Dietary Nitrate Supplementation and Physiological Function in Older Adults

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Blood pressure responsiveness in older adults following dietary nitrate supplementation.

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Abstract

1 in 3 adults in the U.S. have hypertension which is known to increase the risk of heart disease and stroke. Blood pressure regulation is mediated by many factors including but not limited to metabolic factors, pressure/stretch sensing receptors located in the aorta and carotid arteries, endothelial cell function, and kidney function (via water and sodium absorption/excretion). It has been shown that many of these regulatory mechanisms are altered and/or become dysfunctional with aging. Specifically older adults commonly demonstrate a decreased sensitivity in pressure receptors and/or endothelial dysfunction. Additionally with aging, there is an increase in sympathetic nervous system activity and arterial stiffness which can contribute to higher blood pressure. The important vasodilator, nitric oxide (NO), has been implicated as a key signaling molecule involved in several of the mechanisms in blood pressure regulation outlined above. A decreased NO production or bioavailability is thought to contribute to several of the physiologic changes that come with aging. Therefore, the overall aim of this study is to use a randomized, double-blind crossover, placebo controlled study to examine the effects of acute dietary nitrate supplementation (to boost bioavailable NO) on various parameters of blood pressure regulation and responsiveness in normal healthy older adults. Our main hypothesis is that 4 weeks of dietary nitrate supplementation will decrease the blood pressure responses in older adults in response to a variety of physiological stressors.

Research Plan

I. Specific Aims:

Specific Aim 1: Determine whether carotid chemoreceptor sensitivity is altered with healthy aging. We will measure heart rate, blood pressure, and ventilatory (minute ventilation) responses to acute hypoxia in young and older adults. We hypothesize carotid body chemosensitivity to hypoxia is augmented with healthy aging.

Specific Aim 2: Determine whether increasing NO bioavailability via inorganic nitrate supplementation 1) attenuates carotid chemoreceptor sensitivity and 2) decreases blood pressure responsiveness in older adults. We will measure heart rate, blood pressure, and ventilatory (minute ventilation) responses to various physiological stressors in older adults before and after 4 weeks of beet root juice supplementation. We hypothesize carotid body chemosensitivity to hypoxia and blood pressure responsiveness to exercise and exposure to cold will be reduced following dietary nitrate supplementation via beet root juice. In addition, we hypothesize improvements in each of these reflex responses will be associated with the change in circulating nitrate and nitrite levels.

II. Background

Older adults are known to exhibit increased sympathetic nervous system activity (SNA), which is predictive of morbidity and mortality. This increase in SNA is related to elevations in blood pressure (BP) in older adults (17, 18, 19). The exact mechanisms responsible for the increase in sympathetic activation with aging are not completely understood, but may be related to alterations in baroreceptor sensitivity (BRS) (9, 12, 35, 37). Additionally, there is evidence to suggest carotid chemoreceptors in older adults are "overactive" and thus contribute to increases in sympathetic activation. However, controversy exists regarding aging and chemoreceptor activity such that data regarding the effect of aging on carotid chemoreceptor activity is inconclusive. Specifically, older adults have been shown to exhibit increased (7, 12, 30), decreased (13, 16, 25, 30, 40, 44, 46), or preserved (1, 42, 43, 48) carotid body chemoreceptor activity (as assessed by the heart rate and/or ventilatory response to acute hypoxic exposure).

The Carotid Chemoreceptors: The carotid chemoreceptors are primarily known for their oxygen sensing capabilities. More recently, these peripheral chemoreceptors have been found to sense and respond to a variety of other stimuli (e.g. acidosis, hypotension, hyperthermia, etc). In addition, the chemoreceptors have been identified as a potential therapeutic target for sympathetically mediated diseases (e.g. heart failure, hypertension, insulin resistance) such that removal of the carotid bodies can improve prognosis (38). For these reasons, the carotid chemoreceptors may be a viable therapeutic target for many of the comorbidities associated with normal aging.

