AGE- AND SEX-SPECIFIC EFFECTS OF TETRAHYDROBIOPTERIN SUPPLEMENTATION ON PERIPHERAL ARTERY STIFFNESS

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ABSTRACT

Objective: To compare the elastic properties of peripheral arteries in healthy older (vs. younger) men and women and to determine if acute administration of tetrahydrobiopterin (BH₄, a substance that increases bioavailability of the vasodilator nitric oxide) alters these properties in a sex-specific manner. Methods: Resting diameter of the brachial and common femoral arteries (Doppler ultrasound) and blood pressure (Finometer) were measured in 13 men (6 younger, 7 older) and 16 women (7 younger, 9 older). Pressure-strain elastic modulus (E_p) and stiffness index (β), as well as the two determinants of E_p, pulse pressure (PP) and relative diameter change (%), were evaluated under resting conditions following randomized oral administration of placebo or BH₄ (10 mg/kg body weight) on separate visits. Results: Under the placebo condition, aside from younger women having a lower PP (p ≤ 0.05) than older women, there were no age or sex differences in any of the elastic properties measured. Under the BH₄ condition, no effect on arterial distensibility was observed in men (younger or older) or younger women. Older women, by contrast, exhibited BH₄-induced reductions in the E_p of both arteries, and a reduction in stiffness (β) of their brachial artery (all p ≤ 0.05), resulting in older women having significantly more distensible arteries in the presence of BH₄ than younger women. Conclusions: There is an age dependent effect of acute BH₄ administration (10 mg/kg) on the distensibility of peripheral arteries in women, but not men. The increase in peripheral (muscular) artery distensibility observed in older women following BH₄ administration could reflect a reversal of vascular endothelial dysfunction (i.e. decreased endothelium-dependent vasodilation) that healthy women typically experience as they age.
Key Words: arterial stiffness, pressure-strain elastic modulus, brachial artery, common femoral artery, tetrahydrobiopterin
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Ever True to You, Dear Old White & Blue
LITERATURE REVIEW

Overview

Physiological aging is an inevitable part of life that all human beings must
unavoidably journey through. One specific experience occurs within the cardiovascular
system. Cardiovascular and, more specifically, arterial aging are extensively studied
using different tools of measurement and with different substances that create agonistic or
reverse effects. This review will cover the literature that describes the physiology of
ordinary, healthy aging, with a direct look at arterial aging and how exercise can be used
to combat this aging process. What follows will be a discussion about the composition of
an artery, the current understanding of arterial stiffness and vasodilatory effects that both
central elastic and peripheral muscular arteries undergo, as well as an in-depth
explanation of the substance tetrahydrobiopterin and its new, novel therapeutic potential
on vascular function. This section will conclude with what is still unknown in peripheral
muscular artery research and how tetrahydrobiopterin can be added in to this field of
study.

Arterial Aging and Vascular Function

Aging can be described as the changing of many physiological systems over a
period of time. More specifically, it is a progressive functional decline, which limits our
physiological ability and renders us more susceptible to disease. Although the loss of
skeletal muscle mass is the most reported effect of aging, which results in a reduction in
the ability of older individuals to produce force, changes in body composition (increased
body fat, higher cholesterol, etc.), neurological function (slower reflexes, dementia, etc.)
and cardiovascular function are also experienced as one ages.\textsuperscript{1} This section of the review will focus solely on the age-related changes that occur to the arterial system.

Both structural and hemodynamic changes occur throughout the arterial systemic tree as a result of the aging process. Research has been conducted to delineate arterial changes due to primary aging from changes associated with vascular disease progression.\textsuperscript{2} With primary aging, large elastic arteries experience an increase in lumen diameter and a decrease in arterial distensibility (\textit{i.e.} increase in stiffness), popularly measured using the noninvasive index, pulse wave velocity (PWV).\textsuperscript{2} The intima-media wall thickness (IMT) of both central and peripheral arteries increases to adapt to the hemodynamic changes related to local arterial blood pressure\textsuperscript{3} and the stabilization of wall shear stress.\textsuperscript{4} Vascular endothelial function becomes impaired as an individual ages, most commonly seen in a reduction of endothelium-dependent dilation (EDD), and older men and women also experience enhanced peripheral vasoconstriction, seen by lower basal blood flow and decreased vascular conductance.\textsuperscript{3}

