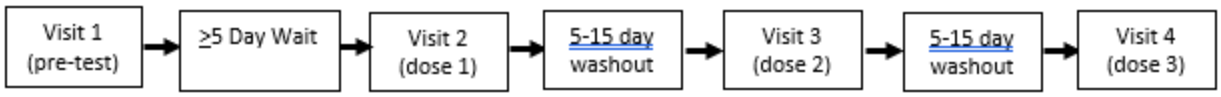
4.0 Enrollment/Randomization

Subjects will be recruited from the community through approved methods including flyers, emails from the Indiana Clinical and Translational Sciences Institute (All IN for Health), and being included in the searchable "All IN for Health" clinical trials database . When potential study subjects contact the study team expressing an interest in the study they will be given additional information regarding the study procedures, requirements, and risks. If they are still interested the study team will complete a brief phone screening to see if they qualify for an in-person screening visit. As previously approved by amendment, potential subjects will be asked if they are part of the IU Health medical system and if so, do they give verbal permission for their records to be reviewed for possible exclusions to participation. If they are not part of the IU Health system or do not give permission, they will still be able to participate in a screening visit if it appears they meet other pre-screening criteria. If it appears they are eligible for a screening visit, they will be scheduled and a consent form will be mailed / emailed to them ahead of the screening visit for review. After the study participant has had several days to review the consent form, a study team member will call to answer any questions the participant may have regarding the consent form. When the subject arrives for the screening visit a member of the research team will thoroughly review the consent with the study participant and answer questions. Study participants will be given as much time as they wish to consider participation before signing the consent form. If the study participant agrees to move forward with the screening visit, the consent will be signed by the study participant and the person who reviewed the consent and obtained the participant's signature. A copy of the signed consent form will be provided. Both the participants and the investigators will be blinded to the order of treatment, which will be randomized using randomization.com.

5.0 Study Procedures

Study Design

Each subject will be studied using a double-blind, placebo-controlled, crossover design. Figure 3 illustrates the flow through the study for an individual participant.



Study Procedures

Participants will be asked to fast for 12 hours prior to each study day.

On Study Day 1 (Pre-test), all study participants will complete a screening / phenotyping examination. The participant will undergo a complete history, physical exam, resting EKG and phlebotomy for screening and phenotyping laboratories (complete blood count, liver and kidney function tests, electrolytes, fasting glucose and insulin). They will also practice the entire isokinetic dynamometer exercise test.

To minimize variation in baseline NO_3^-/NO_2^-/NO levels, participants will be instructed to consume their normal diet (aside from the BRJ supplement) throughout the study. Participants will, however, be asked to avoid consumption of high NO_3^- foods (e.g., beets, spinach, collard greens) the evening prior to testing. This approach is justified based on the short half-life of NO_3^- in plasma (i.e., ~8 h) as well as previous research demonstrating that even a chronic increase in dietary NO_3^- intake up to 2.5 mmol/d (i.e., ~3x normal dietary NO_3^- intake in the US) has no significant influence.[56] Participants will be asked to refrain from chewing gum, alcohol, and caffeine-containing food/drinks for 24 h prior to each remaining visit. Participants will also be instructed to refrain from use of an antibacterial mouthwash throughout the study, since this would limit conversion of NO_3^- to NO_2^- by bacteria in the oral cavity.[57] Participants will be asked to fast for 12 h prior to each remaining study visit.

On Study Day 2, participants will undergo phlebotomy for plasma NO_3^- and NO_2^- measurement, and have their breath NO level determined. (Breath NO will be measured as a biomarker of increases in whole-body NO production in response to dietary NO_3^- intake.[52,53]) The subject will then ingest 3.3 mL/kg of a commercial concentrated beetroot juice (BRJ) supplement (Beet It Sport®, James White Drinks, Ipswich, UK) either: 1) essentially devoid of NO_3^- (placebo); 2) containing (as determined by previous direct measurement) 91 µmol/mL NO_3^- (thus total dose = 300 µmol/kg, or 21 mmol in a 70 kg subject); or 3) an equal mixture of the placebo and NO_3^--containing BRJ products. The placebo, which is prepared the company by extracting NO_3^- from BRJ using an ion exchange resin, is indistinguishable in packaging, color, taste, texture, and smell from the standard product, and does not alter plasma NO_3^-/NO_2^- or breath NO concentrations or the physiological responses to exercise.[29-31] Additional blood and breath samples will be obtained 1 and 2 h later, as well as blood pressure and heart rate, afterwhich time the subject will have the Vmax and Pmax of the knee extensor muscles of their dominant leg determined via isokinetic dynamometry (see below). The timing of all measurements has been chosen to correspond to peak plasma NO_3^-/NO_2^- concentrations and breath NO levels, which reach a maximum 2-3 h after ingestion.[29-31]

On Study Days 3 and 4, participants will be asked to return to undergo the same studies as described above for Study Day 2.