Baroreflex and Chemoreflex Interactions: There are known interactions between the baroreflex and chemoreflex, such that activation of one will blunt responsiveness of the other. For example, when the baroreceptors are stimulated by intravenous phenylephrine (resulting in an increase in blood pressure), the heart rate response to hypoxia is attenuated (49). Similarly, when the carotid chemoreceptors are stimulated with hypoxia, baroreflex sensitivity may be reduced (8). Taken together, not only may altered chemoreceptor sensitivity with aging contribute to chronic sympathoexcitation, it may also explain, in part, why baroreflex sensitivity is reduced in this population. Thus, by attenuating chemoreceptor activation with a nitric oxide (NO) donor, improvements in baroreflex sensitivity may be observed.

Nitric Oxide Bioavailability and Aging: As humans age, there is a decrease in nitric oxide (NO) bioavailability due to endothelial cell dysfunction which causes less enzymatic production of NO through L-arginine-NOS pathway. Inhibition of endothelium-dependent relaxation due to decreased NO bioavailability is the most prominent feature of endothelial dysfunction. Loss of NO bioavailability occurs early in human vascular disease and is a contributing factor to abnormal vasomotion, altered blood flow, and ischemic symptoms (36). Older adults are known to exhibit decreased bioavailability of the important vasodilator, nitric oxide. Interestingly, NO has been shown to have an inhibitory effect on carotid body activity (14, 21, 51, 52). Consistent with this, nitric oxide donors can inhibit sensory discharge in the carotid sinus nerve (21, 38) and depress the ventilatory response to acute hypoxia (14). Additionally, in animal models, injection of NO alters baroreceptor activity (34). It has also been shown that risk of hypertension is decreased when baseline levels of BRS is increased (9). Taken together, decreased nitric oxide

bioavailability with aging may contribute to increased carotid chemoreceptor activity and altered baroreceptor sensitivity.

NO Bioavailability: NOS vs. nitrate-nitrite pathways. It is widely known that the L-arginine-NOS system significantly contributes to the overall production of NO. Until recently the inorganic anions nitrite (NO_2^-) and nitrate (NO_3^-) were considered inert end products of NO metabolism. However, accumulating evidence suggests that NO_2^- and NO_3^- metabolism occurs in the blood and various tissues to form NO (4, 31, 32). Thus, nitrite reduction (i.e. nitrate-nitrite-NO pathway) represents an alternative and differentially regulated system for NO generation that operates in parallel to the classic L-arginine-NOS pathway (33). Therefore, increasing plasma NO_2^- and NO_3^- levels in older adults may enhance NO bioavailability and subsequently improve blood pressure responsiveness to physiological stressors that have previously been demonstrated to be altered with aging and/or involve NO mediated mechanisms.

Benefits of dietary nitrate supplementation: In recent years data have accumulated to suggest that dietary nitrate supplementation can have physiological and therapeutic effects. In healthy young humans acute oral nitrate supplementation improves exercise efficiency and tolerance (2, 3, 27, 28). Dietary nitrite also reverses age-associated vascular endothelial dysfunction and large artery stiffness in mice via restoration of NO bioavailability and reductions in oxidative stress and inflammation (47). Furthermore, a number of studies in animals have reported a protective effect of low dose nitrite against ischemia-reperfusion injuries to various organs (10, 15, 22, 26, 54). Interestingly, acute ingestion of dietary nitrate prevents endothelial dysfunction induced by an acute ischemic insult in the forearm of healthy humans (53). Acute (hours) and short term (1-3 days) dietary inorganic nitrate consumption has also been shown to have favorable effects on isolated measurements of resting blood pressure (BP) in healthy volunteers (23, 28, 29, 53). Additionally, chronic dietary nitrate supplementation can have a sustained effect on BP in rats (41). To our knowledge the medium term effects (4 weeks) of dietary nitrate supplementation on BP have not been examined in healthy adults**.** Moreover, the longer term effects (weeks to months) of dietary nitrate on vascular function and/or arterial stiffness in humans are unknown.

