Dietary Nitrate and Muscle Power with Aging

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Table of Contents:

1.0 Background

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 (1,2). Thus, any intervention that significantly enhances muscle contractile properties 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, with age-related changes in the size, properties, and neural control of muscle likely all playing a role (3). Another important factor, however, may be a fall in nitric oxide (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 (cf. Refs. 4-8 for review). Specifically, during *isometric* contractions NO may, or may not, slightly suppress maximal force production (4-8). This may be due to nitrosation or S-nitrosylation of various proteins (9). During *concentric* activity, however, NO significantly increases the rate of force development, maximal shortening velocity, and maximal *power* of both single muscle fibers and isolated muscles (4-8).

Based on animal studies, these stimulatory effects are thought to be mediated via activation of the classic NO-sGC-cGMP pathway (10), and have been *euphemistically* described by Maréchal and Gailly (5) as a "slow-to-fast" shift *qualitatively* akin to the chronic transformation of muscle fiber type that occurs with, e.g., prolonged electrical stimulation. 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^- (11,12). In skeletal muscle itself, there is a dramatic decrease in interstitial NO_2^- and NO_3^- concentrations (13), as well as a reduction in intracellular NO_3^- content (14) These changes are accompanied by a decline in the activity of the neuronal form of NO synthase (nNOS, or NOS1) (15,16), the primary isoenzyme within muscle responsible for the synthesis of NO from L-arginine, O_2, and NADPH. This is associated with an age-related reduction in flow-mediated vasodilation (17-20), 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 (21). Taken together, these data suggest that decreased skeletal muscle NO production may contribute to the age-associated decline in muscle contractile properties and hence in functional capacity.

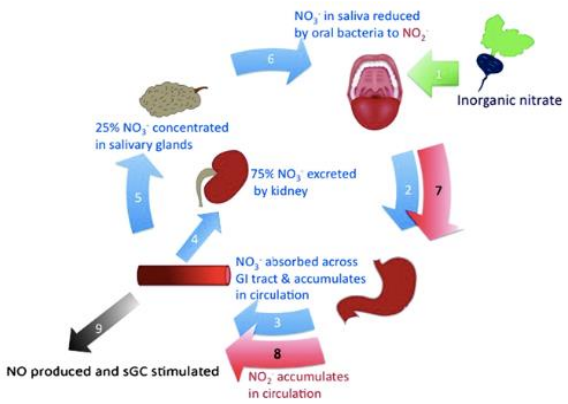


Figure 1. NO-sGC-cGMP signaling via the enterosalivary. From Ref. 22.

In this context, the physiological effects of dietary NO_3^- are of considerable interest. It

is now recognized that NO_3^- in the diet is a significant source of NO in body (22-26) (**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 up to ~25% of basal whole-body NO production (27,28). 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 (22-26), 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 NO_3^-/NO_2^-/NO metabolism (29).

Haider and Folland (30) and Whitfield et al. (31) have reported that dietary NO_3^- intake enhances the rate of force development and peak force output of the human quadriceps muscle during electrically-stimulated contractions. Numerous recent studies (reviewed in 32) demonstrate that dietary NO_3^- can enhance other types of exercise performance in various subject groups. To date, however, only a handful of studies have specifically examined the impact of dietary NO_3^- on exercise capacity in older individuals (33-35). These studies found no effect of consuming 9.6-12 mmol NO_3^-/d for 3-7 days on various measures of *aerobic* exercise performance in healthy older men and women (33,34) or in older individuals with hypertension or heart failure (HF) (35). Similarly, Justice et al. (36) reported that ingesting NO_2^- (*not* NO_3^-) at 1.2-2.4 mmol/d for 10 weeks did not improve VO_2max in middle-aged and older study participant, although it did increase the rate of force development (but not maximal force, i.e., strength) during *isometric* muscle contractions. Importantly, however, *none* of these studies of older study participant measured maximal muscle *power*. The effects of dietary NO_3^- on this critical aspect of muscle contractile function in older study participant are therefore still completely unknown. Moreover, it cannot be automatically assumed that dietary NO_3^- will be equally efficacious in improving muscle function in older persons as we have found in other study participant groups (37-39). For example, in rodents, the effects of dietary NO_3^- are more prominent in fast- vs. slow-twitch muscle (40,41), and some (42-45), but not all (46-48), 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 (49) could limit the beneficial effects of dietary NO_3^- on muscle NO-sGC-cGMP signaling in older persons