Consequently, aging effects and their severity witnessed in the cardiovascular system become “partners” linked with the occurrence of cardiovascular disease (CVD) in the elderly.\textsuperscript{2} What was previously considered normal changes of arterial aging are now seen as predictors of clinical diseases, such as atherosclerosis, hypertension, and stroke.\textsuperscript{2} Simultaneously, arterial aging creates a greater challenge for the body to participate in physical movement, negatively affecting older individuals’ activity levels. However, just as current research indicates that elderly muscle can adapt positively to resistance exercise\textsuperscript{1}, such studies show that cardiovascular function can adapt positively to both
habitual resistance and aerobic activity, helping to delay and/or reduce detrimental cardiovascular risk factors.³

**Exercise Effects on Age-Associated Mechanical and Vasodilatory Responses**

Extensive clinical research has been conducted to recognize the influence that exercise plays on mechanical and morphological factors associated with arterial aging.³ Consistent aerobic exercise decreases central elastic arterial stiffness as well as femoral IMT by normalizing wall shear stress in older adults.³ Aerobic activity also increases EDD in healthy men.⁵ Regular resistance training counteracts the aging-associated decrease in both basal leg blood flow and vascular conductance.³ There is still extensive room for research in this topic, both in isolating and combining aerobic and resistance exercises, to see what physical activity actively combats arterial aging.

**Arterial Geometric Structure and Composition**

The systemic arterial system functions to deliver blood at high pressure from the pulsatile pump (ventricle) to the peripheral artery beds. The arterial tree can be divided into three anatomical regions, each with a separate function. The large, elastic arteries (aorta, carotid, iliac, etc.) receive and store oxygenated blood during systole and expel it to the periphery during diastole. The muscular arteries (femoral, brachial, radial, popliteal, etc.) modify the speed of the pressure and blood flow waves by altering smooth muscle tone. The arterioles are sites of vascular resistance, aiding in the maintenance of mean arterial pressure so that the capillaries receive a steady flow of blood throughout the entire cardiac cycle.⁶
There are three components that make up the arterial wall of all blood vessels—elastin, collagen, and smooth muscle cells. Elastin and collagen promote arterial elasticity, whereas smooth muscle cells cannot be regarded as a true elastic material. The arterial wall itself is divided into three concentric regions: the tunica intima (endothelial layer supported by smooth muscle cells), media, and adventitia (mainly collagen layer attached to connecting tissue). An elastic lamina layer separates each region from another. The tunica media is what differs in composition between elastic and muscular arteries, affecting its mechanical properties. It is comprised of stacked musculo-elastic fascicles in layers of elastin-smooth muscle cells-elastin:collagen:elastin-smooth muscle cells-elastin, and so on; however, the relative amounts of these components differ. Harkness et al. (1957) was the first to compare arterial composition, finding that elastin is the principal overall material in central (elastic) arteries, whereas collagen and smooth muscle cells are the dominant components in the peripheral (muscular) arteries, which has also been seen in later studies.

Attention has also been paid to microvascular arterial aging, where a morphological, age-related increase in collagen and decrease in elastin and smooth muscle cells has been found in central arteries. Elastin, additionally, transforms into a structure not well suited to bear the same stresses that are applied to the arterial wall. As a result, dilation shifts the strain to collagen, increasing arterial stiffness. Peripheral arteries, however, only experience acute changes in smooth muscle tone. They barely (if at all) experience the loss of elastic fibers and laminae orderly arrangement and consequential thinning, splitting, and fragmentation seen to occur in central arteries. It is these structural rearrangements that are linked to an increase in arterial stiffness, as
depicted via an increase in PWV. In contrast, vasoconstriction and vasodilation in central arteries induce passive changes, where as such hemodynamic instances in peripheral arteries are what stimulate an active effect on arterial stiffness.

