THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOLOGY

THE INFLUENCE OF ACUTE DIETARY NITRATE SUPPLEMENTATION ON ENDOTHELIAL FUNCTION IN EARLY POSTMENOPAUSAL WOMEN

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A thesis
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Reviewed and approved* by the following:

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ABSTRACT

Menopause is a pivotal period in a woman's life when cardiovascular risk dramatically increases, driven by ovarian aging and the loss of estrogen. Estrogen's effects on the vasculature are mainly mediated by the production of the vasodilator and vasoprotective molecule, nitric oxide (NO). With the onset and continuation of menopause, women's arteries undergo accelerated aging, characterized by reductions in the endothelial production of NO and a declining ability of conduit arteries (coronary, brachial, etc) to vasodilate. Estrogen replacement has shown beneficial effects on endothelial function in post-menopausal women, yet there are increased risks of adverse events such as stroke or ovarian cancer. These findings have led to the search for safe and effective, non-estrogen therapies for vascular risk reduction in post-menopausal women. Recent reports in our lab reveal that a single dose of nitrate-rich beetroot juice, boosts the production of NO by safely raising nitrite plasma concentrations in older women. Whether nitrate supplementation can augment NO bioavailability and improve endothelial function in more recently postmenopausal women has not been examined. The purpose of the present study, therefore, is to examine the acute effects of nitrate supplementation on vascular endothelial function in a group of recently post-menopausal women. Healthy women will consume either nitrate-rich or a nitrate-depleted placebo beetroot juice on two study visits, in a randomized cross-over, double-blind study. Before and 90 minutes after juice consumption, participants will have the diameter of their right brachial artery measured at rest and following a 5-minute period of forearm blood flow occlusion. Artery diameter images at baseline and during peak dilation are measured using an ultrasound sensor/transducer. These are expressed as the percent change in artery diameter above baseline, FMD%. After the entire study is completed, test order for all subjects will be unblinded and statistical analysis of the results will be conducted. We hypothesize that acute nitrate supplementation will significantly increase brachial %FMD in early post-menopausal women. Once completed, the results of this study will provide insight

into the potential of this natural NO-boosting supplement to improve endothelial function during this period of rapidly declining estrogen production.

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Chapter 1

Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality globally. While overall reductions in CVD development have decreased, there are key sex differences observed with regards to the onset and mortality of CVD. Approximately 35% of women in the United States have CVD, accounting for 52.6% of CVD deaths in 2010(Zhang, 2010) . Coronary heart disease (CHD) is the leading cause of CVD-related deaths and has increased in women 35-54 years of age. In addition, women have a higher mortality rate due to myocardial infarctions compared to men because women experience CVD differently. Ovarian hormones can influence CVD risk factors. Specifically, the onset of menopause and the associated loss of estrogen, as well as changes in other reproductive hormones, can increase risk factors for CVD development (Somani et al., 2019; Witkowski & Serviente, 2018).

Vascular aging, characterized by increased stiffening of arteries and reduced endothelial function, provides an optimal setting for development of CVD. While vascular aging appears to take place with chronological aging in both men and women, reproductive aging in women appears to accelerate this phenomenon. This is largely due to the loss of ovarian function and consequently, changes in reproductive hormones. Endothelial vasodilatory function, an important predictor of CVD and key initiating step in atherosclerosis, which is the main physiological process underlying CVD, is augmented with reproductive aging. Specifically, endothelial

function appears to decline with menopause, independent of chronological aging (Seals et al., 2006, Moreau et al., 2012).

The endothelium is a monolayer of cells in the vasculature, which controls vascular smooth muscle relaxation and constriction, among other vasoprotective functions, maintains vascular homeostasis. The endothelium is able to exert its vasodilatory effects through the production of the potent vasodilator and signaling molecule, nitric oxide (NO). The release of NO induces endothelium-dependent vasodilation (EDV) (Papapetropoulos et al., 1997). Dysfunction of the endothelium is a primary antecedent to atherosclerosis, and is characterized by reduced bioavailability of NO. Additionally, L-arginine, which is a precursor to endothelial derived NO, has been shown to be reduced in postmenopausal women. This relative L-arginine deficiency can lead to endothelial nitric oxide synthase (eNOS, the endothelial enzyme that synthesizes NO) uncoupling, resulting in oxidative stress and consequently endothelial dysfunction (Somani et al., 2019). Endothelial function declines across the life span in both men and women, however key sex differences exist in this observed decline.