Even if it can be shown that dietary NO_3^- acutely improves muscle function in older men and women, it remains to be established whether such benefits can be sustained, and/or whether such effects have a positive impact on a person's daily life. This is not necessarily a given, as it is well known that tolerance develops quite quickly in response to *pharmacological* nitrates, e.g., glyceryl trinitate (nitroglycerin) (49). This does not seem to be true, though, for dietary NO_3^- (50,51) – in fact, animal studies indicate that ingestion of NO_3^- for 5-7 days may lead to positive adaptations in calcium handling proteins and hence force production in muscle that would tend to mitigate age-related declines in these parameters (40). Notably, however, a recent study of humans was unable to reproduce these findings (31).

Our **Preliminary Data** suggest that the effects of dietary NO_3^- on muscle speed and power are maintained (but not enhanced) for at least 14 days with repeated (i.e., daily) dosing in humans (see below).

Preliminary data

Using methods essentially identical to those to be employed in the proposed experiments, including use of a "gold standard", double-blind, placebo-controlled, crossover design, we previously demonstrated that 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 (37). 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 (38). Lastly, we found even greater increases in Vmax and Pmax (of 12±5%, $P=0.09$, and 13±4%, $P<0.05$, respectively) in nine middle-aged patients with systolic heart failure (HF) (52). The greater beneficial effect of dietary NO_3^- in HF patients presumably reflects the fact that, similar to healthy aging, HF results in reduced NO bioavailability (53).

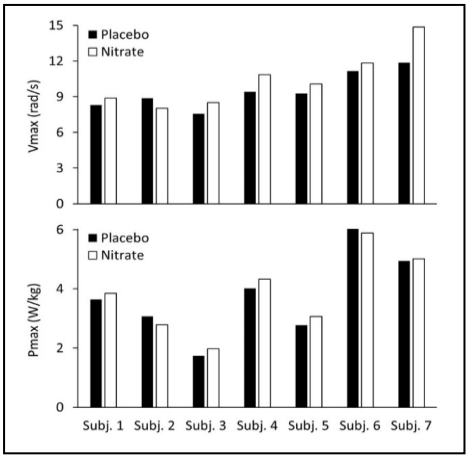
Using the same methods, we have also recently determined the effects of acute dietary NO_3^- intake in seven men and women 61-78 y of age. As shown in **Table 1**, plasma NO_3^- and NO_2^- and breath NO increased ($P<0.05$) by 1309±188%, 268±91%, and 146±19%, respectively, by 2 hours after ingestion, and all three remained elevated for the remainder of the experiment. These observations are consistent with previous research in older study participant demonstrating that plasma NO_3^- and NO_2^- concentrations peak 2-3 hours after ingestion of a NO_3^- load (54), which is comparable to the time course observed in younger study participant (55-57).

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 exer.
Plasma NO_3^- (μmol/L)	Placebo	26±4	24±3	24±3	22±4
	Nitrate	31±7	285±27*	295±27*	282±33*
Plasma NO_2^- (μmol/L)	Placebo	0.21±0.03	0.26±0.06	0.26±0.06	0.20±0.03
	Nitrate	0.27±0.04	0.43±0.13	0.49±0.06*	0.56±0.10*
Breath NO (ppb)	Placebo	23±5	26±4	27±4	23±5
	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 ($P<0.05$).

Fig. 2 Changes in maximal knee extensor speed (Vmax, top panel) and power (Pmax; bottom panel_ in response to dietary NO_3^-; intake in older adults



As shown in **Fig. 2**, dietary NO_3^- intake increased Vmax in six out of seven of these study participants, with the average improvement being 9±3% (P<0.05). Pmax increased in five out of the seven study participants, with the average increase being 4±3% (P=0.15). These preliminary results, which were presented at the 64th annual American College of Sports Medicine meeting in Denver, CO, May 30-June 3, 2017, are similar to those we obtained previously in younger individuals (37), and thus support our primary hypothesis.