**Arterial Stiffness and the Pressure-Strain Elastic Modulus**

**Definition of arterial stiffness**

Originally documented by Charles Roy in 1881, the stiffening of larger elastic arteries, such as the aorta and carotid, as a person ages induced the foundation of William Osler’s somewhat truthful adage at the turn of the twentieth century: “man is as old as his arteries.” Arterial stiffness describes an artery’s ability to deform as a result of its viscoelastic properties. Each artery in the arterial tree experiences a stress, or force per unit area. This stress causes the deformation. The unitless ratio of this bodily deformation to its original form is called the strain. The slope of the linear part of the stress versus strain curve is termed the elastic modulus, and it has the same units as the stress measurement, force per unit area. This stress/strain elastic modulus is more commonly known as Young’s modulus. Using Young’s modulus to explain the difference in material stiffness between collagen and elastin, collagen is over 300 times stiffer than elastin. Therefore, the further an artery is located from the heart, the stiffer that vessel is. However, there are other variations of this stress to strain relationship, recognized by the variables included in the stress/strain ratio.
The pressure-strain elastic modulus

It was recognized that a direct comparison of the values of Young’s elastic modulus from all published work could not be accomplished due to the fact that the arterial wall thickness was not recorded for the arteries in most experiments.\(^{10}\) Peterson \textit{et al.} (1960), however, realized that neither the thickness of the arterial wall nor the circumferential change an artery undergoes during the cardiac cycle has a significant effect on the mechanical behavior of the artery. He went on to describe the ‘pressure-strain’ elastic modulus, \(E_p\), where:

\[
E_p = \frac{\text{Diastolic diameter} \times (\text{Systolic BP} - \text{Diastolic BP})}{\text{Systolic diameter} - \text{Diastolic diameter}}
\]

A change in the \(E_p\) of an artery infers that there is a change in wall thickness\(^{14}\), or change in arterial diameter, over the cardiac cycle. \(E_p\) is the inverse of arterial distensibility.\(^{15}\) The greater the value of \(E_p\), the stiffer (\textit{i.e.} less distensible) the vessel. \(E_p\) has been used extensively in studies that look to compare arterial stiffness between elastic and muscular arteries, compare arteries through the aging process, observe sex-specific differences, and observe differences between subjects with versus without CVD or cardiovascular risk factors.\(^{14,16-21}\)

The stiffness index (\(\beta\))

The stiffness of vascular walls is commonly analyzed using the stress-strain relation (\textit{i.e.} elastic modulus) from the pressure-diameter data collected. However, the absolute values obtained for these indices are directly related to a subject’s blood pressure measurements. An experiment that stimulates changes in subjects’ blood pressures (\textit{e.g.} exercise) influences an inaccuracy in the comparison of the elastic
modulus throughout the different phases of the study. It was for this reason that Kawasaki et al. (1987) introduced the stiffness index, $\beta$, where:

$$\beta = \frac{\text{Diastolic diameter} + (\ln \frac{\text{Systolic BP}}{\text{Diastolic BP}})}{\text{Systolic diameter} - \text{Diastolic diameter}}$$

$\beta$, a unitless measurement, enables the comparison of arterial stiffness independent of distending pressure. This variable has since become a common parameter used in this area of research.\textsuperscript{10,17}

**Other indices of arterial stiffness**

$Ep$ (or any variant of an elastic modulus) and the stiffness index ($\beta$) are not the only variables used to quantify arterial stiffness. As previously mentioned, PWV (the travel of pulsatile-waves by ventricular ejection) is a routinely used measurement to noninvasively gage arterial stiffness. The carotid-femoral PWV (cfPWV) is commonly used to measure central (elastic) arterial stiffness, dominantly aortic stiffness. Arterial compliance, a change in volume over a change in pressure, and arterial distensibility, compliance over the initial volume, are two more measurements of arterial stiffness.\textsuperscript{10} If the arterial segment is being studied \textit{in vivo}, then both compliance and distensibility can be measured using change in diameter in place of volume.\textsuperscript{10} Opposing $Ep$, the more compliant/distensible an artery, the less stiff the vessel. As an example, arterial compliance, distensibility, and cfPWV were all measured in a study conducted by Vermeersch et al. (2008) to find an age-related increase in central arterial stiffness, but not in the periphery, of a middle-aged population.

Relative diameter change (\textit{i.e.}, strain in the $Ep$ computation) is another scale of arterial stiffness. This measurement was used in a recent study to show that vascular
stiffness precedes the development of hypertension, providing evidence of hypertension to be a disease of the arterial wall. Arterial stiffness can also be indicated by an increase in pulse pressure (PP) due to an age-associated increase in systolic blood pressure (SBP) alongside an age-associated stabilization or decline of diastolic blood pressure (DBP) by an individual’s sixth decade of life. The assessment of arterial stiffness is a constantly evolving field of study with many questions still unanswered.

