ACUTE EFFECTS OF DIETARY NITRATE SUPPLEMENTATION IN POSTMENOPAUSAL WOMEN

A Dissertation in
Physiology
by
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ABSTRACT

Women’s arteries are exposed to adverse age- and perimenopause-associated changes in cardiovascular risk factors, leading to accelerated stiffening of central arteries, attenuated functional (exercise) vasodilation, and exaggerated blood pressure reactivity after menopause. Unfortunately, traditional pharmacological management of blood pressure has not been highly effective in older women, and there are only a few low risk, estrogen-based therapies for improving vascular health in this age group. Consequently, there is a clinical need to investigate novel interventions for vascular health in post-menopausal women, particularly dietary interventions that have the potential to augment nitric oxide bioavailability and lower their blood pressure. The studies comprising this dissertation tested the ability of nitrate-rich beetroot juice (140 ml of beetroot juice containing 0.6g of nitrate) to raise circulating concentrations of nitrite (NO\textsubscript{2}) and favorably influence central (aortic) hemodynamics and arterial stiffness at rest (study 1), ischemic forearm exercise tolerance and perceived effort (study 2), and muscle metaboreflex control of blood pressure (study 3) in normotensive, metabolically healthy post-menopausal women (57-64 yr; n = 9). A double-blind, placebo-controlled (BR\textsubscript{nitrate} vs. BR\textsubscript{placebo}; IND#119978), cross-over study design was used, with a 7-day washout period between treatments, and standardized pre-visit instructions to minimize between-visit variation in plasma [NO\textsubscript{2}]. Compared to responses observed during the BR\textsubscript{placebo} visit, BR\textsubscript{nitrate} consumption raised plasma [NO\textsubscript{2}], reduced resting systolic blood pressures (brachial and aortic), delayed the initial rise in perceived exertion during graded ischemic exercise, and attenuated the peak increase in mean arterial pressure during maximal engagement of the exercise pressor reflex (all p < 0.05). Non-significant effects were observed for resting arterial stiffness (pulse wave velocity; p = 0.33), ischemic forearm exercise time to fatigue (p = 0.13) and diastolic blood pressures during metaboreflex isolation (p = 0.18). Collectively, these results provide the first evidence that acute
dietary nitrate supplementation has beneficial cardiovascular effects in healthy older women at rest and during a condition known to promote the conversion of NO$_2^-$ to nitric oxide (ischemic forearm exercise). These studies also raise a new series of questions related to the mechanisms by which dietary nitrate may benefit cardiovascular health and exercise tolerance/blood pressure reactivity in older women, and support the pursuit of larger scale trials of dietary nitrate supplementation to determine whether these effects are in fact clinically significant and sustainable.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic Blood Pressure</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean Arterial Pressure</td>
</tr>
<tr>
<td>HR</td>
<td>Heart Rate</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke Volume</td>
</tr>
<tr>
<td>(\dot{Q})</td>
<td>Cardiac Output</td>
</tr>
<tr>
<td>TPR</td>
<td>Total Peripheral Resistance</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-product</td>
</tr>
<tr>
<td>PECA</td>
<td>Post Exercise Circulatory Arrest</td>
</tr>
<tr>
<td>MSNA</td>
<td>Muscle Sympathetic Nerve Activity</td>
</tr>
<tr>
<td>FF</td>
<td>Free Flow exercise period</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
<tr>
<td>MVC</td>
<td>Maximal Voluntary Contraction</td>
</tr>
<tr>
<td>PWA</td>
<td>Pulse Wave Analysis</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse Wave Velocity</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow Mediated Dilation</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>HFpEF</td>
<td>Heart Failure with preserved Ejection Fraction</td>
</tr>
<tr>
<td>BR\textsubscript{placebo}</td>
<td>Nitrate-depleted Beetroot Juice</td>
</tr>
<tr>
<td>BR\textsubscript{nitr}te</td>
<td>Nitrate-rich Beetroot Juice</td>
</tr>
<tr>
<td>RPE</td>
<td>Rating of Perceived Exertion</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeters</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelial-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>kg</td>
<td>Kilograms</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of mercury</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliters</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>VO$_{2\text{max}}$</td>
<td>Maximal oxygen uptake</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>$E_w$</td>
<td>Left ventricular wasted energy</td>
</tr>
<tr>
<td>AI</td>
<td>Augmentation index</td>
</tr>
</tbody>
</table>
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Chapter 1

Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality (801,000 deaths in 2013) and morbidity (85.6 million people live with ≥ 1 form of CVD) in the United States, with an estimated total annual economic burden of $316.6 billion (Writing Group et al., 2016). Aging is the major risk factor for CVD, with >90% of CVD occurring in middle-aged and older adults. Without effective interventions for CVD, the projected demographic shift (i.e., doubling of the number of older Americans by 2050) will likely lead to a tripling of medical costs in the United States by 2050 (Writing Group et al., 2016).

The prevalence of CVD is lowest in young women, but then exceeds that of men after age 55 to 60 (Writing Group et al., 2016). The accelerated rise in CVD risk with aging in women likely results, at least in part, from the menopause-associated loss of estrogen and its vasodilatory, anti-inflammatory, and anti-atherogenic effects on the vasculature (Miller et al., 2013), all of which have been linked to reductions in the production and/or bioavailability of nitric oxide (NO) (Bryan et al., 2004). Unfortunately, there are few, if any, low-risk estrogen-based therapies for improving vascular health, particularly in middle-aged women without overt CVD (De Meersman et al., 1998; Wassertheil-Smoller et al., 2000). Many post-menopausal women in fact prefer to try natural (e.g., dietary) supplements over medical therapies for cardiovascular health/blood pressure management. Thus, there is a clinical need to investigate non-pharmacological interventions for preserving vascular health in post-menopausal women, particularly interventions that have the potential to augment systemic NO bioavailability and to improve or restore vascular function.
Growing evidence suggests that dietary nitrate, obtained in the diet primarily from vegetables, may contribute to cardiovascular health through its conversion to nitrite (in the oral cavity via nitrate reductases) and ultimately to nitric oxide (Lundberg et al., 2008; Sindler et al., 2014). For example, epidemiological research has linked higher dietary nitrate/nitric oxide status with lower BP, reducing brachial BP more than 4 mmHg with 7-15 days of intervention (Ashworth et al., 2015; Golzarand et al., 2016; Sobko et al., 2010). Potential benefits beyond BP lowering per se, identified mostly from smaller intervention trials of nitrate-rich foods or supplements, include improved endothelial vasodilator function (Ferguson et al., 2013b; Heiss et al., 2012; Joris and Mensink, 2013; Rodriguez-Mateos et al., 2015; Webb et al., 2008), reduced large artery stiffness (Kim et al., 2015), protection against ischemia-reperfusion injury (Bahra et al., 2012; Duranski et al., 2005), and enhanced exercise tolerance (Aucouturier et al., 2015; Bailey et al., 2015; Bailey et al., 2009; Breese et al., 2013; Vanhatalo et al., 2011). These collective findings have led to considerable interest in the use of dietary nitrate supplementation as a natural means of restoring physiological NO signaling in states of chronic NO insufficiency such as aging (Sindler et al., 2013). This alternative pathway/system for NO generation may be particularly beneficial in populations such as post-menopausal women, who exhibit dramatic (accelerated) declines in bioavailable NO. However, most of the dietary nitrate supplementation studies conducted in older and/or clinical populations have included both sexes and have not (to the best of our knowledge) stratified their results to gain insight into the specific effects of dietary nitrate in older women (Hughes et al., 2016; Kenjale et al., 2011).

The studies comprising this dissertation tested the ability of nitrate-rich beetroot juice (140 ml of beetroot juice containing 0.6 g of nitrate) to raise circulating concentrations of nitrite (NO$_2^-$) and favorably influence central (aortic) hemodynamics and arterial stiffness at rest (study 1), ischemic forearm exercise tolerance and perceived effort (study 2), and muscle reflex control of blood pressure (study 3) in normotensive, metabolically healthy postmenopausal women.
Specific Aims and Hypotheses

Specific Aim 1

The purpose of Study #1, “Effects of acute dose of dietary nitrate supplementation on aortic blood pressure and arterial stiffness” was to determine whether an acute dose of dietary nitrate supplementation would have beneficial effects on central hemodynamics and arterial stiffness in postmenopausal women.

Hypothesis 1A: Post-menopausal women have augmented central blood pressure, augmentation index, left ventricular (LV) wasted energy vs. pre-menopausal women.

Hypothesis 1B: Acute nitrate supplementation will lower central blood pressure, augmentation index, LV wasted energy in post-menopausal women.

Hypothesis 1C: An acute dose of nitrate supplementation will lower pulse wave velocity (arterial stiffness) in post-menopausal women.

Specific Aim 2

The purpose of Study #2, “Effects of acute dose of dietary nitrate supplementation on perceived exertion and ischemic exercise tolerance” was to examine the effects of an acute nitrate supplementation on forearm exercise tolerance and endurance time during fatiguing progressive ischemic intermittent handgrip exercise in postmenopausal women.

Hypothesis 2A: An acute dose of nitrate supplementation will increase fatigue resistance (endurance time) during fatiguing intermittent handgrip exercise in postmenopausal women.

Hypothesis 2B: An acute dose of nitrate supplementation will improve perceived effort during fatiguing intermittent handgrip exercise in postmenopausal women.
Specific Aim 3

The purpose of Study #3, “Effects of acute dose of dietary nitrate supplementation on ischemic handgrip exercise induced metaboreflex” was to examine the influences of acute nitrate supplementation on metaboreflex responses during progressive ischemic intermittent handgrip exercise in postmenopausal women.

Hypothesis 3A: An acute dose of nitrate supplementation will normalize augmented blood pressure response (i.e. exercise pressor response, specifically metaboreflex) during fatiguing intermittent handgrip exercise in post-menopausal women.
Chapter 2

Review of Relevant Literature

This chapter will address the literature relevant to the topics comprising this dissertation, notably 1) the effects of cardiovascular aging in women and 2) the efficacy and potential mechanisms of dietary nitrate supplementation in modulating the effects of cardiovascular aging in women.

Cardiovascular Aging in Women

Overview

It is clear that sex differences in the process and outcomes of cardiovascular aging exist. Further, there is evidence to suggest that some of these differences may be less favorable in women. For instance, older women experience greater cardiovascular morbidity and mortality for outcomes such as stroke and heart failure (Writing Group et al., 2016) compared with younger women and age-matched men. While the precise mechanisms underlying these disparities are not fully elucidated, a growing body of literature suggests these disparities may be attributable, at least in part, to 1) accelerated arterial stiffening, 2) impaired microvascular reactivity or 3) exaggerated blood pressure responses to physical stress (e.g., muscle contraction).
Arterial Stiffening

Mechanisms for age-associated arterial stiffening

Arterial stiffness is recognized as a risk factor for cardiovascular disease (Arnett et al., 1994). With advanced in age, central arterial stiffness (i.e. aorta) significantly increases (Mitchell et al., 2004; Wojciechowska et al., 2006), especially in women (Lee and Oh, 2010). This age-associated increase in arterial stiffness can be explained, in part, by structural changes within arterial walls and functional changes of the arteries that occur with advancing age.

For structural stiffening, it is well documented that aging is associated with the formation of advanced glycation end-product (AGE) (Aronson, 2003) that result in crosslinking among collagen molecule within the vasculature, and a corresponding decrease in arterial compliance (Brownlee et al., 1988). Also, AGEs may promote an increase in collagen deposition in arterial walls by altering the balance between collagen formation and collagen degradation (Schnider and Kohn, 1981). Additionally, longstanding pulsatile stress placed on the arteries has been shown to cause fatigue and fracture of arterial elastin fibers, further reducing the overall compliance of the arterial wall (Lee and Oh, 2010). Lastly, calcium content in arterial walls increases with aging and makes the artery more rigid (Lansing et al., 1950). Functionally, declines in flow-mediated dilation (Celermajer et al., 1994; Parker et al., 2006) and responsiveness to infusions of endothelium-dependent dilators (Desouza et al., 2002a; Newcomer et al., 2004) were documented as evidence of endothelial dysfunction with aging. Those observations are mostly due to the damage in endothelial layer resulting decrease in nitric oxide (NO) bioavailability. The net effect of structural and functional stiffening of artery is an increase in the work required by the heart (O'Rourke and Hashimoto, 2007).
**Sex differences in arterial stiffening**

Taken together, aging generally results in stiffer and less compliant central arteries. This positive correlation with age and arterial stiffness has been reported in multiple studies (Mitchell et al., 2004; Wojciechowska et al., 2006). Interestingly, stiffening of large central artery with aging seems to be influenced not only by chronological aging, but also by hormonal (i.e. ovarian) aging. The Framingham study that investigated central arterial stiffness in a community setting, reported not only an aging effect, but also age-mediated sex differences in pulse wave velocity (PWV; Mitchell et al., 2004) which is a direct, noninvasive clinical measure of conduit arterial stiffness. According to the previous reports including the Framingham study, PWV increases with aging, but the age-associated increase in PWV is significantly accelerated in women following approximately the average year of menopause (Writing Group et al., 2016; Zaydun et al., 2006). The loss of estrogen is speculated to be a cause of the significantly accelerated PWV increases in the later decades of life in women, but the exact mechanisms remains unclear.

**Effects of estrogen on arterial stiffness**

Estrogen is a cardiovascular protective molecule and is known to elicit beneficial effects on the cardiovascular system in women. Demonstrated in rats, estrogen decreased stiffness of the carotid artery (Mullick et al., 2001), and prevented collagen accumulation leading to an increase in the proportion of elastic fibers in the aorta (Fischer, 1972). Moreover, Lydrup and Ferno studied whether estrogen receptor α (ER-α) correlates with collagen concentration and stiffness of human uterine arteries, and found that a higher concentration of ER-α is associated with a lower collagen concentration (Lydrup and Ferno, 2003). Therefore, it is clear that estrogen plays
a positive role in conferring arterial compliance, while the loss of the beneficial hormone results in accelerated arterial stiffening leading to unfavorable cardiovascular consequences.

**Consequences of increased arterial stiffness**

Increased arterial stiffness causes an increase in afterload, which places a greater burden on the heart, especially in older women who typically have stiffer and smaller hearts. Due to the prolonged augmented pressure that the left ventricle has to overcome, left ventricular hypertrophy can develop and eventually results in left ventricular dysfunction. In normotensive and untreated hypertensive subjects, arterial compliance was shown to be directly related to echocardiographic LV mass and inversely related to arterial elastance (Chen et al., 1998b). Even separated from augmented blood pressure, arterial stiffness itself was independently related to increase in LV mass (Chen et al., 1998b; Roman et al., 2000). It has also been speculated that ventricular stiffening occurs in response to arterial stiffening (Chen et al., 1998a; Saba et al., 1999) resulting in impaired left ventricular filling. Increased central artery stiffness also exposes small vessels in the brain and renal circulations to abnormally high pulsatile pressures, which over time can result in dysfunction in these organs. Lastly, stiffening of central arteries with aging is shown to reduce baroreceptor reflex sensitivity and impair blood pressure control at rest and during exercise, placing an individual at even greater risk for a cardiovascular event (Michas et al., 2012).
Vascular responses to exercise

Mechanisms of Exercise hyperemia

During exercise, blood flow increases to the active limb, leading to an increase in oxygen delivery in order to meet the oxygen demand of the engaged skeletal muscles. This phenomenon is termed active hyperemia and is primarily regulated at the level of the resistance vessels (microvasculature). There are two factors which determine blood flow in any vascular bed: 1) perfusion pressure, and 2) vascular tone. In order to augment blood flow, either one or both of these factors must be altered. Active hyperemia during exercise can be achieved centrally via increases in cardiac output and blood pressure, as well as peripherally via the release of local vasodilators. The responses to exercise result in either an increase in perfusion pressure and/or a decrease in vascular tone of the exercising limb, overall resulting in an increase in blood flow to skeletal muscle. Perfusion pressure can be increased by elevations in blood pressure (via the exercise pressor reflex) and reductions in venous pressure (via skeletal muscle pump). Also, due to the systemic increase in sympathetic vasoconstriction in the inactive areas such as splanchnic organs, blood can be shunted to working muscle and enhance perfusion pressure to the muscles engaged. On the other hand, vascular tone is controlled by local factors. In active muscle, local vasodilators produced from skeletal muscle, the endothelial layer, and red blood cells cause metabolic vasodilation and abolishes systemically elevated sympathetic vasoconstriction restraining blood flow in the active area. Together, the cardiovascular system manages to alter perfusion pressure and vascular tone during exercise to increase blood flow and meet the metabolic demand of the working muscles.
Aging and Skeletal muscle blood flow during exercise

Age-associated reductions in skeletal muscle hyperemia during exercise have been reported in numerous studies (Kirby et al., 2009; Lawrenson et al., 2003; Poole et al., 2003; Proctor et al., 2003a; Proctor et al., 2004; Proctor et al., 2003b; Schrage et al., 2007; Schrage et al., 2004; Wahren et al., 1974). According to literature, aging appears to shift the balance between vasoconstrictor and vasodilator responses towards a greater vasoconstriction resulting in overall increases in vascular tone and restrained blood flow to the active limb. Interestingly, previous work from our lab and several others have shown blunted blood flow increases in postmenopausal women’s exercising limb compared to older men (Parker et al., 2006; Proctor et al., 2003a; Proctor et al., 2003b) and to younger women (Parker et al., 2008b; Proctor and Parker, 2006). Our lab reported that older women exhibit attenuated increases to leg blood flow and vascular conductance responses during cycling exercise (Proctor et al., 2003a) and single-knee extension at the same work rate and duration (Parker et al., 2008a; Parker et al., 2008b) compared to young women. In addition, Fadel et al. reported impairments in ‘lysing’ of sympathetic vasoconstriction, or functional sympatholysis, during intermittent handgrip exercise that was restored after 1 month of estrogen replacement therapy in postmenopausal women (Fadel et al., 2004).

This blunted hyperemic response during exercise in older women can be explained by multiple mechanisms. First, older women could have a blunted increase in cardiac output. More specifically, post-menopausal women tend to have smaller and stiffer hearts (Burkauskiene et al., 2006; Mukherjee and Sen, 1990; Song et al., 1999; Xu et al., 2003) that has impaired cardiac filling function that can lead to reduced cardiac output during exercise. Secondly, they may have increased sympathetic vasoconstrictor tone. It has been demonstrated that postmenopausal women have a greater muscle sympathetic nerve activity during static exercise when it is compared to age-matched men and younger women (Matsukawa et al., 1998; Ng et al., 1993),

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appointing to greater vasoconstrictor tone that could result in a blunted increase in blood perfusion in exercising muscle. Similarly, they may have increased release of vasoconstrictor substances such as endothelin-1. Miyauchi et al. reported increased circulating endothelin-1 in middle-age women compared to younger women (Miyauchi et al., 1992). This could, again, increase the vasoconstrictor response and cause a blunted increase in blood flow to the exercising limbs. Increases in the metabolic stimulus resulting in an increase in the metaboreflex signal may be a potential mechanism as well. Lastly, an attenuated release of endothelial-derived vasodilator substances is important to consider. With aging and especially in postmenopausal women who experience chronic estrogen deficiency, endothelial layers become damaged and as a result, NO bioavailability declines. This can cause an imbalance in sympathetic vasoconstriction and metabolic vasodilation in active muscle, and result in an override of sympathetic vasoconstriction in the active muscle compared to subjects with better endothelial function, like younger women.

**Augmented sympathetic vasoconstriction in older women**

As discussed above, it has been suggested that postmenopausal women may have an augmented sympathetic vasoconstrictor response during exercise that result in a blunted exercise hyperemic response. As evidence for this argument, studies have shown a greater muscle sympathetic nerve activity (MSNA) in postmenopausal women compared to pre-menopausal women and age-matched men during static forearm exercise (Matsukawa et al., 1998; Ng et al., 1993; Vianna et al., 2012). This suggests that the $\alpha$-adrenergic receptor sensitivity to neurotransmitters from the motor nerve ending may be enhanced in postmenopausal women resulting in greater vasoconstriction in conjunction with a greater stimulus (i.e. MSNA) signal. However, whether the augmented sympathetic vasoconstriction response is from the increase in MSNA or changes in vascular sensitivity, or relative contributions of the two has not been fully
elucidated. Another possible place for the alteration is the production, degradation, re-uptake, and release of the sympathetic neurotransmitter, norepinephrine and epinephrine. The balance between production, release and degradation, re-uptake of the neurotransmitter, norepinephrine and epinephrine could have been shifted which can cause either a greater release or slower removal of the neurotransmitter. The age-related increase in plasma norepinephrine concentration has been shown in numerous papers (Davidson et al., 1984; Goldstein et al., 1983; Jones et al., 1978; Ziegler et al., 1976), but sex differences in the age-associated alteration in plasma catecholamine metabolism during exercise has not been fully elucidated, either.

**Attenuated metabolic vasodilation in older women**

The shift in balance between sympathetic vasoconstriction and local metabolic vasodilation can alter exercise hyperemia. As mentioned above, if sympathetic vasoconstriction is augmented and increased, it will override the local metabolic vasodilation and result in attenuated exercise hyperemia. On the other hand, if the local metabolic vasodilation is attenuated, sympathetic vasoconstriction will override and also result in an attenuated exercise hyperemic response.