Taken together, augmented carotid chemoreceptor sensitivity is known to increase sympathetic nervous system activity and contributes to pathological conditions. In this way, the carotid chemoreceptors have been recently sought as a potential therapeutic target for sympathoexcitatory conditions. Controversy exists regarding the effect of aging on carotid chemoreceptor activity and the potential mechanisms involved. Given nitric oxide is known to attenuate chemoreflex activity and older adults often exhibit reduced nitric oxide bioavailability, it is reasonable to propose carotid chemoreceptor activity is increased with aging and can be reduced with treatment of a NO boosting agent (beetroot juice). Furthermore, given known interactions between the chemoreceptors and baroreceptors, we propose dietary nitrate supplementation (via beet root juice) in older adults will also result in an improvement in baroreflex sensitivity that is correlated with reductions in chemoreflex sensitivity. With these ideas in mind, we seek to systematically

examine carotid body chemosensitivity in older adults and the potential mechanisms behind these observations.

In this proposal, we aim to examine the effects of 4 weeks of dietary nitrate supplementation on peripheral chemoreceptor sensitivity, spontaneous baroreflex sensitivity, and blood pressure responsiveness to a variety of physiological stressors in older healthy adults. We will also examine the effect of dietary nitrate supplementation on measures of central artery stiffness and aortic blood pressure in older adults.

III. Research Design and methods

Subjects:

A total of 25 normal healthy subjects will be recruited for this study. We will recruit 15 older (60-85 years of age) and 10 young (18-35 years of age) adults. The young subjects will serve as a control study group and will not participate in the intervention portion (i.e. dietary nitrate supplementation) of the study. Exclusion criteria include hypertension, hyperlipidemia, smoking, heart disease, diabetes, body mass index (BMI) ≥ 30 kg/m^2, autonomic disorders and other conditions or medications that might normally alter blood pressure responses. They will not be taking any medications except for oral contraceptives (in young women). Subjects who engage in rigorous physical exercise programs (greater than 45 minutes in duration and exceeding 3 times per week) will be excluded. Subjects that have an aversion to beets will also be excluded. Subjects will be pre-screened by telephone and a brief health history will be assessed. Young women will be studied in the early follicular phase of their menstrual cycle (or the low- hormone phase of oral contraceptive use), to minimize any effects of reproductive hormone status, which may make interpretation of results difficult. All females of child bearing potential will have a negative pregnancy test before starting the study. Recruitment will consist of a University-wide email to students, faculty, staff, and retired faculty.

Experimental Procedures:

1. ***Subject Monitoring***. Heart rate will be monitored by 3-lead ECG. Beat-to-beat arterial pressure will be measured using a Nexfin device placed on the middle finger. Respiratory rate and tidal volumes will be measured by turbine (flow-volume turbine, Vacumetrics). End-tidal CO2 and inspired O2 will be measured by capnography.

2. ***Venipuncture and Blood Draw****.* Single venous blood samples (~15-20 mL) from an antecubital vein using aseptic technique will be obtained on each study day for determination of nitric oxide metabolites (nitrate and nitrite) and cGMP. Fasting glucose and lipid concentrations will be determined from the blood sample obtained on the first study day.

3. ***Applanation Tonometry.*** High-fidelity radial artery pressure waveforms will be recorded by applanation tonometry of the radial pulse in the right wrist using a “pencil type” micromanometer (Millar Instruments, Houston, Texas). The radial blood pressure

(BP) and waveforms will be calibrated from the systolic and diastolic brachial artery BP (cuff). A validated, generalized transfer function will be used to generate the corresponding aortic pressure waveform (SphygmoCor system, AtCor Medical, Sydney, Australia). Additionally, ECG gated arterial waveforms will be obtained from the carotid and femoral arteries. Pulse wave velocity (PWV; index of arterial stiffness) will be determined using the Sphygmocor PVX system. The time (*t*) between the feet of recorded pressure waves will be determined as the mean of 10 consecutive cardiac cycles. PWV is calculated by the system software from the distance (*D*; meters) between measurement points and the measured time delay (t): PWV = D/Δt (m/s).