Biopsy samples were obtained from the *v. lateralis* of two of these study participant ~3 h after ingestion of either the placebo or NO_3^- and analyzed for cGMP content. As shown in **Fig. 3**, muscle cGMP was higher in both study participants in the NO_3^- trial. These data support our hypothesis that dietary NO_3^- intake enhances muscle contractile function by increasing muscle NO-sGC-cGMP signaling.

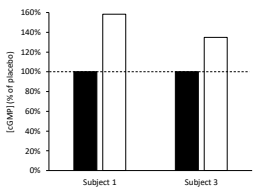


Figure 3. Effect of NO_3^- ingestion on muscle cGMP content.

Another of the study participants (number 5) continued to ingest either the NO_3^- supplement or the placebo daily for a total of 14 days, separated by a 10 day washout period. As shown in **Fig. 4**, their Pmax and Vmax were enhanced almost identically after both acute and chronic ingestion, such that the two dashed curves are superimposable. These data demonstrate that tolerance does not develop in response to repeated ingestion of dietary NO_3^-. Furthermore, since Vmax and Pmax were comparable after 14 vs. 1 day of placebo treatment (i.e., 25 vs. 11 day post NO_3^- treatment), they also indicate that even a 10 day washout period (vs. the 14 days we propose to use) is sufficient to eliminate any carryover effects.

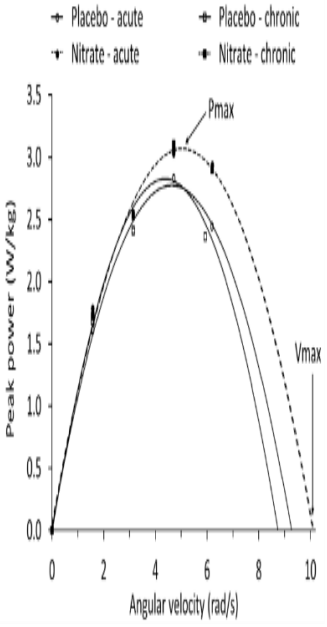


Figure 4. Effects of acute vs. chronic dietary NO_3^- intake on maximal knee extensor power (Pmax) and speed (Vmax) in a 78 y old woman.

In summary, we have demonstrated that dietary NO_3^- improves maximal muscle speed and power in a variety of subject populations, including a small number of healthy, elderly men and women. Furthermore, we have demonstrated that dietary NO_3^- intake results in an increase in muscle NO-sGC-cGMP signaling. Finally, we have demonstrated that *daily* ingestion of dietary NO_3^- for 14 days does not result in tolerance. These preliminary data therefore provide very strong support for the current study.

2.0 Rationale and Specific Aims

We hypothesize that increased dietary NO_3^- intake will enhance muscle NO-sGC-cGMP signaling in older men and women, leading to an increase in maximal speed and power.

Helping drive our hypotheses are the consistent improvements in muscle speed and power that both we and others have observed following dietary NO_3^- intake in healthy younger study participants (37), athletes (38,39), and especially heart failure patients (52).

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 study participant. 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. In preliminary experiments, however, we have not been able to reproduce the O_2-sparing effect of NO_3^- ingestion, and several recent studies have reported that dietary NO_3^- (or NO_2^-) does not significantly improve aerobic exercise function in older men and women (32-35). Similarly, several other recent studies have demonstrated that, contrary to the results of *in vitro* or animal experiments, dietary NO_3^- does not enhance insulin sensitivity in older humans (58-60). We therefore believe that we are well-justified in focusing on changes in muscle contractile properties, especially in light of our preliminary data and 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 (cf. 61 for review). 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.

We considered, but rejected, various alternatives to our "gold standard", double-blind, placebo-controlled, crossover design. This design is the most efficient for testing all of our hypotheses, i.e., it requires the fewest number of study participant to provide adequate statistical power (since each subject serves as their own control). A cross-sectional study would require >10x study participant to provide equivalent power for detecting changes in Pmax.