Study Methods

Measurement of NO_3^- in each batch of BRJ w/ or w/o NO_3^-: The NO_3^- content of each batch of BRJ w/ or w/o NO_3^- will be determined using high performance liquid chromatography (HPLC) (ENO-30, Eicom USA, San Diego, CA).

Measurement of plasma NO_3^-, NO_2^-, and breath NO: Venous blood samples will be obtained prior to consumption of BRJ w/ or w/o NO_3^- and every hour thereafter for 3 hours, plasma rapidly separated by centrifugation, and frozen at -80° C until subsequently analyzed for NO_3^- and NO_2^- concentration using HPLC as described above. Breath NO levels will be measured at the same time points using a portable electrochemical analyzer (NIOX VERO, Circassia Pharmaceuticals, Chicago, IL).

Measurement of skeletal muscle contractile function: A Biodex 4 system will be used to measure each subject's muscle contractile properties as previously described.[29-31] Briefly, subjects will perform maximal knee extensions with their dominant leg at angular velocities of 0, 1.57, 3.14, 4.71, and 6.28 rad/s. (Not all older individuals may be able to achieve the highest velocity – if not, the highest measured angular velocity and associated torque will be used in all subsequent calculations.) The subject will perform 3-4 knee extensions at each velocity, with 2 min of rest allowed between each set of contractions. To eliminate artifacts, data will be "windowed" to isolate the isokinetic phase and smoothed using a 9 point weighted moving average filter. The highest torque generated at each velocity will be used to calculate peak power at that velocity, after which the resulting power-velocity curve will be fit to a polynomial function to determine the subject's maximal knee extensor velocity (Vmax) and corresponding power (Pmax). Subsequently, the subject will perform an "all out" 50 contraction fatigue test (at 3.14 rad/s) to determine whether dietary NO_3^- influences fatigue resistance (i.e., increases average power) during repetitive, maximal activation. Finally, recovery of muscle function will be quantified by measuring restoration of torque during knee extensions performed periodically over the next 10 min.

Risks

Likely: The insertion of an intravenous tube for blood drawing is associated with a small amount of discomfort. The subjects are likely to notice reddish urine or stools after drinking beetroot juice or control. This is normal and not harmful.

Less Likely: The insertion of an intravenous tube for blood drawing is associated with a small risk of bleeding or infection. After the EKG, some people may experience a skin rash but this usually goes away without treatment.

Rare: The risk of arrhythmia and heart attack are rare and the risk of death during exercise testing is extremely rare. The risk of heart attack is <0.1% in all patients undergoing exercise testing (and should be lower in these subjects since they have no coronary artery disease). The risk of death is reported as less than 2/10,000 tests or <0.02%.

Mitigation of Risks

Blood draw: Only trained staff will place IVs and collect blood samples

EKG: trained nurses will prepare the skin for the EKG and place the electrodes.

Exercise test: Every effort to minimize these rare risks by observing and monitoring during testing. Emergency equipment and trained personnel are available to deal with any emergency.

6.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

The following standard definitions will be used for this study:

Adverse event (AE): Any untoward or unfavorable medical occurrence in a human study participant, including any abnormal sign (e.g. abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the participants' involvement in the research, whether or not considered related to participation in the research.

Serious Adverse Event (SAE): Any AE that results is place the participant at immediate risk of death from the event as it occurred, or results in inpatient hospitalization, prolongation of existing hospitalization, persistent or significant disability/incapacity, congenital abnormalities or birth defects, or death.