In a cross-sectional analysis of men and women, it was shown that age-related endothelial dysfunction is attenuated in premenopausal normotensive women compared with men. This study examined the brachial arm blood flow induced by endothelium-dependent vasodilator, acetylcholine, to determine if the onset of menopause is associated with a measurable impairment in endothelium-dependent vasodilation (EDV). Women exhibited only a slight decrease in acetylcholine-mediated vasodilation (0.5% decrease per year) before menopause. However, after menopause EDV declined more precipitously (around 2.1% per year). This

provides evidence that menopause accelerates the age-associated decline in endothelial vasodilator function. The steep decline in endothelial function observed in women that coincides with the time of menopause suggests that the permanent loss of estrogen, as well as changes in reproductive hormones, may underlie these observations. (Somani et al., 2019; Taddei et al., 1996)

In a study performed in women at discrete stages of the menopause transition, it was found that endothelial function, as measured by the brachial artery flow mediated dilation (FMD) technique, decreases beginning at the early stages of perimenopause (Moreau et al., 2012). Perimenopause is the transitional stage into menopause when the ovaries begin reducing estrogen production. Endothelial function and menopausal stage were shown to be inversely related, and this association was independent of age and other cardiovascular disease risk factors. Brachial artery FMD was also associated with circulating estradiol concentration. In the early perimenopausal stage, women appeared to maintain endothelial protection due to ovarian hormone levels, whereas in late perimenopausal women, brachial artery FMD was significantly lower. As circulating estrogen continued to decrease across the menopause, rapid deterioration was seen in endothelial function that worsened with each menopausal stage. This study highlights that CVD risk may potentially be changed in the critical period of late perimenopause to early post menopause, and may represent a window of opportunity to maintain vascular function with novel therapeutic strategies(Moreau et al., 2012).

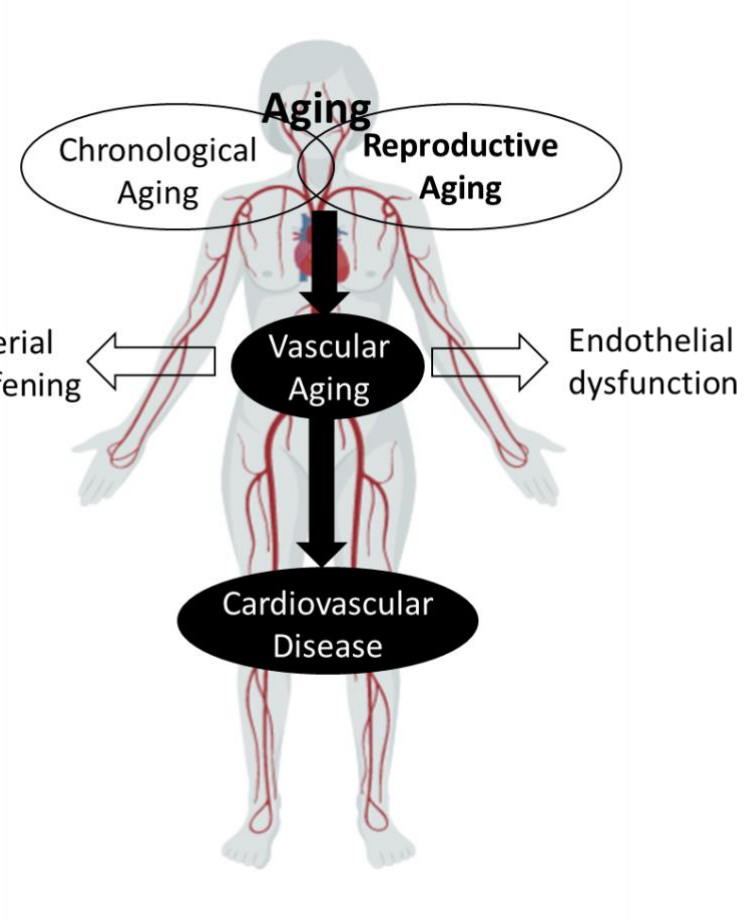

Figure 1. Effects of chronological and reproductive aging on cardiovascular disease development in women