Finally, we note that historically there have been concerns that increased NO_3^- intake may lead to formation of carcinogenic nitrosamines (62). 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 (63). Nonetheless, the safety and efficacy of increased dietary NO_3^- would need to be established in follow-up large-scale, longer-term studies before beet root juice (BRJ) or other NO_3^--rich foods could be widely recommended to older individuals.

Specific Aim #1:	Determine the *acute* effects of dietary NO3- intake on muscle NO-sGC-cGMP signaling and muscle contractile properties
Specific Aim #2:	Determine the cumulative or *chronic* effects of dietary NO3- intake on muscle NO-sGC-cGMP signaling and muscle contractile properties
Exploratory Aim #3:	Assess the effects of dietary NO3- supplementation on perceived fatigue, physical function, and physical activity during daily life via questionnaires and accelerometer data

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, EKG, and routine blood tests
- Agrees to also participate in the FIT study (IRB # 1707550885).

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), neuromuscular disease (e.g., cervical spondylotic radiculomyelopathy, lumbar 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
- Expending greater than 1500 kcal/week in moderate or greater intensity physical activity as defined by the CHAMPS physical activity questionnaire

One hundred study participants will be enrolled to attain sixteen completed participants.

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), NIH sponsored website/email via ResearchMatch.org and newspaper advertisements.

In addition, our study will be listed on the Indiana CTSI study listing called the All IN for Health TrialX iConnect. iConnect is a HIPAA-compliant and secure public facing research recruitment platform provided by the Indiana Clinical and Translational Sciences Institute (Indiana University) consisting of a study trial listing and volunteer registry. This system is licensed by Indiana University from TrialX and is monitored by the Indiana University Information Technology Services (UITS) and University Information Security Office (ISO). Study Teams have access to their study listings and participant referrals based on a secure login. There is a small Administrative Team within the Indiana CTSI that manages the iConnect platform and has access to all study information, referrals, and registry volunteers. This online platform provides researchers with the ability to create a public facing webpage that can contain a brief prescreener and the ability for the public to refer themselves to the trial: (https://research.indianactsi.org). The study listing information can be modified based on the study and targeted population and provides a direct,

easily managed system to enhance communication with the public. Study teams can also use this webpage as the landing or referral page for any marketing materials that are being used (either by listing the hyperlink or creating a scannable QR code). We have included a screenshot of our study listing for your review and approval. Please see attachment titled "Screenshot iConnect". In addition, potential participants can complete a pre-screener that will determine their preliminary eligiblilty (see attachment "Pre-screener for iConnect.")

The study listings are open to the public and any persons who visit our study page may submit their contact information to us through the listing. We can access that information by logging in to our iConnect dashboard. We may respond to and contact interested volunteers directly through the platform or by university phone or email. We will use the following guide for any email responses for phone conversations with the volunteers. For our standard messaging, please see attached "iConnect Study Team Reply Email Message."

In addition to the study listing(s), potential participants will be identified from the Indiana CTSI All IN for Health voluntary registry IU IRB Protocol Number 1105005444. This registry was created so that interested individuals could create a volunteer profile to receive information about ongoing studies. Persons in this registry have provided their self-reported health information for the purposes of being matched to appropriate research studies. The registry volunteers have signed an electronic consent/authorization so that their information can be used and they can be contacted for this reason.

We can also match participants in the registry and are able to send out a message to them through the system in a de-identified manner. We may use the search and filter tool in the system to identify a group of volunteers who match our study criteria based upon the information that the volunteers have included in their profiles. We may then send an outreach message to those de-identified matched volunteers through the online system. If we send an outreach message to matched volunteers, we will use standard messaging (please see attached "iConnect Volunteer Outreach Email Message Template." Matched volunteers who receive our outreach message and indicate they are interested and agree to be contacted specifically for this study will approve for their contact information to be accessible by us through the iConnect portal. If we contact those interested matched volunteers by email or phone, we will use the language from Template C [file name of "iConnect Study Team Reply Email Message" for any email responses for phone conversations with the matched volunteers.