Local metabolic vasodilation involves numerous vasoactive substances such as blood $PO_2$ and $PCO_2$, hydrogen ions, osmolarity, adenosine and adenine nucleotides, potassium, lactate, histamine, kinins, phosphates, prostaglandins, muscle-derived nitric oxide and ATP released from red blood cells (Clifford and Hellsten, 2004; Gonzalez-Alonso et al., 2006; Hellsten et al., 1998; Juel et al., 2000; Stamler et al., 1997; Vanhoutte and Mombouli, 1996) as well as nitric oxide released from arterial endothelium (Endo et al., 1994; Gilligan et al., 1994; Green et al., 2005; Radegran and Saltin, 1999; Schrage et al., 2004; Shoemaker et al., 1997). All of these act as vasodilators during exercise causing exercise hyperemia and increased oxygen delivery to meet
the oxygen demand in the exercising limb. Importantly, the endothelial layer of the artery is required for the production of certain vasoactive substances (i.e. NO). Thus, aging, which results in a damaged endothelial layer, will reduce bioavailability of endothelial mediated vasodilators such as NO. NO can be generated when vasoactive substances such as bradykinin, acetylcholine, and ATP bind to receptors (Furchgott and Martin, 1985) on the endothelium, where endothelial nitric oxide synthase (eNOS) is located. The known mechanism for the NO mediated vasodilation is via activation of guanylate cyclase in the smooth muscle cells (Murad, 1986). NO can also participate in indirect vasodilation by stimulating the release of prostaglandins from endothelial cells (Salvemini et al., 1993; Vassalle et al., 2003). With aging, however, the endothelial layer is damaged from the longstanding pressure it has experienced chronically, and loses its function to convert L-arginine to NO, a potent vasodilator. Therefore, the damaged endothelial layer will result in decreased NO bioavailability in the system resulting in reduced vasodilation, particularly at sites where the metabolic demand is increased such as in situation of exercise.

**Augmented Blood Pressure Responses to Exercise**

Blood pressure responses during exercise are determined by three different neural controlling mechanisms and those include: 1) central command, 2) arterial baroreflex resetting, and 3) the exercise pressor response (Alam and Smirk, 1937; McCloskey and Mitchell, 1972; Murphy et al., 2011). These three mechanisms are activated at the onset of exercise and work together to maintain appropriate range of blood pressure for adequate perfusion of essential tissues such as brain, heart, and skeletal muscle during exercise. If any one or more of the three mechanisms is compromised, blood pressure response to exercise can be augmented more than physiologically necessity. This augmented blood pressure response to exercise can exert deleterious consequences as it increases stress imposed on the vasculature and also increases the
mechanical work of the heart, resulting in a greater likelihood of cardiovascular events. Therefore, it is critical to tightly control and regulate the neural mechanisms which govern the blood pressure response during exercise and maintain homeostatic levels of blood pressure.

The first mechanism, central command, is a feedforward neural control mechanism that functions at the onset of exercise. It causes a rapid increase in sympathetic activity and establishes an intensity dependent basal cardiovascular apparatus by increases in heart rate and blood pressure (McCloskey and Mitchell, 1972; Rowell and O'Leary, 1990; Strange et al., 1993; Thornton et al., 2002). Another determinant for blood pressure responses during exercise is by resetting of the arterial baroreflex (Murphy et al., 2011; Raven et al., 2006). Previous work in both animal and human studies have shown workload dependent resetting of baroreflex to a higher arterial pressure during exercise (Murphy et al., 2011; Raven et al., 2006). This resetting allows blood pressure to increase more than the regular baroreflex set point for blood pressure and allows for increases to perfusion pressure to contracting skeletal muscle. Also, due to this resetting, appropriate systemic blood pressure can be maintained even with the massive metabolic vasodilation occurring at contracting skeletal muscle. Lastly, the exercise pressor response originating from the active exercising limb can also determine the overall blood pressure response during exercise. The exercise pressor response consists of two reflexes termed the “mechanoreflex” and “metaboreflex.” The mechanoreflex is activated by mechanical changes in the muscle and tendon while the metaboreflex is activated by metabolic stimuli in the contracting muscle. Both reflexes cause a dramatic rise in blood pressure when activated, and enhance hyperemia in exercising muscle to match the increased metabolic demand.
Metaboreflex control of blood pressure during exercise

To expand on the metaboreflex, this response originates from the stimulation of thin afferent group III and IV nerve fiber in the working muscle (Fadel et al., 2004; Kaufman et al., 1983; Kaufman and Rybicki, 1987; McCloskey and Mitchell, 1972). These nerve fibers can be stimulated by metabolites such as bradykinin (Stebbins and Longhurst, 1985), ATP (Li et al., 2008), diprotonated phosphate (Sinoway et al., 1994), arachidonic acid byproducts (Rotto et al., 1990), lactic acid (Kaufman et al., 1988), potassium (Kaufman and Rybicki, 1987), and hydrogen ions (Sinoway et al., 1989) and are produced when metabolic activity of the muscle increases resulting in an oxygen demand and supply mismatch and a greater reliance on anaerobic pathways (Sheriff et al., 1990; Sheriff et al., 1987; Wyss et al., 1983). These metabolites bind to appropriate afferent receptors such as acid sensing ion channels (Hayes et al., 2008), transient receptor potential vanilloid 1 (Kaufman et al., 1982), and purinergic ligand-gated ion channels (Hanna and Kaufman, 2003) and stimulates group III and IV fibers. Stimulation of the group III and IV nerve fiber by these metabolites will send signals to the cardiovascular center in the brain and exert increases in sympathetic activity. Additionally, there is evidence suggesting that metabolites produced during exercise can sensitize mechanoreceptors (Adreani et al., 1997). Consequently, systemic sympathetic vasoconstriction takes place and cardiac output (Q) increases via β-adrenergic receptor activation on the heart. According to Darcey’s law, increase in Q and total peripheral resistance (TPR) results in increase in systemic blood pressure.

Aging and Blood Pressure Regulation during Exercise in Older Women

Along with the previously mentioned cardiovascular impairments, which include arterial and myocardial stiffening as well as a blunted exercise hyperemic response, postmenopausal
women exhibit an augmented exercise blood pressure response during exercise, in comparison to premenopausal women and age-matched men (Choi et al., 2012; Fleg et al., 1995; Green et al., 2002; Ogawa et al., 1992; Proctor et al., 2003a; Proctor et al., 2003b). Although the single mechanism leading to the greater increase in blood pressure during exercise has not been identified, the augmented metaboreflex-mediated blood pressure responses during exercise has been speculated as the main factor for augmenting blood pressure responses to exercise in postmenopausal women. Unpublished data from our lab, as well as other published reports, have shown that postmenopausal women have augmented blood pressure response to metaboreflex activation during small muscle exercise compared to premenopausal women (Choi et al., 2012; Sidhu et al., 2015), and the significantly different response in postmenopausal women is driven mostly by the augmented increase in total peripheral resistance measured by the model flow technique (unpublished data).

Effects of dietary nitrate supplementation on cardiovascular health and exercise physiology

Overview

Recently, dietary nitrate supplementation has been recognized by scientists and health care professionals for its cardiovascular benefits. Consumption of dietary nitrate supplementation showed increase in plasma nitrate and nitrite inducing nitrate-nitrite-nitric oxide pathway to increase nitric oxide (NO) bioavailability. Cardiovascular benefits of dietary nitrate supplementation are well documented during the last decade. It is reported that dietary nitrate supplementation lowers resting blood pressures in healthy and hypertensive adults, and also enhances exercise performance and exhibits ergogenic effects. This section will review literature
that reports 1) mechanisms, and 2) cardiovascular beneficial effects on blood pressure and exercise tolerance of dietary nitrate, in form of nitrate-rich beetroot juice.

**Mechanisms and pathways influencing the production of nitric oxide**

The standard NO production pathway utilizes L-arginine and involves NO synthase (NOS). This conventional NO production pathway is NOS-dependent, and is also oxygen dependent since NOS requires oxygen for its action. On the other hand, the alternative NO production pathway, nitrate-nitrite-nitric oxide pathway, that is induced by dietary nitrate doesn’t involve oxygen. In fact, this pathway is sensitive to oxygen tension and gradually activated as oxygen tension falls. Thus, nitrate-nitrite-nitric oxide pathway is most active during hypoxia and acidosis while oxygen-dependent NOS enzyme activities are compromised (Giraldez et al., 1997; Oestergaard L et al., 2007). Due to the activation of complement production pathway, the nitrate-nitrite-nitric oxide pathway, sufficient amount of NO can be produced even at a low level of oxygen tension. This produced NO is rather dependent of nitrite than oxygen-dependent NOS activity (Bryan et al., 2005; Duranski et al., 2005; Zweier et al., 1995a).

When dietary nitrate supplementation (nitrate-rich beetroot juice in following studies) is ingested, consumed dietary nitrate, first, enters to the systemic circulation from the gastrointestinal tract. About 75% of the absorbed nitrate is excreted, and 25% is reabsorbed via the salivary glands and released to oral cavity in saliva (Lundberg and Govoni, 2004; Spiegelhalder et al., 1976). Then, the released nitrate is reduced to nitrite by facultative anaerobic bacteria located on the back of the tongue. Facultative anaerobic bacteria, bacteria that can utilize both aerobic and anaerobic respiration depending on oxygen availability, uses salivary nitrate as an electron acceptor in respiration and reduces nitrate to nitrite in the oral cavity (Duncan et al.,
Nitrite reduced from the reductase activity of facultative anaerobic bacteria, is reduced one more time and becomes NO by a different reductase activity.

Reduced nitrite can be converted to NO by nitrite reductase activity of deoxyhemoglobin. This reaction has been described in number of classic papers (Brooks, 1937; Doyle et al., 1981; Ibsen et al., 2013). Nitrite, ferrous deoxyhemoglobin, and a proton will react together to reduce nitrite to NO (Brooks, 1937; Doyle et al., 1981; Ibsen et al., 2013). As Lundberg and his colleague mentioned in his review, it should be noted that this reaction is not only sensitive to hypoxic condition, but also acidic condition due to the need of a proton (Lundberg et al., 2008).

This finding is also followed by a recent study investigating mechanisms and conversion of dietary nitrate supplementation. According to Cosby and colleagues, conversion rate of NO from nitrite has reciprocal relation with hemoglobin oxygen saturation, and the conversion is hypoxia-regulated mechanism (Cosby et al., 2003).

In addition to deoxyhemoglobin, deoxymyoglobin and other enzymes such as xanthine oxidoreductase can reduce nitrite to NO. Deoxymyoglobin uses same reaction as deoxyhemoglobin to reduce nitrite to NO. Deoxyhemoglobin has a 30 times faster rate than hemoglobin, so in exercising skeletal muscle and subendocardium of the heart, it can rapidly convert nitrite to NO (Lundberg et al., 2008). Xanthine oxidoreductase also converts nitrite to NO at low oxygen tensions and pH values (Godber et al., 2000b; Li et al., 2001; Millar et al., 1998; Zhang et al., 1997).

To sum up, through the nitrate-nitrite-nitric oxide pathway, consumed dietary nitrate (NO$_3^-$) is reduced to nitrite (NO$_2^-$), and then to nitric oxide (NO), a potent vasodilator and cardio-protective molecule. This series of reductions of dietary nitrate to nitric oxide increases NO bioavailability and is greatly enhanced in hypoxic and acidic conditions. Moreover, since the classic NO production pathway (i.e. L-arginine pathway) is NOS dependent, dietary nitrate
supplementation that triggers the nitrate-nitrite-nitric oxide can be particularly helpful for populations with endothelial dysfunction, such as older adults.

**Blood pressure lowering effects of dietary nitrate supplementation at rest and during exercise**

Numerous publications reported cardiovascular benefits of dietary nitrate supplementation in form of nitrate-rich beetroot juice. Intriguingly, most propounding benefits of beetroot juice is the blood pressure lowering effect. It is well documented that acute and chronic dose of dietary nitrate supplementation in form of a nitrate-rich beetroot juice lowers resting blood pressure (Bahra et al., 2012; Bailey et al., 2009; Hobbs et al., 2012; Kapil et al., 2010a; Kelly et al., 2013; Lansley et al., 2011a; Lansley et al., 2011b; Vanhatalo et al., 2011; Webb et al., 2008; Wylie et al., 2013) and 24-hour ambulatory blood pressure (Webb et al., 2008) in healthy adults. Similarly, nitrate-rich beetroot juice lowered resting blood pressures in healthy older adults (Hughes et al., 2016), and even patient populations such as hypertensives (Kapil et al., 2015), peripheral arterial diseases (PAD; (Kenjale et al., 2011), and heart failure with preserved ejection fraction patients (HFpEF; (Eggebeen et al., 2016).

Furthermore, nitrate-rich beetroot juice lowers not only peripheral blood pressure as mentioned, but also central blood pressure in healthy young and older adults (Hughes et al., 2016). Central blood pressure or aortic blood pressure along with augmentation index is known to be a better predictor of major cardiovascular events (Huang et al., 2011; Pini et al., 2008; Roman and Devereux, 2008) and left ventricular hypertrophy (Roman et al., 2010) than conventional brachial blood pressure. Hughes et al. reported that the acute dose of beetroot juice lowered aortic blood pressure without observing any effect on aortic wave reflection characteristics (Hughes et al., 2016). They claime(Lee et al., 2015)d that the lack of differences is mostly due to the acute dose
of the beetroot juice that is too low of a dose to cause any structural change to alter the vascular structure. This produces an evidence that the beetroot juice can be used as a potential therapeutic supplement.

The blood pressure lowering effects of nitrate-rich beetroot juice have been studied during exercise, mostly large muscle dynamic exercise, as well. In healthy young rat model, short term beetroot juice supplementation lowered blood pressure response to treadmill exercise when they are compared to a control group (Ferguson et al., 2013b). In addition, Lee et al. studied the effects of chronic beetroot juice supplementation on BP, TPR, and RPP (rate pressure product) responses to submaximal cycling exercise, and found that 15 days of beetroot juice supplementation lowers blood pressure response to cycling exercise suggesting beetroot juice can enhance O₂ delivery and reduce work of the heart, such that exercise can be performed at a given workload for a longer period of time before the onset of fatigue (Lee et al., 2015).

**Effects on Exercise hyperemia and performance**

Nitric oxide (NO) plays a key contributing role in the modulation of blood vessel tone at rest and under conditions of increased metabolic demand, such as exercise (Heinonen et al., 2011; Joyner and Casey, 2009; Joyner and Tschakovsky, 2003). As a result, there has been a growing interest in the use of dietary interventions that increase bioavailable NO as both cardiovascular health-promoting (Kapil et al., 2010b; Lundberg et al., 2011; Machha and Schechter, 2011) and ergogenic aids (Bailey et al., 2009; Bescos et al., 2012; Jones et al., 2011b; Lansley et al., 2011b; Vanhatalo et al., 2011). In particular, dietary supplementation with inorganic salts or high-nitrate containing foods (e.g., beetroot juice) has recently grown in popularity for its potential exercise performance-enhancing effects. Many of the studies performed in humans have largely focused on the influence of dietary nitrate on exercising muscle metabolism, such as a putative ability to
reduce the oxygen cost of muscle contraction (Bailey et al., 2010; Bailey et al., 2009; Bentley et al., 2014; Bescos et al., 2012; Jones et al., 2011a; Jones et al., 2011b; Larsen et al., 2011).

However, evidence from a rodent model suggests that dietary nitrate supplementation may also improve the perfusion of active skeletal muscles during exercise (Ferguson et al., 2013a). Ferguson et al have recently demonstrated that consumption of nitrate-rich beetroot juice augments hindlimb skeletal muscle blood flow and vascular conductance during treadmill running in rats (Ferguson et al., 2013b). In humans, two studies have investigated the effects of intra-arterially infused doses of sodium nitrite on forearm exercise hyperemia, but these studies involve supra-physiological doses of nitrite (Johnsson, 1967; Maher et al., 2008). Therefore, it is unclear whether increasing plasma nitrite within the physiological range by dietary nitrate supplementation can impact vascular function during exercise.

With regard to the exercise performance, numerous papers have reported exercise performance enhancing effects of beetroot juice. It has been shown in many studies that the Beetroot juice intervention improves exercise time to exhaustion and time-trial performance (Bailey et al., 2009; Cermak et al., 2012; Porcelli et al., 2016). Also, short term beetroot juice intervention attenuated muscular fatigue during exercise in healthy young adults (Hoon et al., 2015) and improved running performance independent of aerobic fitness in healthy young men (Shannon et al., 2016). However, some showed no effects of beetroot juice on exercise performance (Kelly et al., 2014; Muggeridge et al., 2013). In addition, it has been documented that exercise performance enhancing effect of beetroot juice is less apparent in well-trained subjects even with very high dose (~19.5mmol) (Hoon et al., 2013; Peacock et al., 2012; Porcelli et al., 2016; Wilkerson et al., 2012).
Summary

This review of the relevant literature covering effects of cardiovascular aging in women and the efficacy and potential mechanisms of dietary nitrate supplementation in modulating the effects of cardiovascular aging in women reveals the following: 1) three characteristics of cardiovascular aging in women includes central arterial stiffening, attenuated blood flow response during exercise, and augmented blood pressure response during exercise, and 2) dietary nitrate supplementation increases NO bioavailability via activation of nitrate-nitrite-nitric oxide pathway and lower resting blood pressure and attenuate blood pressure response to exercise as well as its ergogenic effects. However, the potential beneficial effects of dietary nitrate supplementation in modulating the impairments of cardiovascular aging in women has not been studied.
Chapter 3

Effects of acute dietary nitrate supplementation on aortic pressures, stiffness, and wave reflections in post-menopausal women

Introduction

Central aortic blood pressure is a stronger predictor of cardiovascular events than brachial blood pressure (Jankowski et al., 2008; Pini et al., 2008; Roman and Devereux, 2008; Safar et al., 2002). and it increases with advancing age leading to unfavorable alterations in ventricular-vascular coupling (McEniery et al., 2005). Heightened resting aortic blood pressures and the progressive stiffening of large arteries (e.g. aorta) in aging population (Ben-Shlomo et al., 2014; Mitchell et al., 2004; Wojciechowska et al., 2006) particularly in women (Lee and Oh, 2010), appear to be important underlying mediators of increased cardiovascular disease risk. Aging also alters indices of aortic wave reflection which in turn increases left ventricular wasted energy (Casey et al., 2008) and reduces efficiency of heart. For this reason, many researchers have been investigating various interventions to improve vascular function and hemodynamics in aging population targeting increasing bioavailability of nitric oxide, a potent vasodilator, that is reduced with advancing age.

However, conventional pharmacological interventions to increase NO bioavailability are very costly (e.g. BH₄) and target traditional NO production pathway (i.e. L-arginine pathway) which requires many co-factors that can be deficient in the diet (e.g. BH₄). Therefore, necessity of developing cost-effective non-pharmacological intervention that can improve vascular function and hemodynamics in aging population escalated.
Dietary nitrate supplementation is a natural means of increasing bodily stores of the vasoprotective molecule, nitric oxide. Miller et al. reported cardiovascular benefits of dietary nitrate supplementation in form of nitrate-rich beetroot juice which raises plasma nitrate and nitrite in a greater extent than adding nitrate-rich food to the diet in older adults (Miller et al., 2012). When beetroot juice is ingested, through the nitrate-nitrite-nitric oxide pathway, nitrate (NO$_3^-$) is reduced to nitrite (NO$_2^-$), and then to nitric oxide (NO), a potent vasodilator and cardio-protective molecule (Lundberg et al., 2008; Sindler et al., 2014). This conversion of nitrate to nitric oxide increases NO bioavailability. Because the classic NO production pathway (i.e. L-arginine pathway) is NOS dependent, dietary nitrate supplementation that triggers the nitrate-nitrite-nitric oxide pathway to produce NO can be particularly beneficial for a population with endothelial dysfunction, such as older adults especially postmenopausal women who exhibit profound declines in bioavailable NO.

Despite the potential benefits of this dietary intervention for older population, the effects of dietary nitrate supplementation on central hemodynamics in older women are not well understood. A recent study by Hughes et al in a mixed sample (9 men, 3 women) of older adults reported an acute effect of nitrate rich beetroot juice on peripheral and aortic BP, but did not include a true (nitrate depleted) placebo and did not measure PWV (Hughes et al., 2016). Thus the influence of dietary nitrate supplementation, per se, on aortic BP and central hemodynamics in older women remains unknown.

Thus, this study was conducted to 1) examine aging effects on central blood pressures, augmentation index, and cardiac wasted energy in women, 2) test the ability of nitrate-rich beetroot juice to reduce peripheral and central blood pressure, augmentation index, and left ventricular wasted energy, and 3) test the ability of nitrate-rich beetroot juice to reduce arterial stiffness in postmenopausal women. We hypothesized that 1) postmenopausal women would have augmented central blood pressures, augmentation index, LV wasted energy compared to pre-
menopausal women, 2) acute nitrate supplementation will lower central blood pressure, augmentation index, LV wasted energy in post-menopausal women, and 3) acute nitrate supplementation will lower pulse wave velocity (arterial stiffness) in post-menopausal women.