Studies in both animal models and in women have demonstrated the direct and indirect effects of estrogen in improving NO bioavailability and consequently vascular endothelial function. Estrogen is a hormone produced in the ovaries and released into the bloodstream and has the ability to exert many effects on the cardiovascular system. Specifically, estrogen is a known vaso-protective hormone as it increases NO bioavailability by directly promoting NO synthesis and has direct and indirect antioxidant properties and anti-inflammatory effects. One of the mechanisms related to estrogen-induced increases in NO generation include increased

transcription and expression of the enzyme involved in catalyzing the synthesis of NO, endothelium NO synthase (eNOS). Additionally, estrogen can directly activate eNOS via a PI3K/phosphokinase B (PKB/AKT)-mediated signaling pathway, as well as by increasing free intracellular calcium concentrations in endothelial cells. While estrogen can have direct anti-oxidant effects due to its phenolic structure and scavenging abilities, estrogen mainly attenuates oxidative stress by lowering genomic expression of oxidative proteins and upregulating anti-oxidant enzyme expression via interaction with estrogenic nuclear receptors (Novella et al., 2012). Additional protective effects of estrogen include lowering endothelin-1, a potent vasoconstrictor and pro-inflammatory peptide secreted by the endothelium (Chakrabarti et al., 2008).

While various forms of estrogen treatment in postmenopausal women have proven to be effective in restoring endothelial function, the potential for increased risk of stroke and ovarian cancer with extended use warrants investigation into alternative strategies to maintain endothelial function in postmenopausal women. This has led to the search for safe, non-estrogen therapies for improving endothelial function in post-menopausal women. A recent report in our lab revealed that a single dose of nitrate-rich beetroot juice, boosts the production of NO by safely raising nitrite plasma concentrations in older postmenopausal women. Whether or not nitrate supplementation can augment NO bioavailability and improve endothelial function in more recently postmenopausal women has not been examined. If found to be safe and effective, nitrate-rich beetroot juice could provide a potential alternative to estrogen replacement therapy for women during this period of rapidly declining estrogen production(Kim et al., 2019).

Consumption of beetroot juice and other nitrate-rich products (green leafy vegetables, supplements, etc.) is a promising non-pharmacological (and non-estrogen) strategy for increasing NO levels and improving endothelial function, particularly in clinical populations and older adults. When ingested, dietary nitrate (NO_3^-) is converted to NO through an endothelium-independent pathway referred to as the entero-salivary nitrate-nitrite-nitric oxide ($NO_3^- \rightarrow NO_2^- \rightarrow NO$) pathway(Duncan et al., 1997)(Hord et al., 2009).

Importantly, this pathway is facilitated by the presence of nitrite reductases (anti-oxidants, polyphenols, etc) which are also present in beetroot juice that enhance the conversion of nitrite to NO. In the US the average consumption of dietary nitrate is about 40-100 mg/d, but has the possibility to increase to about 1200 mg/d through intake of more high-nitrate containing foods(Duncan et al., 1997). Previous work has shown that short term dietary supplementation with nitrate reduces blood pressure in healthy young volunteers, but little is known of its effects on older adults. The contribution of dietary nitrate to increase NO bioavailability provides rationale to help reverse endothelial dysfunction secondary to NO insufficiency, as observed in estrogen deficient women.

With this information as a background, the purpose of the present study is to evaluate the acute effects of a single dose of dietary nitrate supplementation, in the form of beetroot juice, on vascular endothelial function in a group of recently postmenopausal women.

Chapter 2

Methods

Study Participants

We will recruit 12-13 early postmenopausal women who are within 1-5 years following their last menstrual cycle, as defined by most recent guidelines (Stages of Reproductive Aging Women; STRAW criteria). Participants will be normotensive, not taking hormonal therapy, BMI<35, non-smokers, free of overt chronic disease as assessed by a clinician reviewed medical history questionnaire and venous blood chemistry (hematologic, liver, and kidney function) and who have not donated blood or blood products in the past 3 months. We are using this population because we hypothesize there is greater opportunity for modification of the endothelium, which may not be true for late postmenopausal women who are also exposed to the effects of chronological aging.

Study Methods

For this study, endothelial function will be assessed in the brachial artery using the flow-mediated dilation (FMD) method. Brachial artery FMD is a non-invasive technique that correlates well with coronary (heart) endothelial function and is an early indicator of atherosclerosis and CVD risk (Johnson et al., 2019). This procedure involves measuring the artery at rest and following a 5-minute period of forearm blood flow occlusion. Artery diameter images at baseline and during peak dilation are measured using an ultrasound sensor. These are expressed as the percent change in artery diameter above baseline, FMD%.