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 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. Subjects will be asked

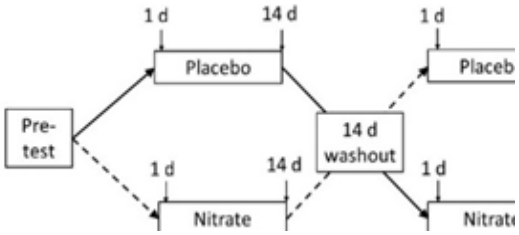
to fast for 12 hours prior to their screening visit, before they sign the consent form. When the subject arrives for the screening visit, a member of the research team will thoroughly review the consent with the study participant and answer questions. Study participants will be given as much time as 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 by the study statistician.

A separate consent form for participation in the Fit Core (IRB study #1707550885) will be administered by the Fit Core staff according to their policies and procedures.

5.0 Study Procedures

Study Design

A diagram of the flow of the study for each participant is shown in **Figure 6.**



Each participant will be studied using a double-blind, placebo-controlled, crossover design.

The dose of NO_3^- to be given, which is roughly the equivalent of 3-4 whole beets or 2 cups of cooked spinach (64), has been chosen based on our previous results (37,38,52) and preliminary data, as well as a recent study by Wylie et al. (55) demonstrating that, acutely (i.e., as a single dose), at least 8.4 mmol are needed to elicit an improvement in exercise performance but additional improvement does not result from a dose of 16.8 mmol. The placebo, also to be purchased from James White Drinks Ltd., is prepared by extracting NO_3^- from beetroot juice (BRJ) using an ion exchange resin and is indistinguishable in packaging, color, taste, texture, and smell from the standard product. Importantly, this placebo does not alter plasma NO_3^-/NO_2^- or breath NO concentrations or the physiological responses to exercise.

Study Procedures

On Visit 1 (Pre-test), all study participants will complete a screening examination: questionnaires will be completed regarding their physical activity and perceived fatigue and physical function. The participant will undergo a complete history and physical exam by a study physician, an EKG, and phlebotomy for screening and laboratories (complete blood count, liver and kidney function tests, electrolytes, fasting glucose and insulin), and an urine test to assess kidney function. They will also practice the entire

isokinetic dynamometer exercise test. Participants will then be escorted to the Fit Core where Fit Core staff will consent and then complete the Fit Core protocol (IRB study #1707550885).

Participants will be instructed to consume their normal diet (aside from the BRJ supplement) throughout the study. They 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 hours; Ref. 27) 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 U.S. (54) has no significant influence on plasma NO_3^- levels (54). Participants will be asked to refrain from using chewing gum, alcohol, and caffeine-containing food/drinks for 24 hours 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 (65,66). Participants will be asked to fast for 12 hours prior to each remaining study visit.

On Visit 2 (Day 1 pre-crossover), study participants will complete questionnaires (PROMIS® questionnaire) regarding their perceived fatigue and physical function, will have their vital signs assessed, and will undergo phlebotomy for NO_3^- and NO_2^- measurement. They will then ingest 140 mL of BRJ either containing or depleted of 11.2 mmol of NO_3^- (i.e., **BRJ w/ NO_3^-** or **BRJ w/o NO_3^-**). After allowing 2 hours for absorption, they will undergo the isokinetic dynamometer. After completion of the dynamometer assessment, subjects will complete the Fit Core protocol, less the DEXA scan. Blood samples will be obtained at hourly intervals during the study. Heart rate and blood pressure will be measured at the same scheduled times as blood samples after which a final blood sample will be obtained. The sequencing of the measurements have been chosen to minimize potential interference/carryover effects that might influence the data and/or its interpretation.

Following completion of the above tests, the participant will continue to ingest 140 mL of BRJ w/ NO_3^- or BRJ w/o NO_3^- every morning for an additional 13 days, after which the protocol described above will be repeated on the 14th day. To encourage compliance with the experimental protocol and enhance subject retention, the subjects will be initially provided with only a 7 day supply of BRJ. They will also be asked to keep and return the empty BRJ bottles at the next visit.

On Visit 3 (Day 7 pre-crossover), participants will be asked to return the empty BRJ bottles. At this visit, participants will have their vital signs assessed and a blood sample will be obtained to quantify plasma NO_3^- and NO_2^- concentrations. Participants will be asked to rate their perception of fatigue and physical function during the previous week using the NIH-funded Patient-Reported Outcomes Measurement Information System (PROMIS®). The final 7 days of BRJ will be provided. They will also be asked to keep and return the empty BRJ bottles at the next visit.