**Methods**

Ten healthy premenopausal women (age, 22 ± 0.79 years; between 20 ~ 30) and 9 healthy postmenopausal women (age, 61 ± 0.72 years; between 57 - 64 years) were recruited from the local university community. Interested volunteers provided written informed consent prior to enrollment. On the same day, medical screening was performed to determine eligibility. Participants were selected if they were low- to moderately active (i.e., <3 days/week of exercise), free of overt chronic disease as assessed by clinician reviewed medical history questionnaire and venous blood chemistry (hematologic, liver, and kidney function), and met the following criteria: (i) resting blood pressure between 110/60 and 140/90 mmHg, (ii) body mass index between 18.5 and 35 kg/m², (iii) fasting plasma glucose < 100 mg/dL or HbA1c < 6.0%, (iv) fasting plasma low-density lipoprotein < 130 and/or high-density lipoprotein > 40 mg/dL, (v) non-smoker, (vi) not taking any cardiovascular medications, and (vii) had not donated blood or blood products in the past 3 months. Characteristics of participants who completed this study are shown in Table 1. All procedures were approved by the Office of Research Protections at Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki.
Study design

Upon arrival for the experimental visits, a venous blood sample was collected for baseline plasma nitrate and nitrite concentrations. Then, peripheral blood pressure (HEM-705CP, Omron), radial pulse wave analysis, and carotid-to-femoral PWV (SphygmoCor CvM, AtCor Medical) were measured after 10 minutes of quiet supine resting. For the dietary nitrate supplement intervention, subjects consumed a nitrate-rich beetroot juice (BR$_{\text{nitrato}}$; 140 mL Beet-It Organic, James White Juice Company) and nitrate-depleted beetroot juice (BR$_{\text{placebo}}$; 140 mL nitrate-depleted Beet-It Organic, James White Juice Company) on separate visits. The order of intervention was blinded for both subjects and the researcher. Approximately 90 minutes following the juice consumption, a second venous blood sample was taken to determine the post-consumption change in plasma nitrate and nitrite. Then, peripheral blood pressure, radial pulse wave, and carotid-to-femoral PWV were measured again after 10 minutes of quiet supine resting (Figure 3-1).

Pulse wave analysis and pulse wave velocity

Aortic blood pressure and carotid-to-femoral PWV (central PWV) was determined by a tonometry based vascular profiling device (SphygmoCor CvM, AtCor Medical). Briefly, aortic pressure (AP) and augmentation index (AI) were derived from radial pulse wave using the transfer function while left ventricle wasted energy is being calculated using equation $E_w$ (sec*dyne*cm$^2$) = AP (mmHg) * reflected wave duration (msec)* $\pi/2$ multiplied by the conversion factor 1.33 (Nichols, 2005). Carotid-to-femoral PWV was calculated by dividing the calculated distance between the carotid and femoral arteries (determined from the distance between carotid artery to sternal notch and sternal notch to femoral artery) by the pulse transit
time between the 2 sites. Reported PWV values are either the mean of the two similar consecutive measurements or the median of the three consecutive measurements, and the test-retest reproducibility within day was checked with operation index (all visits p > 0.05).

**Plasma [nitrate/nitrite] analysis**

Venous blood samples were drawn into heparin tubes (4mL lithium heparin tubes, BD Vacutainer, Franklin Lakes, N.J., USA) and immediately centrifuged at 3200 r/m (1590 g) and 4°C for 10 min. Plasma was then extracted and stored in -80°C freezer for later analysis of nitrate and nitrite concentration. The ENO-20 analyzer (EICOM, San Diego, Calif., USA) with a sensitivity of 0.1 pmol for nitrate and nitrite was used to measure nitrate and nitrite concentration in the plasma samples. Briefly, plasma was mixed with an equal volume of 100% methanol and centrifuged at 13,000 r/m (12,000 g) for 10 minutes. Samples were then loaded into a 96-well plate. Nitrate and nitrite were then separated via column chromatography and individually reacted with a Griess reagent, synthesizing a diazo compound. The absorbance of this red diazo compound was then read at a wavelength of 540 nm using a visible light detector.

**Statistical analysis**

A student t-test determined differences between pre- and post-menopausal women groups. To assess differences between BR\textsubscript{placebo} and BR\textsubscript{nitrate}, two way repeated measure analysis of variance (ANOVA) was used. Pairwise comparisons followed by paired t-test if group x time interaction was observed. For the comparisons of delta values (changes from pre- to post-juice consumption) for testing effects of the dietary nitrate supplementation, a paired t-test analysis was used. Also, *Cohen’s d* was calculated (mean 1- mean 2/ combined standard deviation) on the
primary outcome to determine effect size. All statistical analyses were performed using SPSS version 21 (IBM, Chicago, IL). Statistical significance was set at p < 0.05. All data are expressed as the mean ± standard error.

Results

Participant characteristics

Participants characteristics are reported in Table 3-1. A student t-test revealed significant differences in age and BMI between pre- and post-menopausal women (p < 0.05). No significant differences were observed in height and weight between pre- and post-menopausal women (p>0.05).

Plasma nitrate and nitrite concentration

Plasma nitrate and nitrite concentrations before beetroot juice consumption were not different between visits (nitrate, p = 0.38; nitrite, p = 0.55). Plasma nitrate and nitrite concentration following 90 minutes of BRplacebo consumption were not raised from baseline (nitrate, p = 0.34; nitrite, p = 0.5). However, plasma nitrate and nitrite concentrations were significantly elevated 90 minutes following consumption of BRnitrate (nitrate 13 folds, nitrite 4 folds; both p < 0.01). Plasma nitrate and nitrite concentrations at the end of the experiment were still significantly elevated from the baseline (nitrate 10 folds, nitrite 4 fold; both p < 0.01; Figure 3-2).
**Age group comparison**

Group data for brachial and aortic blood pressures and central hemodynamics in pre- and postmenopausal women are presented in Table 2. Resting supine brachial SBP, MAP, and central SBP, DBP, and MAP were significantly higher in postmenopausal women compared to pre-menopausal women (Table 2; all p ≤ 0.01). In addition to blood pressures, resting supine augmentation index, and calculated left ventricle wasted energy were significantly elevated in postmenopausal women compared to pre-menopausal women (Table 3-2; all p < 0.01).

**Acute dietary nitrate supplementation effects on resting aortic blood pressures and hemodynamics**

Two-way ANOVA revealed time x intervention interaction for resting supine brachial and aortic SBP and MAP (all p < 0.05) and followed pairwise comparison confirmed that acute dose of BR_nitrate lowered brachial SBP and MAP (Figure 3-3; both p < 0.05) and aortic SBP and MAP (Figure 3-4; both p < 0.05) compared to BR_placebo. Moreover, changes from baseline (pre-juice consumption) and 90 minutes following juice consumption for brachial SBP and MAP (Figure 3-3; both p < 0.01) and aortic SBP and MAP (Figure 3-4; both p < 0.01) were significantly lower with BR_nitrate compared to BR_placebo. However, no beneficial effect of BR_nitrate was observed in brachial and aortic DBP, augmentation index (AI), and cardiac wasted energy (E_w) when the data are analyzed with two-way ANOVA or paired t-test for absolute changes from pre- to post-juice consumption (Figure 3-5; all p > 0.05).
Acute dietary nitrate supplementation effects on pulse wave velocity (PWV)

Data presented in Figure 3-6 are the group data for aortic PWV with BR
placebo and BR
nitrate. No time x treatment interaction was detected by Two-way ANOVA for aortic PWV (Figure 3-6, panel A; p > 0.05). Likewise, a change from pre- to post-juice consumption (ΔPWV) for BR
nitrate was not significantly different from that of BR
placebo in postmenopausal women (Figure 3-6, Panel B; p > 0.05). In addition, PWV and ΔPWV normalized to MAP were not affected by acute dose of nitrate-rich beetroot juice compared to nitrate-depleted beetroot juice, either (data not shown; p > 0.05).

Discussion

The primary purpose of this study was to examine the ability of nitrate-rich beetroot juice to reduce central aortic blood pressures, augmentation index, and left ventricular wasted energy in postmenopausal women. The novel finding for this study is that acute dose of dietary nitrate supplementation, in form of nitrate-rich beetroot juice, lowers both central systolic and mean arterial blood pressures in postmenopausal women. A single dose of nitrate-rich beetroot juice raised plasma nitrate (13 fold) and nitrite (4 fold) concentration which indicates the absorption and bioconversion of consumed nitrate in healthy postmenopausal women. Despite these differences in plasma nitrate and nitrite concentration, a single dose of nitrate-rich beetroot juice had no obvious effects on augmentation index, left ventricular wasted energy, and carotid-to-femoral pulse wave velocity when compared to nitrate-depleted beetroot juice in postmenopausal women.

In present study, an acute dose of dietary nitrate supplementation lowered elevated aortic systolic and mean arterial pressures in healthy postmenopausal women. Although Hughes et al
reported effects of beetroot juice on central blood pressures and hemodynamics in older adults, this study is the first study to investigate effects of beetroot juice on aortic blood pressures and indices of aortic reflected wave in healthy postmenopausal women by themselves. Moreover, unlike the Hughes et al. study, we tested effects of dietary nitrate supplementation, per se, by comparing to that of nitrate-depleted beetroot juice, the true placebo (Hughes et al., 2016). A single dose of nitrate-rich beetroot juice lowered not only brachial blood pressures, but also aortic blood pressures in healthy postmenopausal women.

Interestingly, the directional changes of blood pressures from prior to post juice consumption for BR$_{\text{placebo}}$ and BR$_{\text{nitrate}}$ were opposite (Figure 3-3, 3-4). It has been documented that fluid ingestion results in acute increase in blood pressure in older adults (Jordan et al., 2000) which may be due to impaired baroreflex function with aging as they suggested in the paper. This observation indicates that the blood pressure lowering effect of nitrate-rich beetroot juice is powerful enough to override the small rise in blood pressure that might have occurred due to fluid ingestion in older adults as observed in other studies (Jordan et al., 2000).

Central aortic blood pressure predicts cardiovascular events better than brachial blood pressure (Roman and Devereux, 2008). The Anglo-Cardiff Collaborative Trial reports that aortic blood pressure increases with advancing age leading to unfavorable alterations in functions and structures of both vessels and heart (McEniery et al., 2005). Hughes et al. also published data showing increase in aortic blood pressures with aging, and affirmative effects of beetroot juice on augmented aortic blood pressure in older adults (Hughes et al., 2016). However, it is difficult to conclude that the aortic blood pressure lowering effects observed from their study are solely due to nitrate because of the lack of true placebo. Rather, it is more of an effect of beetroot juice which is also high in anti-oxidants and polyphenols. In comparison, this study investigated effects of dietary nitrate supplementation and found its blood pressure lowering effects not only in brachial but also in aortic blood pressures.
We also sought to confirm reported aging effects on aortic blood pressures and central hemodynamics, and observed higher aortic SBP and MAP were observed in postmenopausal women compared to premenopausal women as reported by other research group (Herbert et al., 2014). Moreover, indices of aortic wave reflection such as augmentation index and cardiac wasted energy were higher in postmenopausal women when compared to premenopausal women. These phenomena can be explained, at least in part, by stiffening of central arteries with advancing age. It is well documented that aging causes structural and function changes in arterial tree, especially in central conduit artery (i.e. aorta). Functionally, endothelial dysfunction becomes more apparent in older adults reducing nitric oxide bioavailability (Celermajer et al., 1994; DeSouza et al., 2002b; Newcomer et al., 2004; Parker et al., 2006). In addition, structural changes in arteries create stiffer artery that causes increase in central arterial stiffness and pulse wave velocity (Lee and Oh, 2010). As a result, reflected wave occurs earlier during systole leading to increase in augmentation index and increase in left ventricular wasted energy.

Furthermore, increased aortic arterial stiffness with aging can influence baroreceptor reflex by reducing baroreceptor sensitivity (Michas et al., 2012). Hence, resting sympathetic tone can be elevated and therefore increase resting brachial and aortic blood pressure.

However, increased augmentation index and cardiac wasted energy were not reversed with nitrate-rich beetroot juice intervention in postmenopausal women. Lack of differences in augmentation index and left ventricular wasted energy with nitrate-rich beetroot juice can be explained by, at least in part, no changes in pulse wave velocity. An acute dose of nitrate-rich beetroot juice may not be enough to cause arterial structure changes that can reduce PWV and influence reflected wave. Therefore, we can speculate that the reduction in aortic blood pressure with nitrate-rich beetroot juice is most likely caused by increasing NO bioavailability leading to an increase in NO storage in intravascular system, not by altering reflected wave indices. Stored
NO in the intravascular system can cause direct vasodilation in microcirculation and, finally, reduce systemic blood pressure (Webb et al., 2008).

**Experimental considerations**

This present study has multiple strengths. First, this study includes the use of a double-blind, randomized cross-over study design with a rigorously screened healthy pre- and post-menopausal women. In addition, this study used a ‘true placebo’ (i.e., nitrate-depleted beetroot juice) to examine cardiovascular benefits of dietary nitrate supplementation as opposed to water or other juices used in other studies. The use of a placebo that differs only in nitrate content, along with standardized pre-visit instructions to avoid caffeine and exercise is arguably the most rigorous means of testing the effects of dietary nitrate supplementation *per se* on vascular function. Also, even with the relatively small (n=8) sample size, this study was powered to access differences in the primary outcome variable (i.e. central blood pressures) and the effect size was large for the primary findings (*Cohen’s d* = 1.49).

There are several limitations of the present study that should be considered alongside these strengths. First, this study was done with acute dose of dietary nitrate supplementation and cannot be extrapolated to chronic effects of the supplementation. Second, we did not perform fluid volume control trial to investigate the effects of fluid consumption. However, the placebo in this study allows us to investigate the effects of nitrate supplementation despite of the amount of fluid subjects are consuming which is the same (140ml) for both BRplacebo and BRnitrate. Third, we did not control for subjects’ diet nor collected diet logs. However, participants were consistently fasted at least 8 hours for all of the experimental visits, and refrained from high nitrate containing food such as spinach and lettuce for a day prior to both nitrate-depleted and -rich visit. Also, the
baseline plasma nitrate concentration for both visits was not significantly different (p > 0.05) which suggests that the baseline nitrate consumption was controlled fairly well.

**Conclusion**

The present study indicates that an acute dose of dietary nitrate supplementation in form of beetroot juice lowered supine resting peripheral and central aortic blood pressures, but does not reduce augmentation index, cardiac wasted energy, and central arterial stiffness in healthy postmenopausal women. These preliminary findings warrant continued investigation of the acute cardiovascular effects of dietary nitrate supplementation in post-menopausal women, including those with overt cardiovascular disease (e.g., hypertension, etc). Although these findings are limited to healthy postmenopausal women, this study provides important information about beneficial effects of dietary nitrate supplementation on resting aortic hemodynamics and blood pressures for future investigations of dietary nitrate supplementation.
Table 3-I: Subject Characteristics. Data are expressed as mean ± S.E.M. * indicates significant (p < 0.05) difference between young and older subjects.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22 ± 1</td>
<td>61 ± 1*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>168 ± 2</td>
<td>163 ± 2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60 ± 3</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Body Mass Index (kg·m²)</td>
<td>21.3 ± 0.86</td>
<td>26.7 ± 1.79*</td>
</tr>
</tbody>
</table>
Table 3-2: Aging comparison of hemodynamics and pressures. Data are expressed as mean ± S.E.M. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; Ai, augmentation index; SVI, subendocardial viability index; Ew, left ventricle wasted energy. * indicates significant (p < 0.05) difference between young and older subjects.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
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</thead>
<tbody>
<tr>
<td>Brachial</td>
<td></td>
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</tr>
<tr>
<td>SBP (mmHg)</td>
<td>105 ± 2.3</td>
<td>116 ± 3.7*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>65 ± 2.3</td>
<td>71 ± 2.4</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>79 ± 2.5</td>
<td>88 ± 2.8*</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>40 ± 2.04</td>
<td>45 ± 1.9</td>
</tr>
<tr>
<td>Aortic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>92 ± 2.3</td>
<td>110 ± 3.7*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>66 ± 2.3</td>
<td>72 ± 2.1*</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>78 ± 2.5</td>
<td>88 ± 2.5*</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>25 ± 1.1</td>
<td>38 ± 2.3*</td>
</tr>
<tr>
<td>Ai @ HR75 (%)</td>
<td>4 ± 3.4</td>
<td>31 ± 1.8*</td>
</tr>
<tr>
<td>SVI</td>
<td>156 ± 32</td>
<td>148 ± 9</td>
</tr>
<tr>
<td>Ew</td>
<td>143 ± 127</td>
<td>3401 ± 600*</td>
</tr>
</tbody>
</table>
Figure 3-1: Experimental Timeline.
Figure 3-2: Plasma nitrate and nitrite concentrations. Comparison of average plasma nitrate and nitrite concentrations (mean ± S.E.M.) before, 90 after consumption of BR_placebo and BR_nitrate, and at the end of the experimental protocol in postmenopausal women. Abbreviations: BR_placebo, nitrate-depleted beetroot juice; BR_nitrate, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR_placebo and BR_nitrate.
Figure 3-3: Peripheral hemodynamics. Comparison of average a) systolic, b) diastolic, c) and mean blood pressure (mean + S.E.M.) pre- and post-consumption of BR\textsubscript{placebo} and BR\textsubscript{nitrate} in postmenopausal women. Individual data for these variables are shown on right side (panel d, e, f). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; BR\textsubscript{placebo}, nitrate-depleted beetroot juice; BR\textsubscript{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR\textsubscript{placebo} and BR\textsubscript{nitrate}. 
Figure 3-4: Central hemodynamics. Comparison of average a) systolic, b) diastolic, c) and mean blood pressure (mean + S.E.M.) pre- and post-consumption of BR\textsubscript{placebo} and BR\textsubscript{nitr}ate in postmenopausal women. Individual data for these variables are shown on right side (panel d, e, f). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; BR\textsubscript{placebo}, nitrate-depleted beetroot juice; BR\textsubscript{nitr}ate, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR\textsubscript{placebo} and BR\textsubscript{nitr}ate.
**Figure 3-5**: Augmentation index and calculated cardiac wasted energy. Average data are expressed as mean + S.E.M.; Comparison of average a) augmentation index at heart rate 75 bpm (Aix@HR75) and b) left ventricule wasted energy (Ew) pre- and post-consumption of BR_{placebo} and BR_{nitrate} in postmenopausal women. Individual data for these variables are shown on right side (panel c and e). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; BR_{placebo}, nitrate-depleted beetroot juice; BR_{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR_{placebo} and BR_{nitrate}.
Figure 3-6: Aortic pulse wave velocity. Average data are expressed as mean ± S.E.M.; Comparison of average a) augmentation index at heart rate 75 bpm and b) left ventricle wasted energy (Ew) pre- and post-consumption of BR_{placebo} and BR_{nitrate} in postmenopausal women. Individual data for this variable are shown on right side (panel b). Abbreviations: PWV, pulse wave velocity; BR_{placebo}, nitrate-depleted beetroot juice; BR_{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR_{placebo} and BR_{nitrate}. 

**a)**

![Graph showing PWV (m/s)](image)

**b)**

![Graph showing ΔPWV (m/s)](image)
Chapter 4

Effects of acute dose of dietary nitrate supplementation on perceived exertion and ischemic exercise tolerance

Introduction

Exercise performed in hypoxic conditions causes a compensatory vasodilatory response in the exercising limbs of healthy adults (Hartley et al., 1973; Rowell et al., 1986; Wilkins et al., 2008). However, the aging process can reduce this nitric oxide mediated compensatory vasodilator response to hypoxic exercise, thereby resulting in an attenuated rise in exercise hyperemia (Casey et al., 2010). Interestingly, a recent study by Casey et al showed that acute dietary nitrate supplementation improves the compensatory vasodilatory response to hypoxic exercise in older adults (Casey et al., 2015).

Dietary nitrate supplementation, especially in the form of nitrate-rich beetroot juice, has received much attention for its ergogenic effects in young individuals. Previous studies have revealed that nitrate-rich beetroot juice improves exercise tolerance to high intensity dynamic exercise (Bailey et al., 2009) and resulted in a higher work rate achievement during submaximal cycling exercise (Lansley et al., 2011a). The mechanisms that explain these ergogenic effects are not fully elucidated, however, consumption of nitrate-rich beetroot juice has been shown to decrease oxygen consumption during moderate intensity exercise by improving mitochondrial efficiency (Larsen et al., 2011) and/or decreasing ATP costs (Bailey 2010). When dietary nitrate (NO$_3^-$) is consumed, it is converted to nitrite (NO$_2^-$) and then to nitric oxide (NO) via nitrate-nitrite-nitric oxide pathway. Nitrite and nitric oxide produced from this pathway can play a role in oxygen utilization by either reducing slippage of the mitochondrial proton pump (Clerc et al.,
and/or receiving electrons at the end of the electron transport chain instead of oxygen (Basu et al., 2008). Furthermore, converted nitrite can be stored in the microvasculature and reduced to nitric oxide when needed to cause vasodilation (Basu et al., 2008; Webb et al., 2008). Finally, with nitrate supplementation, the nitric oxide mediated microvascular vasodilation increases perfusion to the exercising limbs improving oxygen demand and supply curve matching (Webb et al., 2008).

Interestingly, the conversion of nitrite to nitric oxide occurs favorably in areas of the vascular bed where pH and partial pressure of oxygen is low (Dejam et al., 2004; Gladwin et al., 2006; Godber et al., 2000a; Godber et al., 2000b; Li et al., 2001; Lundberg and Govoni, 2004; Millar et al., 1998; Zhang et al., 1997), which involves deoxyhemoglobin in the conversion process. Cosby et al reported that deoxyhemoglobin reduces nitrite to nitric oxide, and also that the pH and partial pressure of oxygen are negatively correlated with the nitrite to nitric oxide conversion rate. They reported a positive association of deoxyhemoglobin concentration with nitrite-mediated vasodilation in humans (Cosby et al., 2003). In the present study, conditions of low pH and low partial pressure of oxygen were induced with progressive ischemic intermittent handgrip exercise. By doing so we attempted to maximize the effects of dietary nitrate supplementation and nitrite-induced vasodilation in the exercising limb.