The 5 minute period of cuff occlusion causes ischemia (lack of oxygen) and a build-up of metabolic byproducts in the forearm muscle below the cuff. Release of the cuff produces a transient, compensatory increase in blood flow (reactive hyperemia) which causes an increase in endothelial shear stress in the upstream brachial artery and a subsequent dilation (widening) of the brachial artery. Artery diameter images at baseline (rest) and during reactive hyperemia are measured using an ultrasound sensor and expressed as the percent change in artery diameter above baseline. In people with a functional endothelium the rapid blood flow induces shear stress on the artery and the endothelium responds by releasing nitric oxide to cause vasodilation. Peak diameter is usually found to be within 40-60s post deflation (Thijssen et al., 2009).

Study Design

Each subject will consume either nitrate-rich or a nitrate-depleted placebo beetroot juice on two study visits, in a randomized cross-over, double-blind study The first visit we take baseline measurements of heart rate and blood pressure. Next, the brachial artery is visualized using a Doppler ultrasound machine and sensor. While the participant is laying flat on a padded gurney, a blood pressure cuff is placed around the forearm immediately below the antecubital fossa. The FMD test (discussed above) is then conducted. Next, participants will consume a one-time dose of 140 mL nitrate-rich or nitrate depleted juice and rest for 90 minutes. Previous studies have shown that 90 minutes is sufficient time to digest the nitrate, which is why that time point was chosen. Following consumption, patients will have another measurement of BP, HR and FMD analysis. The ultra sound images will be used for offline analysis with edge-detection software.

On the second study visit, patients begin with the same procedures as in visit one: HR, BP, and FMD protocol. They then consume whichever drink supplement they did not receive during the previous visit and finish with a final measurement of HR, BP, and FMD.

My role in this study is to assist in these study visits and to help analyze the brachial artery ultrasound images. Specifically, I will use a customized software analysis program (Quipu^{TM}) to track the longitudinal wall borders and blood flow and shear velocities for the calculation of the %FMD responses.

Figure 2. Schematic of study design

Image and Statistical Analysis

Using the longitudinal images of the brachial artery and automatic wall tracking I am able to analyze the change in diameter along with the blood velocity. Blood velocity is measured by the Doppler shifts, which measures the fastest moving blood cells. With this information I can also analyze shear stress through our specific shear rate formula.

Statistical Analysis

The comparisons will be accomplished by using paired t-tests to look at differences in endothelial function pre- and post-nitrate consumption.

Anticipated Results and Significance

We hypothesize that acute nitrate supplementation will significantly increase brachial %FMD in early post-menopausal women. Once completed, the results of this study will provide insight into the potential of this natural NO-boosting supplement to improve endothelial function during this period of rapidly declining estrogen production

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ACADEMIC VITA

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Thesis Supervisor: Dr. David Proctor

Work Experience

The Pennsylvania State University, University Park, PA September 2018- Present
Commons Desk Clerk
Worked with a team to assist students with obtaining their mail, receiving temporary keys, and loaning equipment from the university. Communicated issues with mailing services to students and companies.

The Deli Restaurant, State College, PA September 2017- March 2018
Hostess
Greeted guests and assigned them to tables suitable for their needs to provide a personal dining experience. Organized reservations and handled walk-in guest overflow by communicating delays and maintaining the waitlist.

Presentations

The Scripps Research Institute, Jupiter, FL August 11, 2017

The Scripps Undergraduate Research Symposium

Presented my final conclusions from my summer research through a poster presentation to Scripps faculty and staff.

Service

Housing Transitions, State College, PA August 2018- May 2019

Weekly Volunteer
Assisted the homeless shelter with various activities such as answering phones, organizing donations and files, among other things to aid the residents of the shelter.

Mount Nittany Medical Center, State College, PA March 2019- January 2020

Weekly Volunteer
Assisted both the patients and the hospital medical staff through volunteering. Achieved by collecting vitals, discharging patients, returning blood samples to the laboratory, retrieving medical equipment, and maintaining an upbeat environment for the patients.

Additional Skills

Language Proficiency: Spanish

Proficient in speaking, reading and writing