On Visit 4 (Day 14 pre-crossover), participants will be asked to return to undergo the same studies as described above for Study Day 2. In addition, participants will be asked to rate their perception of fatigue and physical function during the previous week using the NIH-funded Patient-Reported Outcomes Measurement Information System

(PROMIS®). The study team member will also complete BRJ accountability for the 2nd, 7-day consumption period by collecting the empty BRJ bottles

Then the study participant will undergo a 14 day (minimum) washout period consuming neither either **BRJ w/ NO_3^-** nor **BRJ w/o NO_3^-**. After this, the study participant will be crossed-over to receive whichever treatment they did not receive first.

On Visit 5 (Day 1 post-crossover), the study participant will undergo the same procedures as described for Study Day 2.

On Visit 6 (Day 7 post-crossover), the study participant will undergo the same procedures as described for Study Day 3

On Visit 7 (Day 14 post-crossover), the study participant will undergo the same procedures as described for Study Day 4.

During each 14 day intervention period (i.e., between Study Days 2 and 4 and 5 and 7 inclusive), an Actigraph wGT3X-BT activity monitor will be worn at the hip during all waking hours except when bathing or swimming. The Actigraph measures ambient light and also accelerations in all three directions, from which total activity counts, active time, sedentary time, sleep, etc., are estimated/quantified.

Subjects will complete a log indicating the times that they wore the activity monitor and return it to the study staff at each visit.

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^-: 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.

Measurement of skeletal muscle contractile function: A Biodex 4 system will be used to measure each subject's muscle contractile properties as previously described (30,33). Briefly, study participant 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 achieved 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 minutes 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 using the manufacturer's software. 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 with a parabolic function to determine the subject's Pmax and Vmax (i.e., Y-

maximum and 2nd Y-intercept of fitted parabola, respectively) as previously described (30,33).

Measurement of perceived fatigue, physical function, and physical activity: Perceived fatigue and physical function will be assessed using PROMIS® Short Forms 8a and 20a, respectively , which are physical functioning questionnaires. Actual physical function will be quantified by the FIT Core using a suite of tests, i.e., usual gait speed, fast gait speed, Short Physical Performance Battery, 30 s chair stand performance, 6 min walk distance. Physical activity will be quantified using ActiGraph wGT3X-BT activity monitors (ActiGraph, Pensacola, FL). On Study Day 1, each participant will be given a fully-charged monitor and directed to wear the monitor on their right hip (superior to the iliac crest) for the next 7 days, removing it only when sleeping or during aquatic activities (i.e., bathing, swimming), and to complete a log of when the monitor is worn. On day 7, they will exchange the monitor for another, to be worn for the next 7 days. Upon return of the monitors, the data will be downloaded using the ActiLife 6 software, divided into 60 second epochs, and analyzed to quantify both total and bouted (i.e., >10 min duration) sedentary behavior, light physical activity, and moderate-to-vigorous physical activity (<100 counts/min, 100-2020, and >2020 counts/min, respectively). Total counts will also be recorded, since this may be more closely associated with the health benefits of physical activity (68). Results will be considered valid only if each monitor is worn for at least 10 hours/day over at least 4 days (including at least one weekend day) each week. Subsequently, the subject will perform an "all out" 50 contraction fatigue test (at 3.14 rad/s) to determine whether dietary NO3- 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

Beetroot Juice: Beetroot juice consumption or the consumption of the BRJ placebo may cause stool and/or urine to appear pink. This does NOT indicate bleeding. You may experience mild gastrointestinal distress (cramps, bloating) or diarrhea following ingestion of the BRJ. In rare cases there is a theoretical increased risk of upper GI cancers if a compound called "nitrosamine" is made from nitrates by the body. However, in large studies of many people followed up for many years, a fruit and vegetable-rich diet (where the acceptable daily intake of nitrates was exceeded several-fold) was not associated with any increase in cancer or mortality. The 2003 Joint Food and Agricultural Organization/World Health Organization Expert committee concluded that there was no evidence that nitrates are carcinogenic to humans.