Ratings of perceived exertion (RPE; 6-20, Borg scale (Borg, 1990)) is a subjective self-reported measure for effort, strain, and fatigue experienced during exercise (Utter et al., 1997). Even though it is a psychological parameter, its relation with a number of physiological responses during exercise have been shown to be a valid representation of effort. Previous studies have shown that the higher RPE is associated with increases in ventilation, oxygen uptake, metabolic acidosis, and reduction of muscle carbohydrate storage during exercise (Borg, 1998; Noble et al., 1983; Skinner et al., 1973). Moreover, RPE is positively correlated with power output and
exercise performance (de Morree and Marcora, 2013) revealing its association to consequences during exercise.

Therefore, in this study, we sought to study the effects of dietary nitrate supplementation on exercise tolerance during progressive ischemic handgrip exercise. It was hypothesized that the acute dietary nitrate supplementation in the form of nitrate-rich beetroot juice would 1) reduce perceived exertion and 2) improve exercise endurance time in postmenopausal women during progressive ischemic intermittent handgrip exercise.

Methods

Subjects

Healthy postmenopausal women (age, 61 ± 0.7 years; between 57 - 64 years) were recruited from the local university community. Interested volunteers provided written informed consent prior to enrollment. On the same day, medical screening was performed to determine eligibility. Participants were selected if they were low- to moderately active (i.e., <3 days/week of exercise), free of overt chronic disease as assessed by clinician reviewed medical history questionnaire and venous blood chemistry (hematologic, liver, and kidney function), and met the following criteria: (i) resting blood pressure between 110/60 and 140/90 mmHg, (ii) body mass index between 18.5 and 35 kg/m2, (iii) fasting plasma glucose < 100 mg/dL, (iv) fasting plasma low-density lipoprotein < 130 and/or high-density lipoprotein > 40 mg/dL, (v) non-smoker, (vi) not taking any cardiovascular medications, and (vii) had not donated blood or blood products in the past 3 months. Characteristics participants that completed this study are shown in Table 1. All procedures were approved by the Office of Research Protections at Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki.
Study Design

Upon arrival on experimental visits, venous blood sample was collected for baseline plasma nitrate and nitrite concentrations. For the dietary nitrate supplement intervention, subjects either consumed beetroot juice (BR\textsubscript{nitrate}; 140 mL Beet-It Organic, James White Juice Company) or a placebo juice (BR\textsubscript{placebo}; 140 mL nitrate-depleted Beet-It Organic, James White Juice Company) on separate visits. Both participants and investigators were blinded to the order of supplementation. Approximately 90 minutes following juice consumption, a second venous blood sample was taken to determine changes in plasma nitrate and nitrite. Following the venous blood draw, participants performed the experimental protocol.

Experimental protocol

Subjects were in a semi recumbent position and instrumented with a finometer (Finometer MIDI, Finapres Medical Systems), and ECG. Using a customized handgrip device, subjects performed maximum voluntary contractions to determine 10% of maximum grip strength which was the target force for the intermittent handgrip exercise. Figure 4-1 shows the experimental protocol (Figure 4-1). The protocol started with 3 minutes of quiet rest for baseline data collection, followed by intermittent handgrip exercise (50% duty cycle, 30 contractions per minute, 1s contraction and 1s relaxation) until exhaustion. Four minutes into the intermittent handgrip exercise, an arm cuff placed around the upper arm was gradually inflated to occlude blood flow at a rate of 20 mmHg/min. This intermittent handgrip exercise was performed three times on one control visit and two experimental visits: one with BR\textsubscript{placebo} and once with BR\textsubscript{nitrate}. On control visit, subjects were familiarized with the progressive ischemic intermittent handgrip exercise, and practiced exercising until volitional fatigue and reporting a rating of exertion (RPE).
During both experimental visits, subjects were asked to give a rating of perceived exertion on a scale from 6 to 20 every minute. Approximately 5 seconds prior to the termination of exercise, the arm cuff was inflated to 250 mmHg for 3 minutes for post exercise circulatory arrest. Forearm blood flow was measured by Doppler ultrasound during the experimental protocol.

**RPE Threshold and slope analysis**

To examine the effects of nitrate-rich beetroot juice on participant’s ratings of perceived exertion and pain, the onset of initial RPE change and the slope following the threshold was compared between BR<sub>placebo</sub> and BR<sub>nitrate</sub>. The threshold was defined as the duration of time from the start of the upper arm cuff inflation to the initial change in RPE or RPP of the end of the free flow handgrip exercise. Also the slope of the RPE and RPP was calculated after the initial changes of the RPE and RPP, threshold, and compared between BR<sub>placebo</sub> and BR<sub>nitrate</sub> (Figure 4-2).

**Plasma [nitrate/nitrite] analysis**

Venous blood samples were drawn into heparin tubes (4mL lithium heparin tubes, BD Vacutainer, Franklin Lakes, N.J., USA) and immediately centrifuged at 3200 r/m (1590 g) and 4°C for 10 min. Plasma was then extracted and stored in a -80°C freezer for later analysis of nitrate and nitrite concentration. The ENO-20 analyzer (EICOM, San Diego, Calif., USA) with a sensitivity of 0.1 pmol for nitrate and nitrite was used to measure nitrate and nitrite concentration in the plasma samples. Briefly, plasma was mixed with an equal volume of 100% methanol and centrifuged at 13,000 r/m (12,000 g) for 10 minutes. Samples were then loaded into a 96-well plate. Nitrate and nitrite were then separated via column chromatography and individually reacted
with a Griess reagent, synthesizing a diazo compound. The absorbance of this red diazo compound was then read at a wavelength of 540nm using a visible light detector.

**Statistical analysis**

A paired sample t-test determined differences between BR\textsubscript{placebo} and BR\textsubscript{nitrate}. All statistical analyses were performed using SPSS version 21 (IBM, Chicago, IL). Statistical significance was set at \( p < 0.05 \). All data are expressed as the mean ± standard error.

**Results**

**Participant characteristics**

Participant characteristics are reported in Table 4-1. Subjects’ peak grip force was similar to values that are reported by other groups (Massy-Westropp et al., 2011).

**Plasma nitrate and nitrite concentration**

Prior to juice consumption, baseline plasma nitrate and nitrite concentrations were not significantly different between visits (nitrate, \( p = 0.38 \); nitrite, \( p = 0.55 \)). Plasma nitrate and nitrite concentration following 90 minutes of BR\textsubscript{placebo} consumption were not raised from baseline (\( p > 0.05 \)), however plasma nitrate and nitrite concentrations were significantly elevated 90 minutes following consumption of BR\textsubscript{nitrate} (nitrate 13 fold, nitrite 4 fold; both \( p < 0.05 \)). At the completion of the study visit plasma nitrate and nitrite concentrations remained elevated from post-90-minute BR\textsubscript{nitrate} consumption (nitrate 10 fold, nitrite 4 fold; both \( p < 0.05 \)).
Time to volitional fatigue

Time from the start of the progressive ischemic handgrip exercise to volitional fatigue was measured in seconds. Although an increasing trend in time to fatigue was observed with an acute dose of $\text{BR}_{\text{nitrate}}$ (vs. $\text{BR}_{\text{placebo}}$), the increase was not statistically significant ($547 \pm 55$ s vs. $572 \pm 61$ s; $p = 0.13$; Figure 4-3, panel a). Individual data show that 5 of 9 healthy postmenopausal women had longer time to fatigue with $\text{BR}_{\text{nitrate}}$ when it is compared to that of $\text{BR}_{\text{placebo}}$ (Figure 1, panel b). Furthermore, relations between changes in plasma nitrite concentration and time to fatigue with an acute dose of $\text{BR}_{\text{placebo}}$ and $\text{BR}_{\text{nitrate}}$ were compared, but the slopes were not different (data not shown; $p = 0.51$).

RPE Threshold and slope analysis

Self-reported RPE ($\text{BR}_{\text{placebo}}$ vs. $\text{BR}_{\text{nitrate}}$, $7.6 \pm 0.4$ vs. $7.0 \pm 0.3$; $p = 0.13$) at the end of the free flow exercise was not different between $\text{BR}_{\text{placebo}}$ and $\text{BR}_{\text{nitrate}}$. However, a RPE threshold which is a time from the end of the free flow exercise to initial change greater than 1 unit in RPE was significantly delayed with $\text{BR}_{\text{nitrate}}$ compared to that of $\text{BR}_{\text{placebo}}$ (Figure 4-4, panel a; $p < 0.05$). Individual data show that 7 of 8 subjects had greater RPE thresholds when they consumed $\text{BR}_{\text{nitrate}}$ compared to $\text{BR}_{\text{placebo}}$ visit (Figure 4-4, panel b). In addition to the RPE threshold, rate of RPE changes after threshold with $\text{BR}_{\text{placebo}}$ and $\text{BR}_{\text{nitrate}}$ is analyzed and compared, but no effects of dietary nitrate supplementation were observed (Figure 4-5).
Discussion

Summary

The primary purpose of this study was to examine the effects of nitrate rich beetroot juice on perceived exertion during progressive ischemic intermittent handgrip exercise in postmenopausal women. Our most interesting finding was that the time to initial changes in rating of perceived exertion (RPE) during the ischemic handgrip exercise was delayed with nitrate-rich beetroot juice in postmenopausal women compared to that of nitrate-depleted beetroot juice. Although the difference was not statistically significant, nitrate-rich beetroot juice had an increasing trend in time to exhaustion compared to that of nitrate-depleted beetroot juice in postmenopausal women. However, the rate of changes in RPE after threshold was not different between the treatments, and no difference in RPP threshold and rate of changes were observed with nitrate-rich beetroot juice.

An acute dose of nitrate-rich beetroot juice delayed the RPE threshold compared to that of nitrate-depleted beetroot juice. This is the first study to investigate the effects of nitrate-rich beetroot juice on perceived exertion and pain during progressive ischemic handgrip exercise in postmenopausal women. When the nitrate-rich beetroot juice is consumed, it goes through a series of reductions and converts nitrate to nitric oxide which causes direct vasodilation in the microcirculation. Thus, the observed delay in RPE threshold in this study can be explained, partially, by direct vasodilation effects of nitric oxide converted from nitrite in the microvasculature. It can be speculated that the microvasculature vasodilation increases perfusion to the exercising forearm, increasing the driving pressure of oxygen (Ferreira et al., 2006) which may result in less phosphocreatine breakdown, and eventually increasing exercise tolerance (Haseler et al., 1998; Vanhatalo et al., 2010b).
We examined the effect of dietary nitrate supplementation on ischemic exercise tolerance (supplement data), and also evaluated the potential order effect of a novel test utilized for this study, progressive ischemic handgrip exercise model, on ischemic exercise tolerance. Four of the 8 subjects were able to exercise to beyond 60% reduction in blood flow on both BR\textsubscript{placebo} and BR\textsubscript{nitrate} visits. Three subjects improved their ischemic exercise tolerance and were able to perform to a greater percent of blood flow reduction under conditions of dietary nitrate supplementation while 2 subjects showed reduction in ischemic tolerance exercised to less blood flow percent reduction. Lastly, examination of ischemic exercise tolerance between visit 2, 3, and 4 revealed no clear order effect.

Although no statistical differences were detected for endurance time between the two treatment groups, nitrate-rich beetroot juice tended to increase time to exhaustion in postmenopausal women during progressive ischemic intermittent handgrip exercise. The lack of significance could be due to the small sample size in this study. Notably, when the data for 2 hypertensive women who participated in this study (data not shown) were added to this analysis, the difference in endurance time became statistically significant (p < 0.01) and also showed that the threshold onset for initial RPE change was delayed with nitrate-rich beetroot juice (p < 0.01). This was expected since xanthine oxidase activity in hypertension patients is reported to be higher (Newaz et al., 1996) which may cause greater bioconversion of nitrite to nitric oxide and have greater physiological effects of nitric oxide. Further investigation into this is required to elucidate the effects of nitrate-rich beetroot juice in hypertensive patients.

**Experimental considerations**

This study includes the use of a double-blind, randomized cross-over study design as well as a rigorous screening protocol for healthy pre- and post-menopausal women. Notably, a ‘true
placebo’ (i.e., nitrate-depleted beetroot juice) was used to accurately assess the cardiovascular benefits of dietary nitrate supplementation. The use of a true placebo that differs only in nitrate content, along with standardized pre-visit instructions to avoid caffeine and exercise, is arguably the most effective means of testing the effects of dietary nitrate supplementation on vascular function. The present study is also one of a few studies that did not control for participant intake of nitrate containing foods prior to supplementation. As a result, findings from the current investigation have greater translational implications than dietary controlled studies. Even with the relatively small sample size, our effect size value ($d = 1.12$) suggested high practical significance for our primary finding (i.e. the time to initial rise in perceived exertion during graded ischemic exercise; Figure 2). Due to limited resources when conducting this study, our group was unable to recruit a larger sample, however, more subjects will be recruited in the future.

There are several limitations of the present study that should be considered alongside these strengths. First, this study explored the effects of an acute dose of dietary nitrate supplementation and therefore our findings cannot be extrapolated to chronic effects of supplementation. Secondly, no microvascular perfusion data are reported in this study. Lastly, we did not control for participants’ diet nor collected diet logs. However, participants came fasted 8 hours for all of the experimental visits, and also asked to refrain from high nitrate containing food such as spinach and lettuce for a day prior to the experimental visit.

**Conclusion**

An acute dose of nitrate-rich beetroot juice delayed the time to the initial change in perceived exertion during progressive ischemic handgrip exercise in postmenopausal women. However, the subsequent rate of change in perceived exertion was not different between nitrate-rich and –depleted beetroot juice. Moreover, no differences were detected in thresholds and rates
of change in perceived pain with nitrate-rich beetroot juice compared to a nitrate-depleted beetroot juice. Importantly, nitrate-rich beetroot juice supplementation demonstrated a trend toward prolonged endurance time during progressive ischemic handgrip exercise in postmenopausal women. This observed trend may have translational implications for improving exercise tolerance in older women and should be further explored in future studies involving a larger participant pool.
Figure 4-1: Experimental Protocol
Figure 4-2: RPE threshold and rate of change after threshold analysis. One subject’s data are shown to help understand the threshold and rate of change after threshold analysis.
Table 4-1: Subject Characteristics. Data are expressed as mean ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Postmenopausal women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163 ± 2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Body Mass Index (kg·m²)</td>
<td>26.7 ± 1.8</td>
</tr>
<tr>
<td>Peak Grip Force (kg)</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>
Figure 4-3: Plasma nitrate and nitrite concentrations. Comparison of average plasma nitrate and nitrite concentrations (mean ± S.E.M.) before, 90 after consumption of BR_{placebo} and BR_{nitrate}, and at the end of the experimental protocol in postmenopausal women. Abbreviations: BR_{placebo}, nitrate-depleted beetroot juice; BR_{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR_{placebo} and BR_{nitrate}.
Figure 4-4: Time to volitional fatigue. Average data are expressed as mean ± S.E.M.; Abbreviations: BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. A) White bar represents an average time to subjects’ volitional fatigue after acute dose of BR<sub>placebo</sub> consumption, and black bar represents the same after acute dose of BR<sub>nitrate</sub>. B) Each line represents each subject. *p < 0.05 vs. BR<sub>placebo</sub>. 
Figure 4-5: Threshold for Ratings of perceived exertion. Average data are expressed as mean ± S.E.M.; Abbreviations: BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. A) The white bar represents an average RPE threshold of BR<sub>placebo</sub>, and the black bar represents an average RPE of BR<sub>nitrate</sub>. B) Each line represents each subject. *p < 0.05 vs. BR<sub>placebo</sub>
**Figure 4-6:** Rate of change after threshold of RPE. Average data are expressed as mean + S.E.M.; Abbreviations: BR<sub>placebo</sub>, nitrate-depleted beetroot juice; BR<sub>nitrate</sub>, nitrate-rich beetroot juice. A) The white bar represents an average RPE threshold of BR<sub>placebo</sub>, and the black bar represents an average RPE of BR<sub>nitrate</sub>. B) Each line represents each subject. *p < 0.05 vs. BR<sub>placebo</sub>
Chapter 5

Effects of acute nitrate supplementation on ischemic exercise-induced pressor responses in post-menopausal women

Introduction

Abnormally high blood pressure (BP) responses during exercise testing are prognostic for future hypertension (Goble and Schieken, 1991; Manolio et al., 1994), adverse cardiovascular events (Allison et al., 1999; Kohl et al., 1996; Kurl et al., 2001; Laukkanen et al., 2006; Lewis et al., 2008; Mundal et al., 1996; Skretteberg et al., 2013), and mortality (Filipovsky et al., 1992; Kjeldsen et al., 2001; Mundal et al., 1996; Skretteberg et al., 2013; Weiss et al., 2010). Older (post-menopausal) women appear disproportionally affected, with exercise-induced increases in BP frequently exceeding those of premenopausal women and age-matched men (Choi et al., 2012; Fleg et al., 1995; Green et al., 2002; Proctor et al., 2003a; Proctor et al., 2003b). Post-menopausal women also display exaggerated pressor responses during ischemic (isometric handgrip) exercise, as well as during circulatory arrest following exercise (PECA), suggesting they may have an enhanced muscle metaboreflex (Choi et al., 2012) and altered autonomic control of blood pressure. Given the poor rates of pharmacological BP control in post-menopausal women (Burt et al., 1995; Wassertheil-Smoller et al., 2000) and the paucity of (low risk) estrogen-based BP lowering options (De Meersman et al., 1998; Fadel et al., 2004; Pines et al., 1996), there is a clinical need to investigate novel interventions aimed at improving BP regulation in this rapidly growing cohort.
Dietary nitrate supplementation is emerging as a promising non-pharmacological intervention for lowering blood pressure and improving cardiovascular health in a variety of populations, including older adults (Hughes et al., 2016; Siervo et al., 2013). Its beneficial cardiovascular effects are thought to be exerted from conversion of consumed dietary nitrate (NO$_3^-$) to nitrite (NO$_2^-$), and then to nitric oxide (NO) via nitric oxide synthase (NOS) independent pathway (i.e. nitrate-nitrite-nitric oxide pathway) (Lundberg et al., 2008). NO$_3^-$ supplementation not only reduces blood pressure at rest, but may also have favorable effects on blood pressure and other physiological responses (peripheral resistance, oxygen consumption) during exercise. Such effects have been reported following acute beetroot juice consumption in younger adults (Bond et al., 2014; Lee et al., 2015), in older patients (Kenjale et al., 2011; Zamani et al., 2015) and in rodents (Ferguson et al., 2013b, 2014). While blood pressure lowering effects of acute nitrate supplementation during exercise appear possible in healthy older adults (Kelly et al., 2013), to the best of our knowledge no studies have exclusively studied these effects in older women or focused on how this intervention impacts the mechanisms that actually regulate BP.

The primary purpose of the present study, therefore, was to determine the effects of acute nitrate supplementation on reflex increases in blood pressure evoked by progressively restricting blood flow to the exercising forearm (via an upper arm cuff) in a group of healthy post-menopausal women. The use of a single, low intensity intermittent work intensity (10% of peak grip strength), coupled with gradual increments in cuff pressure (20 mmHg/min) was chosen to elicit a more gradual engagement of the exercise pressor reflex than is typically used (i.e., 2 min static hold), followed by post exercise circulatory arrest (PECA) in an effort to isolate the contribution of the muscle metaboreflex. By using an experimental model that progressively limits blood flow to exercising muscle up to the point of fatigue, we hoped to maximize the conversion of nitrite to nitric oxide via this pathway (Lundberg et al., 2008) and test whether
acutely nitrate supplementation attenuates the systemic BP responses during progressive metaboreflex activation (graded cuff inflation) and isolation (PECA) in healthy post-menopausal women. We hypothesized that dietary nitrate supplementation would attenuate the reflex increases in blood pressure during graded muscle ischemia and metaboreflex isolation given its reported beneficial effects on contracting muscle perfusion (Casey et al., 2015; Ferguson et al., 2013b) and multiple processes influencing muscle O$_2$ demand and thus metabolite accumulation (Affourtit et al., 2015; Bailey et al., 2010; Bailey et al., 2009; Bentley et al., 2014; Bescos et al., 2012; Jones et al., 2011a; Jones et al., 2011b; Larsen et al., 2011). We further hypothesized that acute nitrate supplementation would shift the components of the exercise pressor reflex in these women (smaller rise in TPR vs. placebo).

**Methods**

Ten healthy postmenopausal women (age, 61 ± 1 years; between 57 - 64 years) were recruited from the local university community. Interested volunteers provided written informed consent prior to enrollment. On the same day, medical screening was performed to determine eligibility. Participants were selected if they were low- to moderately active (i.e., <3 days/week of exercise), free of overt chronic disease as assessed by clinician reviewed medical history questionnaire and venous blood chemistry (hematologic, liver, and kidney function), and met the following criteria: (i) resting blood pressure between 110/60 and 140/90 mmHg, (ii) body mass index between 18.5 and 35 kg/m$^2$, (iii) fasting plasma glucose < 100 mg/dL or HbA1c < 6.0%, (iv) fasting plasma low-density lipoprotein < 130 and/or high-density lipoprotein > 40 mg/dL, (v) non-smoker, (vi) not taking any cardiovascular medications, and (vii) had not donated blood or blood products in the past 3 months. All procedures were approved by the Office of Research
Protections at Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki.