Vascular Endothelial Function, Nitric Oxide, and Vasodilatory Effects

Vascular endothelial cells synthesize and release a wide array of biological molecules that play an active role in arterial hemodynamic and structural function, and proper function is pivotal for cardiovascular success. Unfortunately, with aging comes the development of vascular endothelial dysfunction. The occurrence of platelet and leucocyte adhesion within the arteries leads to the attraction of inflammatory markers and is detected by EDD reduction. Chemically-induced (via endothelial nitric oxide synthase agonist acetylcholine) or mechanically-induced (via brachial artery ultrasound with flow-mediated vasodilation (FMD)) techniques are commonly used to measure EDD and endothelial function.

Though prostaglandins and endothelium-derived hyperpolarizing factors (EDHF) also exist in the endothelial cells and contribute to EDD, vasodilatory response is primarily mediated by the endothelial production and release of nitric oxide (NO). Synthesized from L-arginine by activity of nitric oxide synthase (NOS), NO activates smooth muscle cell relaxation, enforcing vasodilation. Wall shear stress assists in the regulation of smooth muscle tension in the artery, with a high shear stress acting as one
of the major physiological stimuli that activates NOS in the vascular endothelial layer.\textsuperscript{25} Additionally, smooth muscle cells never lose their sensitivity to NO with age; rather, age-associated decline in EDD is facilitated by an oxidative stress-dependent decline in NO bioavailability.\textsuperscript{25} However, reduced EDD does not occur due to a lack of production or activity of eNOS. In an animal study, Yang \textit{et al.} (2009) observed that eNOS expression is not significantly different between ages; rather, a reduced level of tetrahydrobiopterin (BH$_4$), an essential cofactor for NOS, is detected in older mice due to decreased expression levels of enzymes involved in BH$_4$ biosynthesis. This BH$_4$ deficit is detrimental to NO bioavailability.

\textbf{Tetrahydrobiopterin}

\textit{History and molecular structure of tetrahydrobiopterin}

(6R) 5,6,7,8-tetrahydobiopeterin (BH$_4$), a pterin\textsuperscript{27}, was first shown to act as an essential cofactor in the metabolic conversion of phenylalanine to tyrosine by phenylalanine hydroxylase in the liver.\textsuperscript{28} Later studies found BH$_4$ to play a similar cofactor role for two other amino acid hydroxylases, tyrosine hydroxylase\textsuperscript{29} and tryptophan hydroxylase\textsuperscript{30}, all of which denoted BH$_4$ to be a crucial molecule in the biosynthesis of neurotransmitters epinephrine, norepinephrine, dopamine, and serotonin.\textsuperscript{31} Years later, it was discovered that BH$_4$ is also an essential cofactor for all three NOS isoforms- neuronal (nNOS), cytokine-inducible (iNOS) and endothelial (eNOS).\textsuperscript{31}
Tetrahydrobiopterin bioavailability & eNOS function

BH₄ acts as an allosteric cofactor, creating structural stabilization of eNOS dimers and increasing substrate affinity and subsequent binding and conversion of L-arginine to NO. BH₄ must be in its reduced form to function as a cofactor for eNOS. When BH₄ bioavailability declines, multiple molecular and mechanistic changes occur.

Oxidative stress is augmented with age, noted by an increase in superoxide bioactivity and other free radicals in aging human skeletal muscle. This effect decreases BH₄ bioavailability by affecting different factors, one of which is the oxidation of BH₄ to its inactive form dihydrobiopterin (BH₂). BH₂ is a direct competitor of BH₄ for eNOS binding. However, while BH₄-bound eNOS produces NO, BH₂-bound eNOS promotes uncoupling and superoxide production, which is linked to the development of vascular disease. Furthermore, oxidized BH₄ increases the synthesis of peroxynitrite (generated from NO and superoxide), which in effect, is a powerful oxidizer of BH₄. Additionally, oxidative stress depletes NADPH, which is a required reducing agent in both BH₄ de novo synthesis and BH₄ recycling.