Questionnaires: You may experience emotional discomfort when answering some questions in the health questionnaires. If any particular question makes you uncomfortable, you may discuss its importance and the need to answer it with the specially trained interviewer. You may choose not to answer any question with which you still feel uncomfortable.

Needle Stick: Slight pain, bruising or bleeding can occur. Rarely, infection can occur. Very infrequently, faintness may occur. To minimize the discomfort and risks associated with blood draw, only trained staff will collect blood samples.

Exercise Test: Your muscles may feel fatigued during the exercise test. You may also develop soreness in your muscles or joints. Very rarely, an exercise test, such as the neuromuscular function test, may be associated with serious complications including, but not limited to: fainting and disorders of the heart beat (too fast or too slow) which may require hospitalization; heart attack, stroke, or death; and muscle or joint pain. We will make every effort to minimize these rare risks by observing and monitoring during testing. However, no guarantees can be made. Emergency equipment and trained personnel are available to deal with any emergency.

EKG: some people may experience a skin rash but this usually goes away without treatment.

Fit Core: the risks for the Fit Core are described in that protocol (IRB study #1707550885).

Another risk of this study is the possible loss of confidentiality, which is minimal. Even though the risk is small, a link exists between your protected health information and your sample. In addition to the risks listed above, there may be some unknown or infrequent and unforeseeable risks associated with participation in this study. You will be informed in a timely manner of any new information, findings or changes to the way the research will be performed that might influence your willingness to continue your participation in this study.

<u>Mitigation of Risks</u>

Questionnaires: Subjects may feel uncomfortable answering portions of the questionnaires. If so, they may discuss the importance of questions and the need to answer them with a member of the research team. They may choose not to answer any question with which they still feel uncomfortable.

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

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.

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

Fit Core: the mitigation of risks for the Fit Core are described in that protocol (IRB study #1707550885).

These instructions will be provided both verbally and in written form.

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.

7.0 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

8.0 Statistical Considerations

Data analyses: The primary outcomes are muscle contractile function (i.e., Vmax and Pmax) and muscle cGMP concentration on day 1 and day 14, as separate outcomes. H_0 is that they are not different while on BRJ w/ NO_3^- versus on BRJ w/o NO_3^- and H_a is that they are greater while on BRJ w/ NO_3^-. This hypothesis will be tested using a mixed model analysis of variance approach, with treatment (NO_3^-, placebo), sequence (NO_3^--to-placebo, placebo-to-NO_3^-), and intervention period (1^{st}, 2^{nd}) as fixed effects and subject as a random effect to account for repeated measurements. Carryover will be evaluated by first testing the effect of sequence, i.e., does the effect of treatment depend on

sequence. Assuming no carryover, analysis of treatment will proceed using both sets of data. The perceived fatigue, physical function (both obtained from the Fit Core protocol), and accelerometer data will be analyzed using functional and time series data analysis techniques. A two-sided $P<0.05$ will be considered significant. SAS 9.4 will be used for data analysis (SAS Institute, Cary, NC).

Sample size calculations: Samples size calculations were performed based on our preliminary data on NO_3^--induced changes in Pmax in seven older study participant. Based on the observed effect size of 0.47 and assuming an α of 0.05, N=10 would be required to achieve a power (1-β) of 0.80 and N=16 would be required to achieve a power of 0.90. To allow for screen failures and potential withdrawals, we will therefore enroll **100** study participants (to finish 16).

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 the muscle (or plasma) samples, and 2) existing stable isotopic methods only measure the rate of NO production via the NOS pathway. We will, however, measure changes in plasma NO_3^- /NO_2^- levels in response to dietary NO_3^-. We therefore do not consider the absence of direct measurements of muscle NO to be a significant limitation.

A potentially more important limitation to this research is the lack of pilot data for certain measurements. Thus, although we will have adequate power to detect increases in Vmax and Pmax), we cannot be assured that we will be able to detect differences in all of our endpoints. However, the proposed experiments would still provide the data needed to design a larger study that is adequately powered to detect changes in all variables.

9.0 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.

10.0 Follow-up and Record Retention

Study recruitment will be ongoing. The duration of the entire study is expected to be 24 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.

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