**Study design**

Upon arrival on the experimental visits, venous blood sample was collected for baseline plasma nitrate and nitrite concentrations. Then, subjects underwent dietary nitrate supplement intervention, and consumed nitrate-rich beetroot juice (BR$_{\text{nitr}}$; 140 mL Beet-It Organic, James White Juice Company) and nitrate-depleted beetroot juice as a placebo (BR$_{\text{plc}}$; 140 mL nitrate-depleted Beet-It Organic, James White Juice Company) on separate visits. The order of intervention was blinded for both subjects and the researchers. Approximately 90 minutes following the juice consumption, a second venous blood sample was taken to determine the post-consumption change in plasma nitrate and nitrite. Then, subjects were seated in a semi recumbent position on a gurney and instrumented with experimental equipment. On the exercising arm, a blood pressure cuff (Hokinson) for gradual occlusion of blood flow was placed while an automated blood pressure cuff (HEM-705CP, Omron) and finger plethysmography (Finometer MIDI, Finapres Medical Systems) were placed on the non-exercising arm. A 3-lead ECG was also placed on the subject’s chest to collect electrocardiogram data. After instrumentation, the subject performed maximal voluntary contractions (MVC) with handgrip dynamometer (Stoelting Co.) to determine the subject’s maximum handgrip strength. A subject performed 3 MVCs with 1-minute break in between trials, and the highest force of the 3 MVCs was used to set a 100% effort of handgrip force for the intermittent handgrip exercise. Following recovery, a subject performed experimental protocol, and when the experimental protocol was completed, one more venous blood sample was collected to confirm the level of plasma nitrate and nitrite with beetroot...
juice. All study visits started between in the morning between 7 am to 10 am, and subjects were fasted a minimum of 8 hours.

**Experimental protocol**

After 10 minutes of quite rest about 15 seconds of resting brachial artery image was collected, and then resting blood velocity, ECG, and other hemodynamic data from finger plethysmography were collected for 3 minutes. Following 3 mins of resting data collection, the subjects performed intermittent handgrip exercise at a rate of 30 contractions per minute (1s contraction and 1s relaxation) at 10% of their MVC. The first 4 minutes of exercise was free flow exercise without blood flow occlusion, and it was used to ensure oxygen supply and demand matching and blood flow steady state achievement. Then, the blood pressure cuff on subject’s upper arm was progressively inflated at a rate of 20 mmHg/min (5 mmHg increase every 15 seconds. The target force (10% of their MVC) was displayed to provide visual feedback and to allow subjects to maintain the appropriate force output. Subjects continued to exercise until volitional fatigue. As soon as the subject stopped handgrip exercise, upper arm blood pressure cuff was instantly inflated to 250 mmHg and stayed inflated for 3-minutes for post-exercise circulatory arrest (PECA) period. After PECA, the upper arm blood pressure cuff was deflated and the 3-minute recovery data were collected.

**Vascular response to exercise**

Diameter and blood flow velocity of the brachial artery were measured using Doppler ultrasound (HDI 5000, Philips; Bothell, Wash., USA). For blood flow velocity, the artery was insonated at a constant angle of 60° with the sample volume adjusted to cover the width of the
artery. Velocity measurements were sampled in real time (400 Hz) using a data acquisition system (Powerlab, AD Instruments; Castle Hill, Australia). Mean blood velocity was calculated from the final 30 s of the third minute during each bout. High-resolution diameter measurements (6 MHz probe) were taken from 15-s recordings performed during baseline rest and immediately following the measurement of flow velocity during each exercise bout. Images were recorded directly to computer using a video capture device and software (Dazzle Video Creator, Pinnacle, Mountain View, Calif., USA). Brachial artery diameter was determined as the average across the cardiac cycle using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Coralville, Iowa). Forearm blood flow during the experimental protocol was calculated by multiplying the cross-sectional area of the resting brachial artery with mean blood velocity using the equation $\pi \left(\frac{\text{diameter}}{2}\right)^2 \times \text{velocity} \times 60$. All vascular analyses were conducted by a single investigator who was blinded to the participant and supplement order.

Hemodynamic variables

A finger plethysmograph (Finometer MIDI, Finapres Medical Systems) was placed on the non-dominant, non-exercising hand. Beat-to-beat blood pressures were measured by the finometer and the values are verified before and after the experimental protocol by an automated pneumatic blood pressure cuff (HEM-705CP, Omron) placed on the same arm. Cardiac output ($\dot{Q}$), stroke volume (SV), and total peripheral resistance (TPR) were calculated from a software (Beatscope) utilizing Modelflow technique to estimate those variables. Modelflow technique has been validated against other standard techniques for those variable such as pulsed-Doppler cardiography, and inert gas rebreathing (Bogert and van Lieshout, 2005; Ogoh et al., 2003; Stok et al., 1993; Sugawara et al., 2003; Wesseling et al., 1993).
**Plasma [nitrate/nitrite] analysis**

Venous blood samples were drawn into heparin tubes (4mL lithium heparin tubes, BD Vacutainer, Franklin Lakes, N.J., USA) and immediately centrifuged at 3200 r/m (1590 g) and 4°C for 10 min. Plasma was then extracted and stored in -80°C freezer for later analysis of nitrate and nitrite concentration. The ENO-20 analyzer (EICOM, San Diego, Calif., USA) with a sensitivity of 0.1 pmol for nitrate and nitrite was used to measure nitrate and nitrite concentration in the plasma samples. Briefly, plasma was mixed with an equal volume of 100% methanol and centrifuged at 13,000 r/m (12,000 g) for 10 minutes. Samples were then loaded into a 96-well plate. Nitrate and nitrite were then separated via column chromatography and individually reacted with a Griess reagent, synthesizing a diazo compound. The absorbance of this red diazo compound was then read at a wavelength of 540nm using a visible light detector.

**Statistical analysis**

Comparison for resting supine blood pressures upon arrival of each visit was done by paired t-test to confirm the subjects have relatively similar resting blood pressures. To assess differences between BR$_{\text{placebo}}$ and BR$_{\text{nitrate}}$, two way repeated analysis of variance (ANOVA) was used. Pairwise comparisons followed by paired t-test if group x time interaction was observed. For the comparisons of delta (changes from pre- to post-juice consumption), a paired t-test analysis was used. Also, Cohen’s d was calculated (mean 1 – mean 2 / combined standard deviation) on the primary outcome to determine effect size. All statistical analyses were performed using SPSS version 21 (IBM, Chicago, IL). Statistical significance was set at p < 0.05. All data are expressed as the mean ± standard error.
Results

Subject characteristics and baseline resting values

Ten volunteers met inclusion criteria and completed the familiarization study visit. One of these participants did not return for the beetroot study visits because she was uncomfortable with ischemic forearm exercise. A second participant was excluded because a technical problem with the finometer finger cuff during one of her beetroot study visits prohibited the subsequent analysis of her blood pressure recordings. Scheduling a return visit for this participant was unsuccessful so her data had to be excluded from this analysis. Characteristics for the eight women who completed this study with complete data are shown in Table 5-1. Values for BMI and fasting blood parameters (lipids, glucose, uric acid and hemoglobin) reflect a normal- to moderately-overweight, but metabolically healthy group of older women. Peak isometric grip strength (27.4 ± 1.7 kg) is in the 70th percentile for US women in this age range (Massy-Westropp et al., 2011).

Nitrate supplementation raised plasma NO$_3^-$ and NO$_2^-$ concentrations

Ingestion of the beetroot juice supplement and its nitrate-depleted placebo were well-tolerated by all participants, with no adverse side effects reported. Baseline (pre-juice consumption) concentrations of NO$_3^-$ averaged 61 µmol and 45 µmol at the beginning of the placebo and nitrate study visits (p = 0.38 between visits; Figure 5-1), respectively, suggesting that participants complied with our pre-study visit instructions (no consumption of high nitrate containing foods or drinks for 24 hours prior to either visit). The corresponding NO$_2^-$ concentrations at baseline were also similar between study visits (p = 0.55). These NO$_2^-$
concentrations were comparable with previous reports (Hughes et al., 2016) suggesting the procedures we used to process (rapid centrifugation), transfer and store (-80°C) the blood samples prior to analysis were conducted with a high degree of precision.

Plasma NO$_3^-$ and NO$_2^-$ remained low 90 minutes after consuming the placebo beetroot juice (BR$_{\text{placebo}}$) ($p = 0.17$ and $p = 0.25$ vs pre-consumption). Plasma NO$_3^-$ and NO$_2^-$ concentrations after consuming BR$_{\text{nitrato}}$, by contrast, were 13-fold and 4-fold higher compared to baseline (both $p < 0.05$). These findings confirm absorption and conversion of the ingested nitrate and suggest the participants complied with our pre-study instructions (no tooth brushing or mouthwash). Plasma NO$_3^-$ and NO$_2^-$ concentrations remained significantly elevated (10-fold and 4-fold, both $p<0.05$) at the end of these 3- to 4-hour study visits.

Nitrate supplementation did not alter pre-exercise or mechanoreceptor-associated hemodynamics

Figure 5-1 shows finometer-derived hemodynamics at rest (pre-exercise, recumbent position) and during the 4 min period of intermittent handgrip contractions (10% of peak force, 30/min, no cuff occlusion aka “free-flow” exercise; data not shown) on the BR$_{\text{placebo}}$ and BR$_{\text{nitrato}}$ study visits. Nitrate supplementation had no statistically significant influence on systolic, diastolic or mean BP at rest ($p = 0.32$, $p = 0.11$ and $p = 0.13$, respectively) or during the 4 minutes of “free flow” exercise ($p=0.31$, $p=0.49$, $p=0.34$, respectively). While there was a clear trend toward lower TPR values at rest and throughout the free flow exercise period on the BR$_{\text{nitrato}}$ visit, these differences did not achieve statistical significance ($p = 0.24$ vs. BR$_{\text{placebo}}$). Nitrate supplementation also had no effect on the steady state (final minute) exercise hyperemic responses to this very light workload (all $p > 0.19$).
Nitrate supplementation attenuated the peak MAP response to ischemic handgrip exercise

Figure 5-3, 5-4, and 5-5 shows finometer-derived blood pressure adjustments during the BR\textsubscript{placebo} and BR\textsubscript{nitate} visits. These data were plotted as a function of % exercise time to fatigue due to the fact that BR\textsubscript{nitate} tended to extend the duration of ischemic exercise (relative to BR\textsubscript{placebo}) in these participants (p = 0.13). These data were compared as (1) absolute BP values (Figure 5-3) and (2) the delta increase in BP above that measured during the final minute of free-flow exercise (Figure 5-4, 5-5), thus providing a comparison of the pressor responses to nitrate supplementation from a common metabolic starting point i.e., the final minute of free flow exercise where forearm muscle O\textsubscript{2} delivery vs. O\textsubscript{2} demand should be well-matched.

As expected, blood pressures steadily increased with progressive cuff inflation during both visits in all participants (main effect of time all p < 0.05). However, the presence of nitrate in the beetroot juice did not significantly attenuate (relative to BR\textsubscript{placebo}) systolic or diastolic BP responses across progressive cuff occlusion stages (0 to 80%) expressed as a % of exercise time to fatigue. Heart rate and Modelflow-derived variables (stroke volume, TPR; Figure 5-6, 5-7, 5-8) during graded muscle ischemia also appeared to be unaffected by acute NO\textsubscript{3}\textsuperscript{-} supplementation. The only hemodynamic effect of NO\textsubscript{3}\textsuperscript{-} to achieve statistical significance was the relative ΔMAP response at fatigue (BR\textsubscript{placebo} 30 ± 5% vs. BR\textsubscript{nitate} 25 ± 3%; p = 0.04). This effect of nitrate was driven mainly by a lower peak diastolic BP response (27 ± 4% vs. 21 ± 3%; p = 0.10) since peak systolic BP responses on the two visits were nearly identical. Plotting each of the above variables vs. absolute time in minutes/seconds led to subject drop-out after approximately 6 minutes (i.e., shortest time to fatigue), but examining the data in this way did not alter these results.
Nitrate supplementation attenuated diastolic BP during metaboreflex isolation (PECA)

Figure 5-3, 5-4, and 5-5 also shows hemodynamic adjustments during PECA and recovery. As expected, heart rate and blood pressures decreased rapidly with the removal of central command (volitional effort) in all participants (main effects of time all p < 0.05). The reductions in heart rate and systolic BP during PECA were similar during the BR\textsubscript{nitrate} and BR\textsubscript{placebo} visits. However, diastolic BP during PECA tended to decrease to a greater extent on the BR\textsubscript{nitrate} visit (p = 0.18 vs. BR\textsubscript{placebo}). When examined over the entire PECA phase (area under the curve across all 3 min), this diastolic BP difference was significant (p = 0.03 vs. BR\textsubscript{placebo}). Following release of the cuff (3 min of recovery) all hemodynamic variables decreased rapidly toward resting, pre-free flow exercise values.

Discussion

The results of this study show that acute ingestion of NO\textsubscript{3}\textsuperscript{-} rich beetroot juice, relative to a true placebo, attenuates the mean (predominantly diastolic) blood pressure response during maximal engagement of the exercise pressor reflex in healthy post-menopausal women. During PECA, diastolic pressures remained lower in these women when they were supplemented with NO\textsubscript{3}\textsuperscript{-}. However, in contrast to our hypothesis, NO\textsubscript{3}\textsuperscript{-} supplementation did not appear to alter the reduction in forearm muscle perfusion or the reflex pressor responses evoked by graded cuff occlusion in these women. There were also no obvious effects of acute NO\textsubscript{3}\textsuperscript{-} supplementation on exercising forearm hyperemia during low level intermittent contractions (i.e., unrestricted blood flow at 10\%MVC) or the associated mechanoreceptor-mediated pressor responses. Overall, these findings suggest a mild, but persistent systemic vasodilator effect of acute nitrate supplementation in post-menopausal women that does not appear to alter the exercise pressor reflex per se, but
may reduce cardiac afterload via a reduced TPR. To the best of our knowledge, this is the first study to examine the effects of inorganic nitrate supplementation on the exercise pressor reflex or its components in any population.

Effects of nitrate supplementation on cardiovascular adjustments during graded cuff occlusion

A primary hypothesis in this study was that NO$_3^-$ supplementation would enhance muscle perfusion and/or reduce muscle O$_2$ demand during progressive cuff occlusion in these subjects, thereby slowing metabolite accumulation/the drop in pH and delaying activation of the metaboreflex. Although muscle perfusion (brachial artery flow) progressively decreased, and systemic BP clearly rose (5-3, 5-4, and 5-5) with graded (20 mmHg/min) cuff occlusion in all subjects, we were unable to identify a clear “threshold” increase in blood pressure (vs. time or vs. % endurance time) in these subjects. We were also unable to assess interstitial pH in the forearm as originally intended, because our near infrared spectroscopy (NIRS) device failed to detect pH consistently under these conditions (discovered during initial pilot testing). Further challenges to addressing this metaboreflex delay hypothesis, at least from the standpoint of examining the blood flow reduction-vs-pressor response relationship, resulted from 1) the relatively low brachial artery blood flow elicited with this 10% MVC stimulus in these women (resulting in a small absolute range of blood flow values on the x-axis), 2) missing brachial artery blood flow data at random time points and on random study visits/subjects (resulting from occasional loss of Doppler image quality/adequate transducer angle of insonation for these 30+ minute studies), and 3) the fact that some subjects reached complete occlusion (zero brachial blood flow) and continued exercising on one visit, but stopped before reaching zero flow on the other. Despite these technical challenges, there was no evidence that acute NO$_3^-$ supplementation altered in a
consistent way the reduction in forearm muscle blood flow, or the reflex pressor responses evoked by graded cuff occlusion in these women.

**Effects of nitrate supplementation on cardiovascular responses at peak ischemic exercise tolerance**

The increases in systolic and diastolic pressure observed at peak ischemic exercise (average of 90-100% of endurance time) were, as expected, quite large. Systolic BP increases (delta increase above free-flow exercise) averaged 38 and 35 mmHg, respectively (BR_placebo, BR_nitrate) while diastolic pressure increases averaged 16 and 13 mmHg, respectively (BR_placebo, BR_nitrate). A novel finding was the smaller rise in MAP at peak ischemic exercise tolerance during the BR_nitrate visit (p=0.04), a response that primarily reflected an attenuated (p=0.10) diastolic BP response.

It is unclear how nitrate supplementation attenuated peak MAP as our subjects reached the point of local muscle fatigue; such mechanisms are complex and likely involve interactions between central command, baroreflexes, and mechanoreceptors, as well as metaboreceptors. An attenuated rise in diastolic BP, however, could reflect a more general systemic vasodilator effect associated with increased bioavailable nitric oxide. This could result from increased nitric oxide acting centrally (inhibiting sympathetic outflow via the cardiovascular control centers) or within the peripheral vasculature, serving to attenuate sympathetic vasoconstrictor outflow and buffering surges in sympathetic outflow. These possibilities are highly speculative, but the latter would be consistent with the mechanism by which estrogen is thought to disrupt sympathetic vascular transduction in pre-menopausal women (Hart et al., 2012).
Experimental considerations and limitations

Our study had several limitations. First and foremost was the small sample size. This may have limited our ability to detect a statistically significant effect of nitrate supplementation, particularly for outcome variables that were more variable or for which the effects of a single dose of nitrate were small. However, considerable efforts were undertaken to reduce inter-subject variability by controlling pre-visit dietary nitrate intake (8-hour fast, avoidance of high nitrate foods, caffeine, etc), maintaining oral bacterial conversion in the mouth (restrictions on mouthwash and teeth brushing) and confirming the absorption and conversion of nitrate (plasma nitrate and nitrite using gold standard methods). Second, all blood pressure measurements in this study were based on a finger plethysmograph (Finometer). While often criticized for its absolute validity, this method generally tracks relative changes in BP quite well (Sugawara et al., 2003). Proof of the latter can be seen in the consistency of the systolic BP responses to free flow exercise, graded ischemia, PECA and recovery in these subjects across two study visits one week or more apart (see figure 5-3, 5-4, and 5-5). This consistency not only highlights the suitability of using finger plethysmography for small muscle exercise/muscle metaboreflex testing, but also highlights the consistency with which our experimental protocols were conducted and the low probability that our participants’ underwent any habituation following the initial screening. A third consideration was our reliance on finger blood pressure waveform analysis (Beatscope/Modelflow) to estimate stroke volume/cardiac output and TPR. While also limited with respect to absolute values, this is a widely used method in the muscle metaboreflex literature (dozens of peer-reviewed publications) that acceptably tracks changes in these parameters. Future studies should, nonetheless, include absolute measures of ventricular volumes. A final limitation was our inability to utilize NIRS to assess intramuscular pH or oxygenation during graded cuff ischemia.
Summary and conclusion

Overall, these findings suggest a mild, but persistent systemic vasodilator effect of acute nitrate supplementation in post-menopausal women that does not appear to alter their exercise pressor reflex per se, but may reduce their reliance on increases in TPR during intense stimulation of skeletal muscle afferents. These effects were observed with a single (140 ml) dose of beetroot juice in a group of post-menopausal women with relatively low resting blood pressure for their age. Future studies should further investigate the effects of both acute and chronic (daily) nitrate supplementation in post-menopausal women. These studies should extend to patients with chronic disease, particularly conditions common to older women that are characterized by a hypoperfusion of active skeletal muscles (e.g., peripheral artery disease, diastolic heart failure).
Table 5-1: Subject Characteristics. Data are expressed as mean ± S.E.M. Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

<table>
<thead>
<tr>
<th>Postmenopausal women</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164 ± 2</td>
</tr>
<tr>
<td>BMI (kg·m$^2$)</td>
<td>25.9 ± 1.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>208.5 ± 8.7</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>71.7 ± 8.2</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>118 ± 7.6</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>91.1 ± 2.5</td>
</tr>
<tr>
<td>Uric Acid (mg/dL)</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.4 ± 1.2</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.8 ± 0.2</td>
</tr>
<tr>
<td>Physical Activity level (MET-wk)</td>
<td>2066 ± 381</td>
</tr>
<tr>
<td>Peak grip strength (kg)</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>
Table 5-2: Pre-juice consumption peripheral hemodynamics comparison for experimental visits. Data are expressed as mean ± S.E.M. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; PP, pulse pressure. Supine blood pressures were measured after 10 minutes of supine rest.

<table>
<thead>
<tr>
<th></th>
<th>BRplacebo visit</th>
<th>BRnitrate visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>121 ± 5.4</td>
<td>125 ± 5.0</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73 ± 3.0</td>
<td>78 ± 3.0</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>92 ± 4.3</td>
<td>96 ± 3.7</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>47 ± 2.6</td>
<td>46 ± 3.2</td>
</tr>
</tbody>
</table>
**Figure 5-1:** 3-minute rest (pre-exercise, post juice consumption) period peripheral hemodynamics comparison for experimental visits. Data are expressed as mean ± S.E.M. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; PP, pulse pressure.
Figure 5-2: Plasma nitrate and nitrite concentrations. Comparison of average plasma nitrate and nitrite concentrations (mean ± S.E.M.) before, 90 after consumption of BR\textsubscript{placebo} and BR\textsubscript{nitrate}, and at the end of the experimental protocol in postmenopausal women. Abbreviations: BR\textsubscript{placebo}, nitrate-depleted beetroot juice; BR\textsubscript{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR\textsubscript{placebo} and BR\textsubscript{nitrate}. 