Tetrahydrobiopterin clinical experimentation

There is recent clinical evidence that supports the cardiovascular benefit of BH₄ supplementation in the improvement of vascular endothelial function. Eskurza et al. (2005) found that BH₄ administration improved EDD in older sedentary men, while it showed no effect on young sedentary men and habitually exercising older men. These results demonstrate that BH₄ administration to sedentary older individuals may preserve EDD with the same effect as habitual exercise. BH₄ has also been seen to improve EDD
in smokers, diabetics, hypertensive subjects, and patients with hypercholesterolemia, and subjects with coronary artery disease.\textsuperscript{32}

\textbf{Focus of Present Thesis}

A great deal of research has focused on either arterial structure or arterial hemodynamics specifically, usually concentrating mainly on central (elastic) arteries. Studies need to examine the relation of arterial stiffness and endothelial function as individuals age. The peripheral (muscular) arteries need to be examined more exclusively as well due to the fact that vasodilatory/vasoconstrictive drugs have a more profound effect on these vessels.\textsuperscript{10} This present research focuses on analyzing not just the age-associated change in peripheral arterial stiffness, but also how factors such as age and sex influence the mechanical properties of vascular endothelial (dys)function, and if the administration of BH\textsubscript{4} alters the functional dynamics of arteries. These findings should have an important impact on preventing CVD and its risk factors in older individuals through new drugs that ameliorate both hemodynamic and structural arterial components.
INTRODUCTION

Stiffening of central (elastic) arteries is a hallmark of the vascular aging process. Men experience a linear increase in central arterial stiffness throughout adulthood, while women display a similar linear increase until around the time of menopause, after which this process appears to be accelerated. This age-associated, sex-dependent stiffening of central arteries increases the work of the heart and is a major contributor to the remodeling seen in the aged heart and the rising cardiovascular disease risk associated with advancing age.

Aside from the popliteal artery, most peripheral (muscular) arteries do not appear to stiffen to a significant degree with normal aging. However, most of the research on the elastic properties of peripheral arteries has been conducted on male subjects, mixed sex samples, or diseased populations (i.e., subjects with CV risk factors) and thus the possibility of a sex difference in peripheral artery stiffening with primary aging cannot be ruled out. The primary objective of the present thesis was, therefore, to compare the elastic properties of peripheral arteries in healthy older (vs. younger) women and men. We hypothesized that the stiffness of muscular arteries in the upper arm (brachial) and upper leg (common femoral) would be increased with age in women, but not men. This hypothesis was based on two studies, one which reported an age-associated stiffness in these two arteries (without the consideration of a potential sex difference), and the other which observed greater age-associated stiffness in the femoral artery of women compared to men. Unpublished data from our laboratory (i.e., accelerated increase in femoral-ankle pulse wave velocity with age in healthy women compared to men; Ridout et al.) are also consistent with this hypothesis.
Previous research has demonstrated that arterial stiffness is not a static property, as is commonly assumed, but displays a substantial “reserve” that can be altered acutely by changing the contractile state of the underlying smooth muscle. Alterations in muscular artery elasticity, for example, have been detected in response to vasoconstrictor (e.g., sympathetic activation) and vasodilatory stimuli. Alterations in endothelium-dependent vasomotor tone elicited both acutely (via manipulation of the L-Arginine – eNOS- Nitric Oxide pathway) and chronically (via exercise training-induced alterations) also appear to affect the elasticity/distensibility of muscular arteries.

Collectively, the findings discussed thus far illustrate the important influence that both structural (arterial wall composition, elastin/collagen content) and vasoregulatory/functional (smooth muscle, endothelial) factors have on peripheral artery stiffness in healthy humans. Thus, while primary aging may (women) or may not (men) induce significant alterations in muscular artery stiffness per se, an isolated comparison of stiffness parameters between these subject groups (i.e., in the absence of any acute manipulation of vascular tone) might overlook important age- or sex-dependent differences in muscular artery responsiveness. Therefore, the second objective of this thesis was to determine if acutely increasing systemic vasodilator tone reduces peripheral artery stiffness in an age- or sex-specific manner.