Unanticipated problem: As Defined by DHHS 45 CFR part 46, any incident, experience, or outcome that meets all of the following criteria: 1) is unexpected, in terms of nature, severity, or frequency, given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the study population; 2) is related or possibly related to participation in the research (in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and 3) suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

AEs will be graded according to the following scale:

- Mild: An experience that is transient, and requires no special treatment or intervention. The experience does not generally interfere with usual daily activities. This includes transient laboratory test alterations.
- Moderate: An experience that is alleviated with simple therapeutic treatments. The experience impacts usual daily activities. Includes laboratory test alterations indicating injury, but without long-term risk.
- Severe: An experience that requires therapeutic intervention. The experience interrupts usual daily activities. If hospitalization (or prolongation of hospitalization) is required for treatment it becomes an SAE.

Attribution of AEs and SAEs will be categorized as:

- Not related: The AE is clearly not related to the study procedures (i.e., another cause of the event is most plausible and/or a clinically plausible temporal sequence is inconsistent with the onset of the event).
- Possibly related: An event that follows a reasonable temporal sequence from the initiation of study procedures, but that could readily have been produced by a number of other factors.
- Related: The AE is clearly related to the study procedures.

Reporting of AEs, SAEs, and unanticipated problems will follow the guidelines of the IU Standard Operating Procedure for Reporting Unanticipated Problems and Noncompliance. Specifically, the following events will be reported promptly (i.e., within five business days) to the IRB:

- *AEs (including SAEs)* that are assessed by the PI or coinvestigators as (1) unexpected, (2) related or possibly related to participation, AND (3) suggests that the research places subject(s) or others at greater risk of harm than was previously known;
- *Major protocol deviations* that may, in the opinion of the PI, (1) impact subject safety, (2) affect the integrity of the data, OR (3) affect study participant' willingness to participate in the study;
- *Noncompliance*, which includes any action or activity associated with the conduct or oversight of the research that fails to comply with federal or state regulations, institutional policies governing human study participant research, or the requirements or determinations of the IRB.

Unanticipated problems that do not meet the criteria for prompt reporting will be reported at time of protocol renewal to ensure the IRB has a full understanding of the conduct of the research

Study Withdrawal/Discontinuation

A participant may withdrawal from the study at any time verbally or by providing this request in writing as described in the informed consent document. As outlined in the consent document, if the participant/patient wishes to withdraw consent, the PI will:

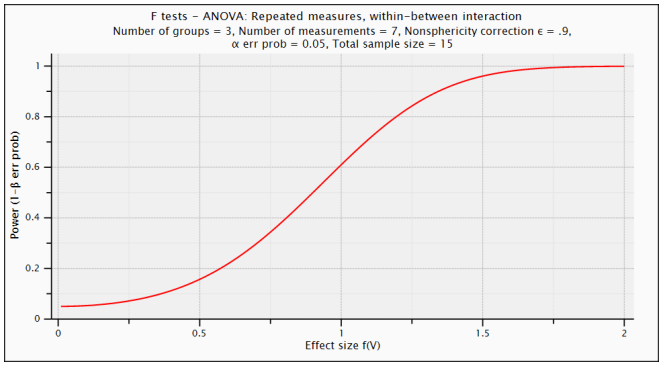
- no longer use and take reasonable steps to destroy all blood/tissue samples and information
- not take back any research / analyses already completed

7.0 Statistical Considerations

Statistical analyses: Normality of data distribution will first be tested using the D'Agostino-Pearson omnibus test. Analysis of individual endpoints will then be conducted using a mixed model analysis of variance (ANOVA) approach, with dose, trial number, and sequence as fixed effects and subject as a random effect to account for repeated measurements. As a preliminary analysis, carryover will be evaluated by testing the effect of sequence, i.e. does the effect of dose depend on trial. Assuming a sufficient washout period, i.e., no carryover effect, analysis of dose will proceed using all trials. Dose effects will be determined from ANOVA model results. A P value of <0.05 will be considered significant.

Calculation of sample size/power analyses: Our proposed sample size (i.e., n=20) for this pilot, proof-of-concept study has been chosen primarily based on budgetary considerations and the 1 y duration of the project. G*Power© version 3.1.9.4[59] was used to determine the sample size required to provide a power (i.e., 1-ß) of 0.90 assuming an effect size (i.e., Cohen's d) of 1.5. This effect size is based on the n=7 healthy older subjects that we have studied to date and is roughly the same as that we have previously observed for the effects of dietary NO_3^- on muscle contractile function in younger subjects.[29,30] These calculations demonstrated that with α=0.05, n=15 would be sufficient to provide the chosen power. The power that a sample size of n=15 would provide to detect other effect sizes is shown in Figure 4. Based on these calculations, we will enroll 20 subjects to allow for subject drop-out.