4. ***Spontaneous Baroreflex Test****.* Heart rate will be measured via ECG and matched to beat-to-beat arterial pressure recording over a 10-minute resting period. Respiratory rate and tidal volume will be measured during this time to determine the influence of respiratory rate and volume on heart rate. Computer software will be used to determine all sequences of three or more successive heart beats in which there are concordant increases or decreases in systolic BP and RR interval. A linear regression will be applied to each of the sequences, and an average regression slope calculated for the sequences detected during each recording period. This slope represents the cardiac baroreflex sensitivity (in milliseconds per millimeter of mercury) by the spontaneous baroreflex method (SBR slope).

5. ***Hypoxic ventilatory response****.* The sensitivity of the carotid body chemoreceptors will be assessed using a hypoxic ventilatory response test (completed in duplicate, separated by a minimum of 15 minutes of quiet normoxic breathing). End-tidal CO2 levels will be clamped at eupneic values throughout hypoxia by using a partial rebreathe system. Hypoxia will be achieved by titrating the level of inspired oxygen using an anesthesia gas blender [2 gas reservoirs (21% oxygen, 10% oxygen)] to achieve an arterial oxygen saturation of ~80% SpO2 (lasting ~2-6 minutes). This will be followed by 2 minutes of normoxic recovery. Ventilation, expired gases, and arterial oxygen saturation (finger pulse oximetry) will be continuously measured. Chemosensitivity will be assessed as the slope of the linear regression line for minute ventilation (L/min), heart rate (beat/min), or blood pressure (mmHg) versus arterial oxygen saturation (%) (Figure 1).

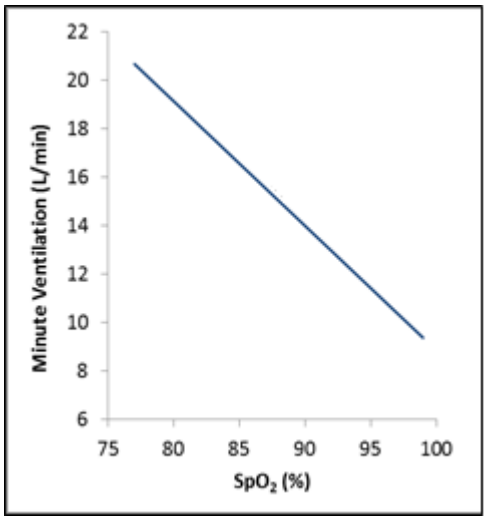


Figure 1. Regression line used to determine chemosensitivity.

6. Metaboreflex test. Subjects will be studied while in the supine position under standardized conditions. Maximal voluntary contraction (MVC) of the nondominant hand will be measured by having subjects squeeze a handgrip device at maximal effort three times; the average value will be used as the MVC. The MVC will be used to calculate a relative work rate of 30% MVC for the experimental protocol. Subjects will then rest quietly for 10 min after which an isometric handgrip test will be performed for 2 min. During the last 3 s of the isometric handgrip test, an occlusion cuff placed on the exercising arm above the elbow

will be rapidly inflated to suprasystolic pressure (240 mmHg) and remain inflated for 2 min and 15 s (post exercise ischemia, PEI). The additional 15 s of post exercise ischemia is incorporated to account for the potential effects of the initial decrease in BP that occurs when exercise is immediately stopped.

8. ***Rhythmic Forearm Exercise****.* Rhythmic forearm exercise will be conducted with a handgrip dynamometer at a light (~4 kg) and moderate (~8 kg) level of exercise (4 minutes each), as previously described (6). In addition, this technique has been adapted to facilitate beat-to-beat measurements of brachial artery blood flow via Doppler ultrasound.