** Figure a): Plasma [Nitrate] **

** Figure b): Plasma [Nitrite] **
Figure 5-3: Absolute blood pressure responses with BR$_{\text{placebo}}$ and BR$_{\text{nitrato}}$ during experimental protocol. Data are expressed as mean ± S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; BR$_{\text{placebo}}$, nitrate-depleted beetroot juice; BR$_{\text{nitrato}}$, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR$_{\text{placebo}}$ and BR$_{\text{nitrato}}$. 
Figure 5-4: Absolute changes of blood pressure responses from free flow with BR\textsubscript{placebo} and BR\textsubscript{nitrate} during experimental protocol. Data are expressed as mean + S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; BR\textsubscript{placebo}, nitrate-depleted beetroot juice; BR\textsubscript{nitrate}, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR\textsubscript{placebo} and BR\textsubscript{nitrate}.
Figure 5-5: Relative changes of blood pressure responses from free flow with \( \text{BR}_{\text{placebo}} \) and \( \text{BR}_{\text{nitrate}} \) during experimental protocol. Data are expressed as mean ± S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; \( \text{BR}_{\text{placebo}} \), nitrate-depleted beetroot juice; \( \text{BR}_{\text{nitrate}} \), nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between \( \text{BR}_{\text{placebo}} \) and \( \text{BR}_{\text{nitrate}} \).
Figure 5-6: Components of the pressor responses with BR$_{\text{placebo}}$ and BR$_{\text{nitr}}$. Data are expressed as mean ± S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; BR$_{\text{placebo}}$, nitrate-depleted beetroot juice; BR$_{\text{nitr}}$, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BR$_{\text{placebo}}$ and BR$_{\text{nitr}}$. 
Figure 5-7: Absolute delta (changes from free flow) components of the pressor responses with BRplacebo and BRnitrate. Data are expressed as mean ± S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; BRplacebo, nitrate-depleted beetroot juice; BRnitrate, nitrate-rich beetroot juice. * indicates significant (p < 0.05) difference between BRplacebo and BRnitrate.
Figure 5-8: Relative delta (changes from free flow) components of the pressor responses with BR\textsubscript{placebo} and BR\textsubscript{nitrate}. Data are expressed as mean $\pm$ S.E.M.; Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PECA, post exercise circulatory arrest; BR\textsubscript{placebo}, nitrate-depleted beetroot juice; BR\textsubscript{nitrate}, nitrate-rich beetroot juice. * indicates significant ($p < 0.05$) difference between BR\textsubscript{placebo} and BR\textsubscript{nitrate}.
Chapter 6

Critical Analysis of Findings and Future Directions

Identifying lifestyle interventions that can favorably impact cardiovascular health and physical function in our rapidly expanding aging population is a high biomedical research priority (Seals and Melov, 2014). This is particularly relevant for recently menopausal women (within 10 years of menopause) because this group experiences accelerated changes in disease risk (arterial stiffening, blood lipids, etc) and body composition (bone, fat, and muscle) resulting from the “double whammy” of ovarian and chronological aging. Interventions that successfully slow and/or prevent cardiovascular aging in post-menopausal women could, even in the absence of increases in lifespan, compress the years they spend living with physical and/or cognitive disabilities i.e., increased “healthspan”. The studies comprising this dissertation sought to determine the physiological effects of a dietary intervention (inorganic NO\textsuperscript{3}\textsuperscript{-} supplementation) known to have anti-aging effects on the vasculature and neuromuscular systems in pre-clinical (small rodents) and which has demonstrated clinically promising effects in overt cardiovascular disease states such as human hypertension, heart failure and peripheral artery disease. Overall the acute supplementation of nitrate in healthy post-menopausal women improved several cardiovascular outcomes and showed some benefit to ischemic exercise tolerance, without any demonstrable risks to these participants. The purpose of this chapter is: 1) to critically evaluate the experimental design and methods we used to investigate these outcomes; 2) to discuss the viability of dietary nitrate supplementation as an intervention for post-menopausal women; 3) to place our findings (both significant and not significant) in context with other studies in the literature; and 4) to suggest directions for future study in this area.
Critical evaluation of the experimental design and methods used in this dissertation

The sample population we recruited for these studies was post-menopausal women without overt chronic disease and who were not taking any medications with significant cardiovascular effects. The nine post-menopausal women who completed all study visits were relatively homogenous with respect to age (57 – 64 yrs), menopausal status, habitual physical activity (none were competitive athletes or exercise training at a high level) and ethnicity (all Caucasian). While this limits the generalizability of our findings, it reduced a potential null effect of NO\textsubscript{3}\textsuperscript{-} supplementation that might be observed if we had studied highly active subjects with no endothelial or mitochondrial dysfunction, or included subjects taking medications that could interfere with bacterial conversion of NO\textsubscript{3}\textsuperscript{-} to NO\textsubscript{2}\textsuperscript{-} in the mouth (James et al., 2015).

As discussed in earlier sections of this dissertation, we employed rigorous procedures to ensure there would be significant increases in circulating NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} in these subjects at the time of our experimental measurements i.e., between 90 and 180 minutes. These procedures included 1) our use of an FDA-approved nitrate-rich supplement (Beet-It organic shot) that is manufactured, bottled, and shipped to the U.S. under highly controlled conditions, 2) highly sensitive measurements of plasma NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-} conducted by a highly experienced laboratory (Wake Forest University), 3) administration of a moderately high dose of NO\textsubscript{3}\textsuperscript{-} (0.6 g) that has been demonstrated in multiple studies to lower blood pressure (Hughes et al., 2016; Kelly et al., 2013; Kenjale et al., 2011) and enhance exercise efficiency (Bailey et al., 2010; Jones et al., 2013; Larsen et al., 2011) in healthy, but non-trained adults, 4) detailed pre-visit instructions regarding teeth brushing, exercise, caffeine and high nitrate food intake, and 5) direct observation of ingestion of the supplement (in our laboratory). Evidence of subject compliance and nitrate absorption was observed in the plot of plasma NO\textsubscript{3}\textsuperscript{-} concentrations between the pre-ingestion and post-ingestion time points (Figure 3-2). All 9 post-menopausal women exhibited large increases
in plasma NO₃⁻ at 90 minutes (13-fold increase, p < 0.01) which were relatively well-maintained until the end of the study (10-fold increase), consistent with the pharmacokinetics of this supplement (James et al., 2015; Wylie et al., 2013). Evidence of conversion of the absorbed NO₃⁻ to NO₂⁻ was also observed in all 9 subjects (lower plot of Figure 3-2), consistent with previous studies using beetroot supplements in other cohorts. Collectively, these findings suggest that two 70-ml shots of Beet-It Organic juice (0.6 g of NO₃) results in a “minimum effective increase” in plasma NO₂⁻ / NO₃⁻ in this cohort.

While it is evident that all 9 of our post-menopausal subjects had large increases in circulating NO₃⁻ and NO₂⁻, there was still considerable variability in the physiological responses to this acute NO₃⁻ load. Moreover, we did not observe any significant associations between the rise in plasma NO₂⁻ and any cardiovascular or ischemic exercise outcomes in this group of subjects. Therefore, we cannot determine why some subjects were apparent “responders” on certain outcomes and “non-responders” on others (note that there were no consistent responders or non-responders on all variables). This could reflect between-subject differences in the conversion of NO₂⁻ to nitric oxide which we, like most studies, did not directly assess.

The use of a double blind, cross-over study design and a true (NO₃⁻ depleted) placebo lends additional credibility to the current study and is consistent with recent guidelines published by NIH and other scientific agencies for enhancing the “rigor and reproducibility” of scientific research (Collins and Tabak, 2014). This is particularly important for studies examining the efficacy and safety of dietary supplements. Many studies examining the vascular and ergogenic effects of dietary nitrate supplementation have not used a true placebo and thus cannot definitively attribute observed differences to NO₃⁻ *per se.*
**Is the NO₃⁻→NO₂⁻→NO pathway a viable therapeutic target for post-menopausal women?**

Relatively little is known about the effect of dietary NO₃⁻ supplementation on the NO₃⁻→NO₂⁻→NO pathway in older adults without overt chronic disease. Unfortunately, the capacity to increase plasma NO₂⁻ and NO via consumption of a diet high in nitrate appears limited in older adults. This is based on a 2012 study by Miller et al (Miller et al., 2012) which reported no effects of a high-nitrate diet (3 days) on plasma NO₂⁻ or biomarkers of NO in a group of healthy, normotensive older (72 ± 5 yr) women and men. This was in contrast to the large increase in these parameters observed in the same subjects after 3 days of nitrate supplementation with beetroot juice (consumed with breakfast). No sex differences were reported. This study by Miller et al suggests that targeting the NO₃⁻→NO₂⁻→NO pathway through dietary means, at least in older adults, may augment this pathway to a greater extent when given in the form of a concentrated supplement (e.g, beetroot juice) than increasing consumption of high nitrite containing food alone.

The dose of ingested nitrate needed to elicit physiologically significant effects in healthy older adults, and in particular older women, has not been established. Bondonno et al reported a linear, dose-dependent increase in plasma nitrate, nitrite and RXNO (marker of bioactive NO) in healthy, middle-aged women given four doses (0.1 to 0.4 gm) of a dietary nitrate supplement, but did not report any physiological outcomes (Bondonno et al., 2012). In the present studies we administered approximately twice their highest dose (0.6 g) and observed several physiologically significant effects, lowering central blood pressure at rest and attenuating blood pressure response during fatiguing exercise, providing preliminary support for the use of this (acute) dose in future studies on this population.

To date there have been no reports of any adverse events associated with acutely administering nitrate-rich beetroot juice to older adults. The lack of any such events (significant
hypotension, gastric discomfort, etc) following ingestion of a highly concentrated source of nitrate (0.6 g of nitrate in 140 ml of James White Beet-It organic juice) in the present group of postmenopausal women adds further support to this product’s safety profile. Oral supplementation of nitrate salts (potassium or sodium nitrate), by contrast, increase the risks of methemoglobinemia (potentially lethal) and formation of N-nitroso compounds (potentially carcinogenic in people with Barrett’s esophagus). Supplementation with nitrite (bypasses oral conversion) also carries these potential risks and leads to rapid and more dramatic drops in blood pressure and more common side effects (dizziness and headache). All of these potential risks are minimized with nitrate-rich beetroot juice which 1) has a much longer half-life (resulting in sustained release of nitrite limited by bacterial conversion on the tongue) than nitrite and 2) naturally contains high quantities of anti-oxidants, which limit the formation of nitrosamines in the GI tract.

An important question not addressed in the present dissertation relates to the safety and efficacy of chronic (daily) administration of this supplement in this population. Recent studies that have administered beetroot juice over several weeks (ranging from 1 to 4 weeks) in both younger adults (Lee et al., 2015; Vanhatalo et al., 2010a) and older patients (Eggebeen et al., 2016) have focused on blood pressure outcomes (resting). These studies suggest that daily consumption of nitrate-rich beetroot juice (i.e., range of 0.3-0.5 g/day) can result in a sustained reduction in resting (systolic) blood pressure. No adverse events were reported other than beeturia. Future investigations examining the chronic effects of this supplement on a range of cardiovascular and ergogenic outcomes, particularly in postmenopausal women, are clearly warranted.
Implications of cross-talk between NOS-dependent and NOS-independent pathways

Nitric oxide is a central biological mediator involved in the control of vascular tone, neurotransmission, cellular respiration, cell proliferation, and the immune response (Carlstrom et al., 2015). The endothelial production of NO via the L-arginine eNOS pathway is essential for maintaining cardiovascular homeostasis. However, the eNOS enzyme is 1) dependent on the availability of many co-factors (which can be deficient in the diet), 2) is oxygen-dependent and thus does not function well when oxygen tension declines to low levels (such as during heavy exercise and in hypoxic environments) and 3) decreases with advancing age (due to reduced expression and uncoupling) (Baker et al., 1999; Luque Contreras et al., 2006). Understanding how the newly discovered NOS-independent pathway ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO}$) interacts with the classic L-Arginine eNOS (NOS-independent) pathway, particularly in conditions where the eNOS pathway is acutely (e.g. ischemic exercise) or chronically (e.g. aging) impaired, is an important emerging question in this field.

The quantified oxygen tension at which the complementary nitrate-nitrite-NO pathway activity is enhanced more than the L-arginine-NO pathway has not been clearly defined. However, the contribution of the classic L-arginine-NO pathway and the effects of direct nitrite infusion on the systemic NO production at rest and at low oxygen tension have been studied to better understand the role of complementary nitrate-nitrite-NO pathway in NO production. For such studies, since direct measurement of NO \textit{in vivo} is extremely difficult, plasma nitrate and nitrite concentrations which are formed by oxidation of the NO (Moshage et al., 1995) and ironnitrosyl-hemoglobin which is formed by reaction of NO with deoxyhemoglobin (Dejam et al., 2004) were used as a measure of NO production. For the contribution of NOS-dependent NO production pathway to the systemic NO production, Kleinbongard et al. studied NOS knockout mice and reported that the circulating nitrite level decreased by approximately 70% in NOS
knockout mice compared to sham animals indicating the pronounced activation of NOS-dependent NO production pathway at rest (Kleinbongard et al., 2003). The same group also reported approximately 50% reduction in plasma nitrite concentration in young adults with endothelial dysfunction compared to the healthy control group confirming the greater contribution of the standard L-arginine NO production pathway in healthy human at rest (Kleinbongard et al., 2006). Those findings were accompanied by increases in vascular resistance. On the other hand, direct infusion of nitrite, both supra-(36 μmol/min) and near-physiologic (0.36 μmol/min) intravascular nitrite concentration, in the human forearm increased NO production measured with ironnitrosyl-hemoglobin and increased forearm blood flow before and during exercise, with or without NOS inhibition (Dejam et al., 2004). Additionally, it is clear that the NO produced at low oxygen tension is not NOS-dependent but nitrite-dependent (Bryan et al., 2004; Duranski et al., 2005; Zweier et al., 1995b). According to Cosby and colleagues, the conversion rate of NO from nitrite has a reciprocal relation with hemoglobin oxygen saturation, and the conversion is a hypoxia-regulated mechanism (Cosby et al., 2003). In summary, one can speculate that the nitrate-nitrite-NO pathway works in parallel with the L-arginine pathway to maintain the homeostatic level of NO and is enhanced in hypoxic/low oxygen tension conditions.

Does acute nitrate supplementation impact resting hemodynamics in postmenopausal women?

In Chapter 3 of this dissertation, the effect of dietary nitrate supplementation on central hemodynamics and arterial stiffness were examined.
Resting central and peripheral blood pressure

Previous findings have demonstrated the peripheral blood pressure lowering effects of dietary nitrate supplementation in various populations including healthy adults and different clinical populations (Bahra et al., 2012; Bailey et al., 2009; Hobbs et al., 2012; Kapil et al., 2010a; Kelly et al., 2013; Lansley et al., 2011a; Lansley et al., 2011b; Vanhatalo et al., 2011; Webb et al., 2008; Wylie et al., 2013). In an older population, approximately 5 and 3 mmHg reductions in peripheral SBP and DBP have been observed with nitrate-rich beetroot juice (Hughes et al., 2016; Kelly et al., 2013; Kenjale et al., 2011). Although the magnitude of reduction in SBP with dietary nitrate supplementation was slightly lower in this study compared to two other studies (5 vs. 3 mmHg), SBP was significantly reduced with an acute dose of dietary nitrate supplementation compared to the placebo in postmenopausal women.

Based on previous findings on peripheral BP lowering effects of the dietary nitrate supplementation, we hypothesized that central hemodynamics would be altered with an acute dose of beetroot juice in postmenopausal women. Currently, there is only one published study that investigated the effects of dietary nitrate supplementation on aortic BP in older adults (Hughes et al., 2016). The study reported approximately 7 (systolic) and 4 mmHg (diastolic) reductions in BPs 90 minutes following nitrate-rich beetroot juice consumption in older adults (Hughes et al., 2016) which are greater than the magnitude of our reductions in BPs, 5 (systolic) and 2 mmHg (diastolic). However, their older subjects were mostly men (9 of 12 were men) and also had higher baseline (pre-juice consumption) BP than our postmenopausal women (126 ± 12 vs. 115 ± 4). Regardless of the slightly lower BP reduction with dietary nitrate supplementation compared to the study by Hughes et al., congruent with our hypothesis, we found that an acute dose of beetroot juice significantly lowered aortic BP, as measured using a non-invasive pulse wave analysis device. Moreover, the change in BP from pre- to post-beetroot juice consumption
for the two treatment visits was directionally opposite where it was a negative value for the nitrate-rich beetroot juice visit, but positive for the nitrate-depleted beetroot juice visit. This is indicative of a powerful BP lowering effect of the nitrate supplementation.

In summary, an acute dose of dietary nitrate supplementation reduced both peripheral and central BP significantly compared to placebo. Similar to other studies that reported the effects of dietary nitrate supplementation on BP, greater effects on SBP were observed than on DBP and they led to a decrease in MAP.

Arterial stiffness and indices of aortic wave reflection

Arterial stiffness is defined by the functional and structural components related to the intrinsic elastic properties of the arteries (Amett et al., 1994). In study 1 (Chapter 3), it was hypothesized that increasing NO bioavailability with dietary nitrate supplementation would decrease smooth muscle tone and BP which would improve functional arterial stiffness (Dobrin, 1978; Neutel et al., 1992; O'Rourke et al., 1982; Steele, 1937). We expected that improved functional arterial stiffness would lead to normalization of the age-associated increase in central arterial stiffness. As mentioned in previous paragraph, we successfully lowered central and peripheral BP with an acute dose of dietary nitrate supplementation which indicates direct vasodilation effects of the supplementation. However, no effect of dietary nitrate supplementation on central arterial stiffness was observed in study 1 (Chapter 3). This can be explained, at least in part, with the dosage of the dietary nitrate supplementation in study 1. As there are other factors that influence PWV such as wall thickness and blood density (Gribbin et al., 1979) that are almost impossible to alter with a single dose of any intervention, even with the significant reduction in blood pressures, the acute dose of dietary nitrate supplementation may have not been enough to exert any detectable alterations in PWV. Given that the intervention involved only one acute
dose, significant structural changes that can potentially have a greater impact on arterial stiffness were not expected. Also, as the data in study 1 suggest, the impact of an acute dose of dietary nitrate supplementation on functional stiffness may have not been enough to cause a detectable change in PWV.

Augmentation index (Ai) is a measure of augmentation in aortic BP is an accepted measure of aortic reflection and arterial stiffness (Murgo et al., 1980). When arterial stiffness increases, the reflected wave arrives in the aorta prematurely leading to an increase in aortic BP and therefore an increase in Ai. Cardiac wasted energy is an indication of extra effort sustained by the left ventricle due to augmented aortic pressure. It is well documented that advancing age is associated with increase in Ai and cardiac wasted energy and we were able to confirm that with our pre- and post-menopausal women comparisons for these variables. Then, we expected to observe a decrease in Ai, and therefore cardiac wasted energy with dietary nitrate supplementation. We also found no effect of nitrate rich beetroot juice on augmentation index and cardiac wasted energy, which can partially be explained by the absence of observed alterations in arterial stiffness and the acute dose of the dietary nitrate supplementation.

In 2014, Rammos et al. reported that a 4-week long sodium nitrate intervention lowered PWV and Ai in older adults (Rammos et al., 2014). In addition, a clinical trial with a 4-week long nitrate-rich beetroot juice supplementation in hypertensives showed significant reduction in PWV (Kapil et al., 2015). Similarly, a 4-week dietary nitrate supplementation reduced indices of aortic wave reflection in normotensive and hypertensive older adults (Kapil et al., 2015; Rammos et al., 2014). In an animal study, a 3-week sodium nitrite treatment reduced PWV in older mice compared to the control group and reversed the age-associated increase in PWV (Sindler et al., 2014). Although they are a different type of supplementation (pill vs. nitrate-rich beetroot juice) and a different population (hypertensives, older adults vs. postmenopausal women; animal vs. human model), those studies strongly suggest that chronic dietary nitrate supplementation rather
than an acute dose would improve arterial stiffness and indices of aortic wave reflection in postmenopausal women.

Taken together, it would appear that larger studies examining the effects of short term or chronic supplementation of nitrate-rich beetroot juice on 1) arterial stiffness, 2) reflected wave characteristics and 3) left ventricular effort are warranted in this high risk population. This will allow us to more confidently determine the potential benefits of dietary nitrate supplementation as well as the potential dose required for beetroot juice to be used as an arterial de-stiffening therapy.

**Does acute nitrate supplementation impact progressive ischemic exercise tolerance and BP response in postmenopausal women?**

In Chapter 4 (study 2) and 5 (study 3) of this dissertation, the effects of dietary nitrate supplementation on exercise tolerance and BP response to exercise were examined.

**Rationale for progressive ischemic exercise model**

For the study 2 and 3, intermittent handgrip exercise at a low work intensity (10% of peak grip strength), coupled with gradual increments in cuff pressure (20 mmHg/min) was chosen to elicit a more gradual engagement of the exercise pressor reflex than is typically used (i.e., 2 min static hold). Also, post exercise circulatory arrest (PECA) was followed after the handgrip exercise in an effort to isolate the contribution of the muscle metaboreflex.