Acute supplementation with tetrahydrobiopterin (BH₄), a naturally occurring biological substance that acts as an essential co-factor for the enzyme nitric oxide synthase (NOS), was chosen as the intervention due to the ability of oral BH₄ to increase the bioavailability of the vasodilator substance nitric oxide (NO) and subsequently cause vasodilation in humans. We hypothesized that this intervention would have more
potential to cause systemic vasodilation and attenuate arterial stiffness (i.e., increase artery distensibility) in older women, given their greater tendency, relative to other groups (i.e., younger women, and their male counterparts), for endothelial dysfunction.\textsuperscript{25,48,49}
METHODS

Participant Screening

This experiment was part of a larger prospective study designed to assess the influence of age and sex on neurovascular control of leg blood flow at rest and during exercise. Thirteen young (6 men, 7 women) and 16 older (7 men, 9 women) participants completed the study and were included in the current analysis. All subjects were classified as normotensive (resting blood pressure below 140/90 mmHg) and healthy as evaluated by medical history questionnaire, a physical examination, resting ECG, and blood/lipid/CBC screening. Further testing indicated that these subjects had body fat percentages (DEXA scan) and aerobic fitness levels (graded treadmill test for determination of maximal oxygen uptake) that were consistent with their physical activity status (i.e., normally active, but not highly endurance trained). No subjects were taking medications that could affect cardiovascular function or interfere with BH4 supplementation, including oral contraceptives or estrogen hormone replacement therapy. Young female subjects were studied in days 1-7 of their menstrual cycle to standardize the female hormonal influence. Subjects were asked to refrain from alcohol, exercise, or caffeine for at least 12 hours prior to testing days. All participants provided written, informed consent to participate in the study. This study was approved by the Office for Research Protections at The Pennsylvania State University in agreement with the guidelines set forth by the Declaration of Helsinki.
Experimental Studies

Following the completion of screening visits, each subject participated in two experimental study visits. One visit took place three hours after consuming a single oral dose of BH₄ (10 mg/kg of body weight mixed in orange juice) and the other three hours after consuming a placebo (also mixed in orange juice). The order of these two treatments was randomized and the investigators were blinded to the order of treatment. The protocol, timing, and measurements obtained during these two visits were identical.

Brachial artery data collection

The arterial pressure and diameter measurements used to estimate the pressure-strain elastic modulus of the brachial artery were collected during a supine rest period preceding the assessment of flow-mediated vasodilation (FMD). The purpose of this FMD test was to assess the impact of the BH₄ treatment on endothelial vasodilator function (these data are not reported in the present thesis). These measurements were obtained with the subject lying supine on a padded table.

Arterial blood pressure was continuously measured using an inflatable cuff (Finometer Midi, Finapress Medical Systems, Netherlands) placed on the subject’s right ring/middle finger. Pressure signals were collected online at a sampling frequency of 400 Hz, and saved using a Powerlab system (AD Instruments, Castle Hill, Australia). Systolic and diastolic pressures were measured while the subject underwent an electrocardiogram assessment.

Following the conclusion of arterial blood pressure data collection, subjects remained supine and at rest for approximately 10 minutes. Diameter of the left brachial
artery was then measured using high resolution Doppler ultrasound (HDI 5000, Philips; Bothell, Washington), with images recorded directly to DVD.

Femoral artery data collection

The arterial pressure and diameter measurements used to estimate the pressure-strain elastic modulus of the common femoral artery were collected during the rest period preceding the knee extensor exercise testing (i.e., approximately 10 minutes after the completion of brachial artery data collection with the applied neck suction. Neck suction data are not reported in the present thesis). Diameter measurements of the left common femoral artery were obtained with the subject strapped into a padded seat in the semi-recumbent position. Arterial pressure and diameter during this initial rest period were measured using the same systems used for the brachial artery testing (i.e., Finometer finger cuff and Doppler ultrasound, respectively). However, measurements of arterial pressure were logged continuously while diameter measurements for the femoral artery were obtained and images were recorded directly to DVD.

Estimation of Brachial and Femoral Artery Stiffness

Brachial and femoral artery diameter images recorded directly to DVD were measured across the cardiac cycle using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA). The relative change of artery diameter, also referred to as strain, during the cardiac cycle was calculated as:

\[
\text{relative change of diameter} = \frac{\text{Systolic diameter} - \text{Diastolic diameter}}{\text{Diastolic diameter}}
\]

Pulse pressure (mmHg) was estimated for both brachial and femoral arteries as the difference between Systolic BP and Diastolic BP obtained from the finger (Finometer)
recordings. Resting BP Powerlab recordings taken during the same period as the administration of the ECG test were used to compute brachial artery pulse pressure.