Potential limitations and alternatives considered: A possible limitation of the proposed study is the lack of direct demonstration that dietary NO_3^- results in an increase in NO specifically within muscle. This is because 1) the biological half-life of NO is extremely short (i.e., seconds), precluding measurement of its concentration in human tissue samples, and 2) existing stable isotopic methods only measure the rate of NO production via the NOS pathway, and not the enterosalivary pathway. We will, however, measure changes in plasma NO_3^-/NO_2^- and breath NO (a biomarker of whole-body NO bioavailability) levels in response to dietary NO_3^-. We therefore do not consider the absence of direct measurements of plasma or muscle NO a significant limitation.



A more significant limitation of the proposed study is that, due to budgetary and time constraints, we will be evaluating only two doses of NO_3^-. We will therefore be unable to identify the truly optimal dose (which could fall in-between the two doses to be tested, or higher than the highest dose). As stated previously, however, there is very limited data in the literature regarding the dose-response relationship of dietary NO_3^-, especially with respect to changes in exercise performance. Thus, despite this limitation the information to be gained from this study will represent a significant advance, and in fact is a necessary first step towards the design of larger (e.g., multicenter) studies.

By focusing specifically on changes in muscle contractile properties, we will not be able to determine whether dietary NO_3^- supplementation reduces the O_2 cost of exercise in older subjects. We will also not be measuring the effect of acute and/or chronic dietary NO_3^- intake on muscle blood flow, or on muscle energetics or aerobic exercise performance. We will also not be testing whether dietary NO_3^- intake improves insulin sensitivity. Several recent studies, however, have reported that dietary NO_3^- (or NO_2^-) does not significantly improve aerobic exercise function in older men and women.[60-63] (Despite increasing muscle contractile function.[63]) Similarly, several other recent studies have demonstrated that, contrary to the results of *in vitro* experiments,[64] dietary NO_3^- does not enhance insulin sensitivity in older humans.[65-67] We therefore believe that we are well-justified in focusing on changes in muscle contractile properties, especially in light of the important role that reductions in muscle function play in the aging process.

Because our focus is muscle contractile function, we considered using percutaneous electrical nerve stimulation rather than voluntary exercise. However, this approach inverts the normal orderly recruitment of motor units, preferentially depolarizing larger alpha motor neurons innervating type II muscle fibers.[68] Thus, while potentially magnifying the effects of dietary NO_3^- on muscle speed and power it would significantly diminish the external validity of our findings.

Although the proposed study will provide important new information regarding the effect of NO_3^- dose on muscle contractile function in older individuals, it will not address the mechanism(s) responsible for any improvements that may be observed. While clearly relevant, answering such questions are beyond the scope of the present investigation.

Finally, we note that historically there have been concerns that increased NO_3^- intake may lead to formation of carcinogenic nitrosamines.[69] However, the Joint FAO/WHO Expert Committee on Food Additives has concluded that "Overall, the epidemiological studies showed no consistently

increased risk for cancer with increasing consumption of NO_3^-."[63] Furthermore, diets high in vegetables, such as the DASH diet widely recommended to hypertensive patients, routinely exceed the WHO acceptable daily intake of NO_3^- by >5-fold. In contrast, the amount of dietary NO_3^- shown to improve exercise tolerance in previous studies is only slightly above the WHO limit.[70] Nonetheless, the safety and efficacy of increased dietary NO_3^- would need to be established in follow-up large-scale, longer-term studies before BRJ or other NO_3^--rich foods or could be widely recommended to older individuals.

8.0 Privacy/Confidentiality Issues

All study activities will be performed within areas respective of the participants' right to privacy.

Although there can be no absolute guarantee of confidentiality, every practical precaution will be taken.

Each study participant will be assigned a unique ID. Samples and information will be de-identified using this unique ID.

9.0 Follow-up and Record Retention

Study recruitment will be ongoing. The duration of the entire study is expected to be 12 months.

Any remaining blood samples will be de-identified and discarded. The de-identified data will be retained on computer files indefinitely. Hard copy study documents will be kept in a locked, file cabinet in a locked room. Electronic study information will be stored on a specified, password-protected network that is backed up daily. Only the study team and the relevant personnel will have access. Files will be kept on site until 2 years after study completion, and then sent to a secure archiving facility. Files will be kept for 7 years after study completion per state law requirements.

10.0 References

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