In addition, by using an experimental model that progressively limits blood flow to exercising muscle up to the point of fatigue, ischemic condition is induced to maximize the conversion of nitrite to nitric oxide via the nitrate-nitrite-NO pathway (Lundberg et al., 2008), and we tested whether acute nitrate supplementation attenuates the systemic BP responses during
progressive metaboreflex activation (graded cuff inflation) and isolation (PECA) in healthy post-menopausal women.

**Effects of nitrate supplementation on exercise tolerance**

The study presented in Chapter 4 of this dissertation investigated the effects of dietary nitrate supplementation on exercise tolerance by examining rating of perceived exertion and time to exhaustion during progressive ischemic handgrip exercise. Analysis revealed that an acute dose of nitrate-rich beetroot juice delayed initial changes in the rating of perceived exertion during progressive ischemic handgrip exercise. Based on previous dietary nitrate supplementation studies, we hypothesized that the dietary nitrate supplementation would increase NO bioavailability and result in a direct vasodilation in the microvasculature which would improve perfusion to the working muscle and exercise tolerance. Unfortunately, our attempt to measure indirect microvascular vasodilation with NIRS was unsuccessful; therefore, we cannot identify the mechanism of the observed delay in the rating of perceived exertion change.

Nitrate-rich beetroot juice failed to increase the time to exhaustion in postmenopausal women during progressive ischemic handgrip exercise. However, during the graded occlusion period, more subjects reached zero flow on the nitrate-rich beetroot juice visit than on the placebo beetroot juice visit (5 of 9 subjects vs. 3 of 9 subjects). The lack of significance is most likely due to the small sample size studied. Moreover, the association between physiological alterations that occur in the contracting muscle and the microvasculature of the active limb that may cause a change in perceived effort and exercise tolerance remains elusive.
Effects of nitrate supplementation on BP response to progressive ischemic exercise

In Chapter 5 of this dissertation, the effect of nitrate-rich beetroot juice on the metaboreflex mediated blood pressure response to progressive ischemic exercise was investigated. An acute dose of nitrate-rich beetroot juice reduced the MAP response to progressive ischemic handgrip exercise at fatigue when it was compared to nitrate-depleted beetroot juice in postmenopausal women. The smaller increase in MAP with nitrate-rich beetroot juice at fatigue was most likely a result of the decreased magnitude of DBP changes, not the components of the exercise pressor reflex. The components of the exercise pressor reflex were not significantly altered with dietary nitrate supplementation as compared to the nitrate-depleted beetroot juice visit. These findings suggest a mild, but persistent systemic vasodilator effect of acute nitrate supplementation in post-menopausal women that does not appear to alter their exercise pressor reflex per se, but may reduce their reliance on increases in TPR during intense stimulation of skeletal muscle afferents.

The lack of significance of components of the exercise pressor response with nitrate rich beetroot juice was unexpected. Based on the previous data, we hypothesized that dietary nitrate supplementation would increase NO bioavailability and cause vasodilation in microvasculature reducing the magnitude of increase in total peripheral resistance. Total peripheral resistance was not different at fatigue and during post exercise circulatory arrest. This could be due to a small sample size and/or due to the acute dose of the intervention. Therefore, larger scale, longer intervention studies are needed to investigate the possible mechanism of the beneficial effects on the blood pressure response to exercise. Another consideration was our reliance on finger blood pressure waveform analysis (Beatscope/Modelflow) to estimate stroke volume/cardiac output and TPR. While also limited with respect to absolute values, this is a commonly used method in the muscle metaboreflex literature (multiple of peer-reviewed publications) that closely tracks
changes in these parameters. Future studies should, nonetheless, include absolute measures of ventricular volumes and derive TPR based on the absolute ventricular volume. A final limitation was our inability to fully utilize near-infrared spectroscopy (NIRS) to assess intramuscular pH or oxygenation during graded cuff ischemia. We were only able to collect the index of muscle oxygenation in some subjects (n=4) due to technical difficulties with NIRS. However, even in the subset of subjects (n=4), no treatment effect on tissue saturation index (TSI, %) which is an index of muscle oxygenation measured with NIRS (Portamon; Artinis) was observed (all p > 0.05).

Physiological mechanisms

Although the specific mechanism has not been identified, a review by Jones and other studies suggests two possible mechanisms for the exercise performance enhancement and exercise tolerance improvement with nitrate supplementation (Bailey et al., 2010; Jones et al., 2013; Larsen et al., 2011): improving the efficiency of 1) muscle contraction efficiency (Bailey et al., 2010) and 2) mitochondrial oxidative phosphorylation (Larsen et al., 2011).

Bailey et al. reported that chronic (6-days) dose of dietary nitrate supplementation with beetroot juice lowered estimated ATP turnover rates from PCr hydrolysis and oxidative phosphorylation along with no change in the estimated ATP turnover rates from anaerobic glycolysis (Bailey et al., 2010). Therefore, total ATP turnover rates were reduced during both low- and high-intensity exercise (Bailey et al., 2010) suggesting a lower ATP cost of muscle contraction for the same force production (i.e., improved muscle contractile efficiency). In addition, the intramuscular accumulation of ADP and Pi and the extent of PCr depletion were attenuated with nitrate supplementation (Bailey et al., 2010). Since accumulation of ADP, Pi and PCr depletion induces muscle fatigue (Allen et al., 2008), the attenuation and the delay in the time taken to reach critical concentrations of ADP, Pi, and PCr depletion with nitrate
supplementation may explain the observed improvement in exercise tolerance. Additionally, one can speculate that the microvasculature vasodilation increases perfusion to the exercising forearm, increasing driving pressure of oxygen (Ferreira et al., 2006) which may result in less PCr breakdown, and eventually increasing exercise tolerance (Haseler et al., 1998; Vanhatalo et al., 2010b).

Additionally, nitrate supplementation has been shown to improve mitochondrial efficiency (Larsen et al., 2011). In the study by Larsen et al. treated isolated mitochondria from vastus lateralis muscle of healthy human with sodium nitrate reduced proton leakage and uncoupled respiration increasing mitochondrial P/O ratio (Larsen et al., 2011). Similarly, NO has been shown to reduce slippage of the mitochondrial proton pump (Clerc et al., 2007) and receive electrons at the end of the electron transport chain instead of oxygen (Basu et al., 2008) leading to improvement of mitochondrial efficiency. Improvement in mitochondrial efficiency would be the most plausible mechanism for the beneficial effect of nitrate supplementation during complete ischemic exercise (zero flow) in study 2.

**Future directions**

In the future, a chronic dose study should be conducted to determine the potential benefits of dietary nitrate supplementation on central arterial stiffening and on blood pressure response to exercise in postmenopausal women. Numerous animal and human studies have shown arterial de-stiffening effects with chronic dose of dietary nitrate supplementation (Fleenor et al., 2013; Kapil et al., 2015; Rammoss et al., 2014; Sindler et al., 2014). A chronic dose of the dietary nitrate supplementation not only de-stiffened arteries of human and animals, but also lowered BP and TPR during progressive cycling exercise in healthy young men (15-days; (Lee et
Therefore, studies examining the effects of a chronic dose of dietary nitrate supplementation on 1) arterial stiffness and reflected wave characteristics and 2) blood pressure response to exercise are warranted in this high risk population, postmenopausal women. This will allow us to more confidently determine the potential benefits of dietary nitrate supplementation on resting and exercising hemodynamics as well as the dose required for beetroot juice to be used as an arterial de-stiffening therapy.

In addition to the chronic intervention studies, conducting clinical studies to explore whether the beneficial effects of dietary nitrate supplementation can be extended to and benefit different clinical population is also suggested. Based on the observations from this study, a patient population with prolonged anoxic or ischemic condition such as peripheral arterial diseases, digital ischemia, and even coronary heart disease patients have a great potential to derive the beneficial effects of dietary nitrate supplementation. Moreover, the potential to prevent ischemic damage to function and structure of vessels can be studied more in depth to with the findings being applicable to surgical procedures that involve prolonged blood flow occlusion of limbs such as joint replacement.

Studies investigating the mechanisms for the cardiovascular benefits of the supplement in postmenopausal women can also be conducted. First of all, a non-invasive mitochondrial function test using NIRS (Ryan et al., 2013; Ryan et al., 2012) can be utilized in future studies to test our hypothesis - dietary nitrate supplementation will improve mitochondrial efficiency in the ischemic muscle. Alongside the non-invasive human studies, in vitro studies that measure the P/O ratio of isolated mitochondria from older female animals after a chronic dose of the supplement can be conducted to elucidate the mechanism by which the potential therapeutic effects of the supplement is exerted.
Summary

This series of studies have advanced our understanding of non-pharmacological nitrate supplementation in the context of cardiovascular aging in women. We observed acute benefits of dietary nitrate supplementation on resting central blood pressure, time to change in perceived exertion, and blood pressure responses at fatigue during progressive ischemic handgrip exercise. Collectively, these findings suggest that there are potential beneficial effects of dietary nitrate supplementation for vascular aging and NO-mediated responses in postmenopausal women. These effects of dietary nitrate supplementation such as reducing central blood pressure, delaying threshold for initial perceived exertion change, and reducing the magnitude of ischemic exercise mediated increase in blood pressure at fatigue in postmenopausal women provide evidence of a beneficial cardiovascular effects of the supplementation. Although the mechanisms by which the beneficial effects are exerted remain unclear, studies in this dissertation warrant continued investigation of the cardiovascular effects of dietary nitrate supplementation in post-menopausal women, including those with overt cardiovascular disease.


Appendix A

INFORMED CONSENT

Informed Consent Form for Biomedical Research
The Pennsylvania State University

Title of Project: Acute blood pressure-lowering effects of beetroot juice in postmenopausal women with and without hypertension

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1. Purpose of the study:

Women are more likely to develop high blood pressure (e.g., hypertension) after menopause. Postmenopausal women also experience greater increases in blood pressure in response to physical stressors such as exercise compared with either premenopausal women or men of a similar age. The purpose of this research study is to test the ability of an acute (one-time) dose of beetroot juice to lower resting blood pressure as well as blood pressure responses to exercise in postmenopausal (55-80 years) women with and without hypertension and to compare their results to those obtained from premenopausal women (18-35 years).

2. Procedures to be followed:

The study and its procedures are outlined below
Note: If you are a premenopausal woman, you will not participate in the beetroot juice portion of this study (Postmenopausal visits 3 & 4).

(your initials)

If you are a premenopausal woman, you will be asked to complete the following:

Visit 1 - Screening/Familiarization Visit.

You should also report to this visit in a fasted state without consuming food (except water) for 12 hours prior to arrival. After you check in, a blood sample from a vein in your arm will be taken by a Clinical Research Center (CRC) nurse to measure your lipid profile, blood chemistry, and the number of cells in your blood. Following the blood sample, you will be given a small snack (e.g., granola bar or other carbohydrate food, with water or juice) to help reduce feelings of hunger

(your initials)

You should report to this visit without consuming caffeine for 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival.

(your initials)

During this visit, you will complete a health history and physical activity/fitness questionnaire. Your height, weight, as well as resting heart rate and resting blood pressure will be measured by a trained member of the Vascular Aging and Exercise Laboratory. You will then perform a submaximal (less than “all-out” effort) exercise test that will estimate your aerobic fitness. During this test, you will be asked to step on and off of a low (30 cm or approximately 1 foot high) box for 5 minutes. A metronome will help you to step on and off the box at a set pace. Throughout this test, researchers will measure your heart rate with monitor that straps around your chest. After you complete the exercise test, you will be familiarized with the study protocol and the measurements that will be performed during your subsequent study visits.

(your initials)

Urine Collection: In order for your menstrual cycle hormones to be measured, you will be asked to collect a urine sample every morning for one complete menstrual cycle. During your screening visit, you will receive written and verbal instructions for collecting, storing, and recording urine samples. You will collect urine every morning using a small urine cup that is provided by the lab. You will store urine samples in a freezer, then transport the urine to the lab using supplies that the lab will provide. You will begin urine collection on the first day of your first period following your general screening visit and collect a sample daily until the first day of the following menstrual period. You will keep track of menses and urine collection times on a daily menstrual and urine calendar provided by the
lab. Collection, processing, and recording of urine collection will take about 5 minutes per day.

______  (your initials)

Visit 2 - Baseline Study Visit.

You should report to this visit in a fasted state, without consuming food or caffeine 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival. Upon arrival, you will check in at the CRC in Noll Laboratory, and a CRC nurse will take a 16 mL (~1 tbsp.) blood sample from a vein in your arm. This sample will be used to determine the amount of nitrate and nitrite (by-products of nitric oxide, a naturally occurring substance in your body that causes blood vessels to widen) and estradiol (estrogen) in your blood. Following the blood sample, you will be given a small snack (e.g., granola bar or other carbohydrate food, with water or juice) to help reduce feelings of hunger.

______  (your initials)

A urine pregnancy test will be provided to you by a CRC nurse before the blood sample is taken during this visit. If the pregnancy test reading is negative, you will provide the blood sample and continue with the remainder of the experiments performed in this study visit. However, if the pregnancy test is positive reading, you will not provide a blood sample or continue with the remainder of the study visit, and we will ask that you schedule an appointment with a physician (Ob/Gyn) to confirm the positive test.

______  (your initials)

Next you will be escorted to the Vascular Aging and Exercise Laboratory (201 Noll Lab), where the following procedures will be performed:

**Heart Rate.** Heart rate will be measured by placing three sticky electrodes on your chest and reading the electrocardiogram (ECG) signal. A small-inflatable cuff will also be placed around a finger on your hand to measure changes in your pulse detected at your fingertip. After the electrodes and the finger cuff are applied to you, you will be asked to lie flat on a bed and rest for 20 minutes while your heart rate and finger pulse are recorded.

______  (your initials)

**Pulse Wave Velocity** Pulse wave velocity is a non-invasive measurement that allows us to estimate the “stiffness” of your blood vessels. You will lay flat on a bed with a blood pressure cuff placed around your femoral artery (a blood vessel in your thigh), while a
researcher holds a small (pen-sized) sensor over your carotid artery (a blood vessel in your neck). To make a measurement, the blood pressure cuff placed on your thigh will inflate to a pressure that temporarily prevents blood flow into your leg. The cuff will then deflate over 1-2 minutes, while sensors in the cuff and on your neck measure how fast each pulse of blood travels through your blood vessels. This measurement will be performed 3-4 times.

\[\text{(your initials)}\]

**Resting Blood Pressure.** Your resting blood pressure will be measured by an automated blood pressure machine. An inflatable cuff will be placed on your upper arm, and you will be asked to sit quietly in a chair for at least 5 minutes. The cuff will then be inflated to a pressure that prevents blood from flowing into your forearm. As soon as flow into your forearm is stopped, the cuff will gradually deflate over 1-2 minutes. As the cuff deflates sensors inside of the cuff will detect the blood pressure in your upper arm, and the machine will use a validated equation to also calculate the blood pressure in the artery that leaves your heart (i.e., aorta). This measurement will be performed 3-4 times.

\[\text{(your initials)}\]

**Leg Suction.** You will lie on a bed, and your leg will be placed inside of a closed box up to the middle of your thigh. A sleeve made from a stretchy material (i.e., neoprene) will help to seal your leg inside of the box in a way that provides a snug fit, but does not constrict your leg. Once your leg is sealed inside of the box, you will lie quietly for 5 minutes. Next, a vacuum will apply up to 4 different levels of suction to your leg inside the box for one minute at a time. The amounts of suction used will increase the pressure inside the blood vessels of your leg to similar pressures experienced when you stand up. There will be a 2-4 minute rest period between each level of suction. Each level of suction will be applied between 2-4 times. Some of these times we will measure the amount of blood flowing into your leg inside the box. Some of these times we may measure the amount blood flowing into your opposite leg (i.e., the one not inside the box). The amount of blood flowing into your leg(s) will be measured using a Doppler ultrasound machine that produces sound waves to measure the size of your blood vessel and the speed of your blood. Throughout this procedure, your blood pressure will be monitored with a small, inflatable finger cuff. The information collected from this procedure will allow us to estimate how much your vessels constrict (i.e., get smaller) when they are stretched.

\[\text{(your initials)}\]

**Static Handgrip Exercise.** First, we will place a blood pressure cuff on your upper arm and forearm. The cuff on your upper arm will be inflated to above systolic blood pressure. You will rest with the cuff inflated for a period of 2 minutes, after which the cuff on your forearm will rhythmically inflate and deflate at a rate of 30 inflations/min for a period of 1 minute (upper arm cuff to remain inflated) or your wrist will be flexed and extended by a member of the Vascular Aging lab. After this 3 minute period, the upper arm cuff will be deflated and you will be given a 10-15 minute resting period. Upon completion of the rest period, you will squeeze a handgrip device as hard as you
can (for 1-3 seconds) to determine your maximal grip strength. Your maximal grip strength will be determined as the highest value recorded over three such maximal efforts, each separated by a 1-minute rest period. A sticky plastic sensor will be placed over the muscles in your forearm. This sensor will emit light into your forearm muscles, and measure the amount of oxygen and hydrogen ions (H+, a byproduct of lactic acid) in your muscle tissue. A blood pressure cuff will also be placed on your upper arm and forearm. After sitting quietly for 10 minutes, you will begin to squeeze the handgrip device intermittently at a rate of 30 contractions per minute at a low force (10-20% of your maximal grip strength). You will perform this intermittent exercise twice. The first trial will be performed without the blood pressure cuff inflated and will continue until you report a rate of perceived exertion value of 19 or 20 (on a scale of 20). During the second course of this intermittent exercise, the blood pressure cuff on your arm will be gradually inflated (tightened by approximately 12 millimeters of cuff pressure each minute). The exercise period will stop when you report a rate of perceived exertion value of 19 or 20 (on a scale of 20) or when there is a >90% reduction in blood flow through your arm. During the last five seconds of exercise in both trials, the blood pressure cuff on your upper arm will be inflated to above systolic blood pressure for 3 minutes. During the last minute of this 3 minute period, the blood pressure cuff on your forearm will be rhythmically inflated and deflated for a 1 minute period or your wrist will be flexed and extended by a member of the Vascular Aging lab. Throughout this procedure, your blood pressure will be monitored with a small, inflatable finger cuff.

_____

(your initials)

**Dynamic Leg Exercise.** For this measurement, you will be seated in a supine (i.e., reclined) position. Your left leg will be placed inside of a boot that is attached to the pedal arm of a stationary bicycle, and a blood pressure cuff will be placed on your upper thigh. A sticky plastic sensor will also be placed over a muscle in your thigh to measure the amount of oxygen and hydrogen ions in your leg muscle. After a 10-minute rest period, the blood pressure cuff on your upper thigh will be inflated to a pressure that prevents blood from flowing into or out of your leg, and a researcher will move your lower leg for you in a kicking motion (i.e., passive exercise) for up to 3 minutes. The blood pressure cuff will then be deflated, and you will rest for another 10 minutes. You will then perform voluntary (i.e., active) single-leg knee extensions for 9 minutes. For the first 3 minutes you will perform knee extensions with no resistance. For the last 6 minutes, you will perform knee extensions against a moderate resistance. At the end of the 9-minute exercise bout, the cuff on your upper thigh will re-inflate to a pressure that prevents blood from flowing into or out of your leg for 3 minutes. A researcher will move your lower leg for you in a kicking motion during the last 1 minute of leg occlusion. Throughout this procedure, the amount of blood flowing into your leg will be measured with a Doppler ultrasound machine, and your blood pressure will be monitored with a small, inflatable finger cuff.

_____

(your initials)

**Visit 3 – Estradiol assessment.**

Prior to your second experimental visit (Premenopausal visit 4), you will be asked to return to the lab on one day between days 9-11 of your menstrual cycle for a brief blood
draw. Approximately 10 ml of blood will be taken from an arm vein during this visit. This blood draw will be used to assess the amount of estrogen present in your blood on that day. The results of this test will be used to schedule your second experimental visit (visit 4).

(Your initials)

Visit 4 – Experimental Study Visit.

You should report to this visit in a fasted state, without consuming food or caffeine for 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival. During days 10-14 of your menstrual cycle, you will return to the CRC to repeat the same procedures as you did during visit 2. Procedures and timeline will be identical to those during visit 2.

(Your initials)

If you are a postmenopausal woman, you will be asked to complete the following:

Visit 1 – Screening/Familiarization Visit.

You will bring a copy of your most recent blood test results (lipids, glucose, red blood cell count, etc.) provided to you by your primary care physician. If your most recent blood test indicates a blood glucose level between 110 and 125 mg/dl we will perform a blood draw to determine the average glucose in your blood over a 3 month period (HbA1c). Your willingness to provide this information is voluntary, and all information will be kept confidential.

(Your initials)

You should report to this visit without consuming caffeine for 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival.

(Your initials)

During this visit, you will complete a health history and physical activity/fitness questionnaire. Your height, weight, as well as resting heart rate and resting blood pressure will be measured by a trained member of the Vascular Aging and Exercise Laboratory. You will then perform a submaximal (less than “all-out” effort) exercise test that will estimate your aerobic fitness. During this test, you will be asked to step on and off of a low (30 cm or approximately 1 foot high) box for 5 minutes. A metronome will help you to step on and off the box at a set pace. Throughout this test, researchers will measure your heart rate with monitor that straps around your chest. After you complete the exercise test, you will be familiarized with the study protocol and the measurements that will be
performed during your subsequent study visits.  