9. ***Measurement of Forearm Blood Flow by Doppler Ultrasound****.* Forearm blood flow (FBF) will be determined from the simultaneous measures of brachial artery diameter and mean blood velocity (MBV). The echo-Doppler image of the brachial artery will be obtained in real time using a hand-held 12 MHz linear probe, operating in B-mode. The probe will be positioned over the brachial artery, approximately 9 cm proximal to the medial epicondyle. Brachial artery blood flow is the product of mean blood velocity and artery cross sectional area. Forearm vascular conductance (FVC) will be calculated using blood flow and mean arterial pressure

9. ***Cold Pressor Test****.* After 10 minutes of quiet rest, a 2-minute baseline period will be recorded, followed by immersion of the hand in ice water (0-4 degrees C) for 2 minutes. Heart rate and blood pressure will be averaged over each minute of cold immersion and recovery; these values will be compared to the average values during the pre-stress period.

10. ***Plasma Nitrate/Nitrite and cGMP****.* Plasma nitrate and nitrite levels will be determined from blood samples using a Sievers chemiluminescence NO analyzer (NOA 280i; Sievers Instruments, Boulder, CO). [NO_2^-] will be determined by addition of plasma samples to potassium iodide in acetic acid at room temperature in a gas-sealed purging vessel. To minimize the influence of diet on plasma levels of nitrate/nitrite and measurements of physiologic function, all subjects will be asked to follow a 3 day low nitrate diet prior to each study day. cGMP will be determined using an enzyme immunoassay (Cayman Chemical).

11. ***Dietary Nitrate Supplementation.*** At study entry, the 15 older subjects will be randomized to receive either a beetroot powder containing nitrate (active) or a beetroot powder without nitrate (placebo) (Neogenis Sport, Austin TX) for 4 weeks. After visit 2 and the subsequent 4-week washout period, all subjects will be crossed over to receive either the active or placebo powder (opposite of the one they received at study entry) for another 4 weeks (Figure. 2).

12. ***Data Analysis and Statistics***.

Sample-size/ Statistical Power: The primary aim of this investigation is to assess the potential efficacy of dietary nitrate supplementation for improving physiologic function on blood pressure within older individuals. With this aim in mind, we have designed a randomized, double-blind crossover, placebo-controlled study to assess potential efficacy and provide preliminary data for designing a larger trial in the future. Thus the primary endpoint is the change from baseline to end of treatment in blood pressure responses to various physiological stressors in older individuals.

Data Analysis: The primary variable of interest for this investigation is blood pressure responses to physiological stressors which will be measured at baseline and at the end of each respective 4 week treatment (beetroot juice with nitrate vs. placebo) and analyzed

as change from baseline. For the primary analysis, the change in pressure response from baseline to end of treatment will be compared between treatment groups (beetroot juice with nitrate vs. placebo) using a two-way repeated measures analysis of variance (ANOVA). Exploratory analyses will be performed to assess whether improvements in BP are positively correlated with changes in indices of arterial stiffness and whether improvements in blood pressure responses are associated with changes in vascular function.

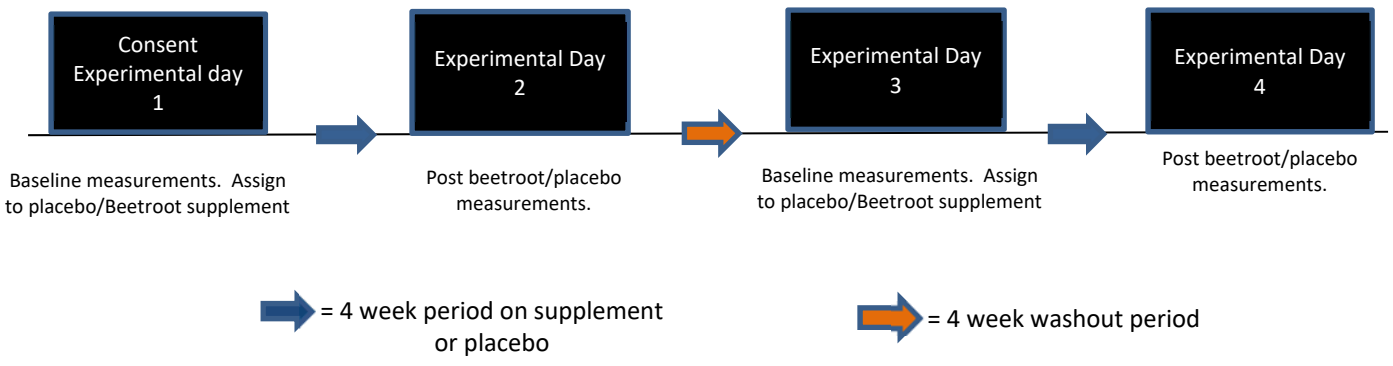
PROTOCOL - To address Specific Aims

Rationale and hypothesis:

As humans age, nitric oxide bioavailability decreases and there is an elevation in resting sympathetic activity (17, 19). This increase contributes to increased arterial stiffness and blood pressure during rest and exercise. Blood pressure responsiveness is also increased due to many factors including a decrease in the sensitivity of pressure receptors located in the carotid arteries and aorta. Recent evidence has shown that acute and short term (days) dietary nitrate supplementation is potentially effective in increasing NO bioavailability (5, 23, 27) and has favorable effects on measures of peripheral blood pressure at rest (23, 24). We will examine whether longer durations of dietary nitrate supplementation (4 weeks) have favorable effects on blood pressure responsiveness to a variety of physiological stressors in older adults.

Approach:

15 healthy older subjects will be studied. All older adults will undergo 4 separate days of testing as well as the placebo/supplementation portion of the study (Figure 2). We will also recruit 10 young adults to serve as a control group to compare baseline (prior to any intervention) data between age groups. The young group will only participate in one day of testing and will not undergo any of the placebo/supplementation portions of this study. Each group on their respective testing days will be subjected to a battery of tests that include a blood draw, spontaneous baroreflex testing, two separate consecutive hypoxic ventilatory response tests, handgrip exercise (both isometric and rhythmic),and a cold pressor test. The older group after undergoing testing on visits 1 and 3 will then be given their placebo/supplementation beetroot packets to be consumed once per day for 4 weeks. On each study day, heart rate will be monitored by 3-lead ECG and beat-to-beat arterial pressure will be measured using a Nexfin device on the middle finger during each test. Arterial O_2 saturation levels will be monitored by pulse oximetry throughout the hypoxic ventilatory tests. A single venous blood draw will be performed at the start of each study day (Figure 3) for the determination of plasma nitrate and nitrite levels.