_____ (your initials)

Urine Collection: In order for menstrual cycle hormone exposure to be measured, you will be asked to collect a urine sample every morning for one 30-day monitoring period. During your screening visit, you will receive written and verbal instructions for collecting, storing, and recording urine samples. You will collect urine every morning using a small urine cup that is provided by the lab. You will store urine samples in a freezer, then transport the urine to the lab using supplies that the lab will provide. You will begin urine collection on a random day, designated by the lab, and collect daily for 30 days. You will keep track of urine collection times on a daily menstrual and urine calendar provided by the lab. Collection, processing, and recording of urine collection will take about 5 minutes per day.

_____ (your initials)

Visit 2 - Baseline Study Visit.

You should report to this visit in a fasted state, without consuming food or caffeine 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival. Upon arrival, you will check in at the CRC in Noll Laboratory, and a CRC nurse will take a 16 mL (~1 tbsp.) blood sample from a vein in your arm. This sample will be used to determine the amount of nitrate and nitrite (by-products of nitric oxide, a naturally occurring substance in your body that causes blood vessels to widen) and estradiol (estrogen) in your blood. Following the blood sample, you will be given a small snack (e.g., granola bar or other carbohydrate food, with water or juice) to help reduce feelings of hunger.  

_____ (your initials)

Next you will be escorted to the Vascular Aging and Exercise Laboratory (201 Noll Lab), where the following procedures will be performed:

**Heart Rate.** Heart rate will be measured by placing three sticky electrodes on your chest and reading the electrocardiogram (ECG) signal. A small-inflatable cuff will also be placed around a finger on your hand to measure changes in your pulse detected at your fingertip. After the electrodes and the finger cuff are applied to you, you will be asked to lie flat on a bed and rest for 20 minutes while your heart rate and finger pulse are recorded.  

_____ (your initials)

**Pulse Wave Velocity** Pulse wave velocity is a non-invasive measurement that allows us to estimate the “stiffness” of your blood vessels. You will lay flat on a bed with a blood pressure cuff placed around your femoral artery (a blood vessel in your thigh), while a researcher holds a small (pen-sized) sensor over your carotid artery (a blood vessel in your neck). To make a measurement, the blood pressure cuff placed on your thigh will
inflate to a pressure that temporarily prevents blood flow into your leg. The cuff will then deflate over 1-2 minutes, while sensors in the cuff and on your neck measure how fast each pulse of blood travels through your blood vessels. This measurement will be performed 3-4 times.

_____ (your initials)

**Resting Blood Pressure.** Your resting blood pressure will be measured by an automated blood pressure machine. An inflatable cuff will be placed on your upper arm, and you will be asked to sit quietly in a chair for at least 5 minutes. The cuff will then be inflated to a pressure that prevents blood from flowing into your forearm. As soon as flow into your forearm is stopped, the cuff will gradually deflate over 1-2 minutes. As the cuff deflates sensors inside of the cuff will detect the blood pressure in your upper arm, and the machine will use a validated equation to also calculate the blood pressure in the artery that leaves your heart (i.e, aorta). This measurement will be performed 3-4 times.

_____ (your initials)

**Leg Suction.** You will lie on a bed, and your leg will be placed inside of a closed box up to the middle of your thigh. A sleeve made from a stretchy material (i.e., neoprene) will help to seal your leg inside of the box in a way that provides a snug fit, but does not constrict your leg. Once your leg is sealed inside of the box, you will lie quietly for 5 minutes. Next, a vacuum will apply up to 4 different levels of suction to your leg inside the box for one minute at a time. The amounts of suction used will increase the pressure inside the blood vessels of your leg to similar pressures experienced when you stand up. There will be a 2-4 minute rest period between each level of suction. Each level of suction will be applied between 2-4 times. Some of these times we will measure the amount of blood flowing into your leg inside the box. Some of these times we may measure the amount blood flowing into your opposite leg (i.e., the one not inside the box). The amount of blood flowing into your leg(s) will be measured using a Doppler ultrasound machine that produces sound waves to measure the size of your blood vessel and the speed of your blood. Throughout this procedure, your blood pressure will be monitored with a small, inflatable finger cuff. The information collected from this procedure will allow us to estimate how much your vessels constrict (i.e., get smaller) when they are stretched.

_____ (your initials)

**Static Handgrip Exercise.** First, we will place a blood pressure cuff on your upper arm and forearm. The cuff on your upper arm will be inflated to above systolic blood pressure. You will rest with the cuff inflated for a period of 2 minutes, after which the cuff on your forearm will rhythmically inflate and deflate at a rate of 30 inflations/min for a period of 1 minute (upper arm cuff to remain inflated) or your wrist will be flexed and extended by a member of the Vascular Aging lab. After this 3 minute period, the upper arm cuff will be deflated and you will be given a 10-15 minute resting period. Upon completion of the rest period, you will squeeze a handgrip device as hard as you can (for 1-3 seconds) to determine your maximal grip strength. Your maximal grip strength will be determined as the highest value recorded over three such maximal
efforts, each separated by a 1-minute rest period. A sticky plastic sensor will be placed over the muscles in your forearm. This sensor will emit light into your forearm muscles, and measure the amount of oxygen and hydrogen ions (H\(^+\), a byproduct of lactic acid) in your muscle tissue. A blood pressure cuff will also be placed on your upper arm and forearm. After sitting quietly for 10 minutes, you will begin to squeeze the handgrip device intermittently at a rate of 30 contractions per minute at a low force (10-20% of your maximal grip strength). You will perform this intermittent exercise twice. The first trial will be performed without the blood pressure cuff inflated and will continue until you report a rate of perceived exertion value of 19 or 20 (on a scale of 20). During the second course of this intermittent exercise, the blood pressure cuff on your arm will be gradually inflated (tightened by approximately 12 millimeters of cuff pressure each minute). The exercise period will stop when you report a rate of perceived exertion value of 19 or 20 (on a scale of 20) or when there is a >90% reduction in blood flow through your arm. During the last five seconds of exercise in both trials, the blood pressure cuff on your upper arm will be inflated to above systolic blood pressure for 3 minutes. During the last minute of this 3 minute period, the blood pressure cuff on your forearm will be rhythmically inflated and deflated for a 1 minute period or your wrist will be flexed and extended by a member of the Vascular Aging lab. Throughout this procedure, your blood pressure will be monitored with a small, inflatable finger cuff. 

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**Dynamic Leg Exercise.** For this measurement, you will be seated in a supine (i.e., reclined) position. Your left leg will be placed inside of a boot that is attached to the pedal arm of a stationary bicycle, and a blood pressure cuff will be placed on your upper thigh. A sticky plastic sensor will also be placed over a muscle in your thigh to measure the amount of oxygen and hydrogen ions in your leg muscle. After a 10-minute rest period, the blood pressure cuff on your upper thigh will be inflated to a pressure that prevents blood from flowing into or out of your leg, and a researcher will move your lower leg for you in a kicking motion (i.e., passive exercise) for up to 3 minutes. The blood pressure cuff will then be deflated, and you will rest for another 10 minutes. You will then perform voluntary (i.e., active) single-leg knee extensions for 9 minutes. For the first 3 minutes you will perform knee extensions with no resistance. For the last 6 minutes, you will perform knee extensions against a moderate resistance. At the end of the 9-minute exercise bout, the cuff on your upper thigh will re-inflate to a pressure that prevents blood from flowing into or out of your leg for 3 minutes. A researcher will move your lower leg for you in a kicking motion during the last 1 minute of leg occlusion. Throughout this procedure, the amount of blood flowing into your leg will be measured with a Doppler ultrasound machine, and your blood pressure will be monitored with a small, inflatable finger cuff. 

_initials_

**Visit 3 - Experimental Study Visit.**

You should report to this visit in a fasted state, without consuming food or caffeine for 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival. Upon arrival, you will check
in at the CRC in Noll Laboratory, and a CRC nurse will take a 16 mL (~1 tbsp.) blood sample from the vein in your arm. This sample will be used to determine the amount of nitrate and nitrite (by-products of nitric oxide, a naturally occurring substance in your body that causes blood vessels to widen) and estradiol (estrogen) in your blood. You will then be escorted to the Vascular Aging and Exercise Laboratory (201 Noll Lab), where the following procedures will be performed:

1. Heart Rate
2. Pulse Wave Velocity
3. Resting Blood Pressure

Next, you will be asked to consume 140 mL (9.5 tablespoons) of either nitrate-rich beet juice (known as the “active” drink) or beet juice with nitrates removed (known as the “placebo” drink). You will not be able to tell which drink you are consuming. You will also be provided a small snack (e.g., granola bar or other carbohydrate food, with water or juice) to help reduce feelings of hunger. You will then be asked to wait in the CRC or Noll lab for approximately 1.5 hours to allow time for the active (or placebo) drink to be fully digested and absorbed. During these 1.5 hours, you will be able to use a personal laptop computer or other portable electronic device. A second (6 mL) blood sample will then be collected after 1.5 hours, to determine if there is an increase in nitrate or its by-products in your blood after consuming the beet juice. You will then return to the Vascular Aging and Exercise Laboratory where the following procedures will be performed.

1. Heart Rate
2. Pulse Wave Velocity
3. Resting Blood Pressure
4. Leg Suction
5. Static Handgrip Exercise
6. Dynamic Leg Exercise

When you complete these procedures, a third (6 mL) blood sample will be taken to determine how well the nitrates and nitrites remain elevated in your blood.

**Visit 4 - Experimental Study Visit.**

You should report to this visit in a fasted state, without consuming food or caffeine for 12 hours prior to arrival. You should not consume any alcohol or dietary supplements for 48 hours prior, or participate in any exercise workouts (e.g., weight lifting or sustained aerobic exercise > 15 minutes) for 24 hours prior to arrival. At least 5 days after visit 3, you will return to the CRC to repeat the same procedures as you did during visit 3. Procedures and timeline will be identical to those during visit 3 except that you will consume whichever drink supplement you did not receive during visit 3 i.e., the active (nitrate-containing) drink or the placebo (nitrate-removed) drink. The order of these two drink supplements will be randomly determined for each participant.
3. Discomforts and risks:

**Blood Sampling.** You may experience some mild discomfort and bruising where the needle is inserted into your arm. This discomfort often goes away once the needle is securely placed in your vein. There is also a very small risk of developing an infection or a clot in your arm. However, these risks are minimized by having a trained nurse perform all blood samples using sterile equipment and techniques.

(your initials)

**Step-based Fitness Test.** This step-based test is designed to provide an exercise intensity of sub-maximal (i.e., less than an “all-out”) effort. However, you may still experience some discomfort with the exercise test such as muscle fatigue, shortness of breath, or get a muscle cramp. You may also experience lightheadedness, chest discomfort, or irregular heartbeats during this test. There is also a small risk of stumbling and/or falling during the test. To minimize this risk, researchers will monitor you closely throughout the test, and will stand nearby to provide support if you lose your balance.

(your initials)

**Heart Rate.** It is possible that the adhesive on the sticky electrodes may irritate your skin. There is also a minimal risk that an allergic reaction could occur from the adhesive.

(your initials)

**Blood Pressure/Pulse Wave Velocity.** There is a risk of temporary discomfort at the sites where blood pressure cuffs are inflated (upper arm/upper thigh). The discomfort might be greater the longer the cuffs are inflated. In addition, you may feel a numb and/or tingling sensation in your hands/feet while the cuff is inflated; however, these feelings go away quickly after the cuff is deflated. During the pulse wave velocity test, you may also experience some temporary discomfort while the researcher holds a small sensor over an artery in your neck. However, this feeling will also go away quickly after the sensor is removed.

(your initials)

**Leg Suction.** You may feel some mild discomfort associated with the leg suction procedure. A vacuum applied to the sealed box will cause blood vessels in your leg to fill with more blood than usual. Thus, you may feel temporary sensations of swelling in your leg. There is also a small risk that you may experience temporary feelings of lightheadedness. The vacuum will tend to pull your leg into the box, and as a result you may feel a moderate pressure pushing against your upper thigh. Any of these feelings will go away immediately after the vacuum is turned off. Moreover, the potential for these risks or discomforts to occur will be minimized by limiting bouts of suction to a short duration (1 min each).

(your initials)
**Doppler Ultrasound.** There is a minimal risk that the ultrasound probe and/or gel will irritate your skin. You may feel minor discomfort (pressure) when the researcher is pressing the ultrasound sensor against your skin (upper arm and upper thigh/groin) to allow the researcher to locate a good image of the underlying artery.

(your initials)

**Near-Infrared Spectroscopy.** There are no known risks associated with use of the near-infrared device. However, it is possible that the adhesive on the sticky plastic sensor may irritate your skin.

(your initials)

**Static Arm Exercise.** You may experience temporary fatigue in the muscles of the exercising arm. It is also possible to experience soreness in these muscles within 24-48 hours following this study visit. There is a small risk that you may develop a bruise from the inflation of the cuff that prevents blood from flowing into or out of your forearm. You may also experience some discomfort and/or a numb, tingling sensation in your arm while the cuff is inflated. These sensations go away quickly after the cuff is deflated. There is also a small risk of the appearance of petechia (small pink blotches on the skin) as a result of the increased venous blood pressure in the occluded forearm during exercise. If present, these small pink dots may persist for several days but are not associated with any pain or long term adverse effects.

(your initials)

**Dynamic Leg Exercise.** You may experience temporary muscle fatigue in the thigh muscles of your exercising leg. It is also possible to experience soreness in these muscles within 24-48 hours following this study visit. There is a small risk that you may develop a bruise from the inflation of the cuff that prevents blood from flowing into or out of your leg. You may also experience some discomfort and/or a numb, tingling sensation in your leg while the cuff is inflated. These sensations go away quickly after the cuff is deflated.

(your initials)

**Consumption of beet juice:** There are no known health risks associated with consumption of nitrate-rich beet juice, a commonly sold health drink/supplement in Europe. The most common side-effect of beet juice consumption is pinkish-colored urine (known as “beeturia”) and/or stool. This can occur after consuming either the nitrate-rich or placebo version of the drink.

(your initials)

**Urine Collection:** There are no known risks associated with the self-collection of one’s urine. Volunteers will be provided screw top and airtight containers to store their urine, and coolers to use for storing and carrying urine samples to the laboratory for analysis.
4. **Benefits to Participants/Society:**

**Possible Benefits to participants:**
Postmenopausal participants will receive more regular monitoring of their resting blood pressure. Throughout the duration of the study protocol, you will have your untreated blood pressure measured by trained research personnel on 3 separate occasions, and these measurements will be provided at no cost you and/or your insurance provider. You will also be provided information about their aortic blood pressure, a measurement which may have greater predictive value for cardiovascular risk than arm blood pressure measurements alone. With your permission, all blood pressure-related information will be shared with your primary care provider.

For younger women, there are no direct benefits to you for participating in this study other than knowing your cardiovascular risk factors (i.e., blood pressure, blood cholesterol, fitness level, etc.).

**Possible benefits to society:**
This research may further our understanding about the potential for nitrate-rich food/dietary supplements to help control blood pressure without (or with less) medication(s) in a population with increased blood pressure-related cardiovascular risk.

5. **Duration/time of the procedures and study:**

The initial screening/familiarization visit (Visit 1) will take approximately 1.5 hours. The baseline study visit (Visit 2) will take approximately 4 hours. The premenopausal estradiol assessment visit (Premenopausal visit 3) will take approximately 30 minutes. The second premenopausal experimental visit (Premenopausal visit 4) will take approximately 4 hours. The following two postmenopausal experimental visits (Postmenopausal Visits 3 & 4) will each take approximately 6 hours.

If you are a premenopausal woman the total duration of all the research sessions will be approximately 10 hours.

If you are a postmenopausal woman the total duration of all the research sessions will be approximately 17.5 hours.

All study visits (Visits 2-4) will be separated by no less than 5 days each. Therefore, it is expected that all postmenopausal participants will be able to complete all 3 study visits over 2-6 weeks following their screening visit.

6. **Alternative procedures that could be utilized:**

There are alternative procedures available that could be used to measure your aerobic fitness level (e.g., maximal graded exercise test), aortic blood pressure (e.g., central artery catheter), limb blood flow (e.g., limb vein catheter), and muscle metabolite concentration (e.g., microdialysis; placing thin fibers through your skin and into your muscle tissue). However, most of these methods are either invasive (catheters, microdialysis) and/or could present additional risk and
discomforts to participants. The procedures we have selected to use in this research study present less risk and potential discomfort to you as a participant, while still providing us with reliable research data and information.

Blood and Urine Sampling: We will utilize a combination of measurements made from blood and urine to study hormones. The collection of daily urine samples eliminates the need for daily blood samples for monitoring reproductive hormones over the course of the menstrual cycle. The fasting blood samples are necessary to track changes in some hormones that cannot be measured in the urine.

7. Statement of confidentiality:

Your participation in this research is confidential. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.). Moreover, data will be stored and secured in the Vascular Aging and Exercise Laboratory (201 Noll Laboratory) in password protected computer files. Any hard copies of data will be stored in locked filing cabinets. In the event of any publication resulting from the research, no personally identifiable information will be disclosed. Penn State’s Office for Research Protections, the Institutional Review Board, and the Office for Human Research Protections as well as the Food and Drug Administration in the U.S. Department of Health and Human Services may review records related to this project.

8. Right to ask questions:

Please contact David Proctor at 814-863-0724 (office) or 814-571-5234 (cell) with questions, complaints or concerns about the research. You can also call these numbers if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

9. Payment for participation:

You will not be paid for the initial screening/familiarization visit (Visit 1).

If you are a **premenopausal woman**, you will be paid a total of $40 for completion of all 4 visits. You will be paid for your study visits as outlined below:
Visit 2: $20
Visit 3 and 4: $20

If you are a **postmenopausal woman**, you will be paid a total of $80 for completing all of your visits (Visits 1-4). You will be paid for your study visits as outlined below:
Visit 2: $20
Visit 3: $30
Visit 4: $30
Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Voluntary participation:

Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise.

Additionally, if you do not comply with the study protocol (e.g., you skip/miss an excessive number of study visits or fail to follow pre-visit instructions) we may not seek your continued participation in this study.

11. Injury Clause:

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

12. Abnormal Test Results:

In the event that abnormal lab test results are obtained during initial screening or subsequently throughout this study, you will be informed as quickly as possible of these results and instructed to contact your private physician for further assessment. The lab test results will be made available to your private physician at your request.

You must be 18 years of age or older to take part in this research study. If you agree to take part in this research study and the information outlined above, please sign your name and indicate the date below.

You will be given a copy of this signed and dated consent form for your records.

______________________________________________
Participant Signature                        Date

______________________________________________
Person Obtaining Consent                    Date

OPTIONAL BLOOD AND URINE STORAGE: In addition to the main part of the research study, there is an optional part of the research. You can participate in the main part of the research without agreeing to take part in this optional part.
Storage of Leftover Blood and Urine Samples for Future Research Studies
As part of this study, we are obtaining blood and urine from you. If you agree, the research team would like to store leftover samples of your blood and urine that is collected so that your blood and urine can be studied in the future after this study is over. These future studies may provide additional information that will be helpful, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact David Proctor at dnp3@psu.edu; 863-0724.

Your leftover samples will be labeled with a code number and stored in a locked laboratory. If you consent to have samples of your blood and urine saved for future research, the period for the use of the samples is unknown but you will be free to change your mind at any time. You should contact David Proctor at dnp3@psu.edu; 863-0724 and let him know you wish to withdraw your permission for your blood to be used for future research. If you do this, any unused blood and urine will be destroyed and not used for future research studies.

Please initial below to indicate your preferences regarding the optional storage of your leftover blood and urine for future research studies.

a. Your samples may be stored and used for future research studies performed in our laboratory to learn about the effects of beetroot juice on blood or urine markers of vascular health.
   ______ Yes    ______ No

b. Your samples may be stored and used for research about other health problems.
   ______ Yes    ______ No

c. Your samples may be shared with other investigator/groups as long as any identifying information (name, birthdate, etc.) is removed.
   ______ Yes    ______ No
**Participant:** By signing below, you indicate that you are voluntarily choosing to take part in this optional part of the research.

<table>
<thead>
<tr>
<th>Signature of Participant</th>
<th>Date</th>
<th>Printed Name</th>
</tr>
</thead>
</table>

**Person Explaining the Research:** Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

<table>
<thead>
<tr>
<th>Signature of Research Personnel</th>
<th>Date</th>
<th>Printed Name</th>
</tr>
</thead>
</table>
VITA

Danielle Jin-Kwang Kim

Danielle Jin-Kwang Kim was born in Seoul, South Korea, on January 10, 1988. In 2004, she came to Galion, OH of the United States as an exchange student for 10 months and went back to Korea to finish up high school. After graduating high school in 2007, she came to the U.S. for her college degree. Between 2007 and 2011 she studied pre-medicine at the Pennsylvania State University in University Park, PA. She stayed at the same school for her doctoral degree and received a Ph.D. in physiology from the Pennsylvania State University in 2016.

EDUCATION

Ph.D., Physiology 2011 – Present
The Pennsylvania State University

B.S., Pre-Medicine 2011
The Pennsylvania State University, University Park, PA

PUBLICATION


TEACHING EXPERIENCE

Instructor – BIOL 141 Spring 2016
Introductory Physiology
Dept. of Biology, The Pennsylvania State University

Graduate Teaching Assistant – BIOL 142/473 2012 - 2015
Physiology Laboratory / Laboratory in Mammalian Physiology
Dept. of Biology, The Pennsylvania State University

Guest Lecture: “Diastolic Heart Failure” – KINES 455 Spring 2016
Physiological basis of Exercise as Medicine
Dept. of Kinesiology, The Pennsylvania State University