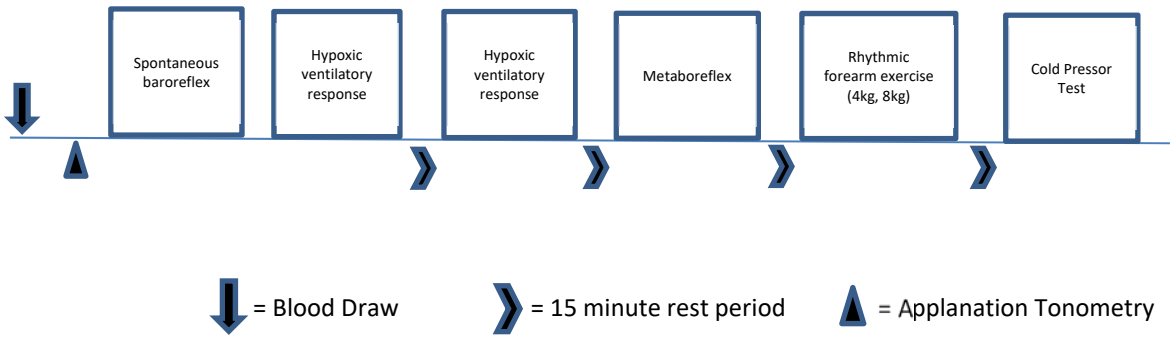


Figure 3. Experimental testing on each study day

Expected results, Interpretation, and Follow-up studies: We predict that following 4 weeks of nitrate supplementation compared to placebo, older adults will demonstrate a decrease in blood pressure responsiveness to each of the physiological stressors employed in the lab. Additionally, we hypothesize that dietary nitrate supplementation will have a beneficial effect on central artery stiffness. If our hypotheses are correct, it would suggest a beneficial therapeutic effect of nitrate supplementation in older adults.

General Issues: All techniques in this proposal have been successfully used by Dr. Casey for many years and are not expected to present difficulties. Additionally, Dr. Kenichi Ueda (Co-investigator) has extensive experience with venous blood draws in humans.

Human Subjects Research

Recruitment of Subjects

The subjects will be recruited from the faculty, staff, and students of the University of Iowa and from persons in Iowa City, Iowa and surrounding communities. Subjects will be eligible based on their health history questionnaire.

Potential Risks.

Risks from participation in these studies are not considered minimal.

1. Venipuncture. Risks of venipuncture in the arm include bruise or clot formation and infection. Additionally, risks associated with a needle stick and blood draws may include feeling light headed or nauseous and/or fainting.

2. Hypoxic ventilatory response tests: The hypoxic ventilatory response will be measured before and after beet root juice supplementation. There are theoretical risks related to prolonged exposure to hypoxia, but acute exposures as planned in this proposal should not pose any risk to the volunteers. Pulse oximetry, blood pressure, capnography, and heart rate monitoring will be used to ensure adequate gas exchange, circulatory parameters, and patient safety when arterial oxygen is altered. We will closely monitor subject arterial oxygen saturation to keep saturation at 70% or higher during the testing. We will have hyperoxia available in the unlikely case saturations fall below 70%.

3. Consumption of Neogenis BeetElite Powder. There is no risk associated with consumption of beetroot juice. However, pink/red urine or stools has been reported.

4. Applanation tonometry. There is no risk associated with arterial tonometry measurements.

5. Forearm exercise. Isometric and/or rhythmic handgrip exercise can lead to minor fatigue in the forearm and soreness for a day or two. There are no long term risks associated with hand gripping exercise.

6. Cold Pressor Test. The cold pressor test may cause some discomfort, but the subjects will be informed that they can discontinue the trial in case of unusual or extreme discomfort. This discomfort lasts only as long as the hand remains immersed in the ice water.

Protection.

Each subject will give written informed consent after all questions have been answered by an investigator or study coordinator. Confidentiality of data will be assured by coding of subject identities, and that coding will be known only to the investigators. No identified individual data will be presented or published, rather group mean data will serve as the basis for comparisons. Participation in these studies is safe, with minimal short-term (days) complications and minimal anticipated long-term risks. Procedures used in this study are performed regularly in the laboratory or clinical setting by the investigators.

Inclusion of Women and Minorities.

The proposed studies will include healthy young (18-35 yr) and older (50-85 yr) men and women of all races and ethnic backgrounds recruited from the faculty, staff, and students of the University of Iowa and from persons in Iowa City, Iowa and surrounding communities. This is a therapeutic protocol. We will strive to enroll an equal number of males and females and recruit minority participants at least to the extent representative of the participation of minority populations in Iowa City and Johnson County.

Inclusion of Children.

Children between the ages 18-21 will be studied. We believe these individuals have physiologically mature blood pressure regulating systems. Additionally, children in this age group can give informed consent for invasive procedures. We will exclude children less than 18 years of age because they are unable to give consent.

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