The pressure-strain elastic modulus, established by Peterson et al. (1960), was calculated for both the brachial artery and the common femoral artery with the following equation:

$$E_p = K^* \frac{\text{Diastolic diameter} \times (\text{Systolic BP} - \text{Diastolic BP})}{\text{Systolic diameter} - \text{Diastolic diameter}}$$

where $K = 133.3$ is the factor for converting mmHg to N/m$^2$.

The beta stiffness index, introduced by Kawasaki et al. (1987) was computed for both the brachial artery and the common femoral artery with the following equation:

$$\beta = \frac{\text{Diastolic diameter} \times (\ln \frac{\text{Systolic BP}}{\text{Diastolic BP}})}{\text{Systolic diameter} - \text{Diastolic diameter}}$$

$\beta$ is a unitless measurement.

**Statistical Analysis**

Independent sample t-tests were used to test for differences in baseline characteristics between age groups amongst each sex. Repeated measures two-way analysis of variance (group x treatment) was used to compare within age groups and supplement conditions within each sex. Sigma Plot software (version 11) was used for all independent sample t-tests and all repeated measures two-way analysis tests. Statistical significance was set at $p \leq 0.05$. All data are expressed as mean ± standard error (SE), unless otherwise noted.
RESULTS

Group Characteristics

Physical characteristics of the four subject groups are shown in Table 1. Older women weighed more and had more body fat than young women, but these characteristics did not differ (p > 0.05) between older and younger men. Aerobic fitness levels were, as expected, lower in the older women (-34%) and older men (-24%) relative to their young counterparts. Oxidized LDL concentration, a systemic indicator of chronic oxidative stress, did not differ between groups.

Age Group Comparisons (Placebo Visit)

Brachial artery

Pressure-strain elastic modulus (Ep) values were similar between young men and older men, as well as between young women and older women, revealing no difference in arterial stiffness with advancing age in either sex (Figure 1A). Comparably, there was no difference in relative diameter change between young and older men (Figure 2A). A noticeable, but non-significant, increase in relative diameter change in the brachial artery (p = 0.066) was observed between younger (2.37% ± 0.25) and older (3.40% ± 0.38) women. Pulse pressure (PP) taken prior to brachial diameter measurements again showed no difference between age in men, but a greater, though not significant (p = 0.114), PP value in older women was spotted in relation to the younger women (Table 2). This increase in PP created the increase in relative diameter change, and therefore, when PP was factored out by the stiffness index (\(\beta\)), the \(\beta\) value for older women was close to
significantly lower (p = 0.069) than for young women (Figure 3A). β did not differ in value among young and older men.

*Common femoral artery*

No difference in $E_p$, relative diameter change, or $\beta$ was observed between young men or women and their respective older counterparts (Figures 1B, 2B & 3B). PP recorded simultaneously with common femoral arterial diameter measures did not differ with age in men, but it did increase substantially ($p \leq 0.05$) between young and older women (Table 2). However, with no difference in $E_p$ or stiffness ($\beta$) between young and older women, the observed PP difference has relatively no effect on the elastic properties of the artery.

*Age Group Comparisons (Effects of BH$_4$)*

*Brachial artery*

No change in $E_p$, relative diameter change, or $\beta$ was seen within young or older men in response to BH$_4$ oral supplementation (Figures 1A & 4A-B; Figures 2A & 5A-B; Figure 3A). Although young women experienced an increase ($p \leq 0.05$) in PP in response to BH$_4$ (Table 2), they too experienced no obvious BH$_4$ response-related change in $E_p$, relative diameter change, or $\beta$ (Figures 1A & 4C; Figures 2A & 5C; Figure 3A). Older women exhibited a decrease ($p \leq 0.05$) in $E_p$ and $\beta$ (Figures 1A & 4D; Figure 3A) and an increase ($p \leq 0.05$) in relative diameter change (Figures 2A & 5D) in response to BH$_4$ consumption. Under the BH$_4$ condition, older women have more distensible vessels (lower $E_p$ and $\beta$ values and greater relative diameter change, all $p \leq 0.05$) than those of young women.
Common femoral artery

Like the brachial artery, common femoral artery Ep, relative diameter change, and β of both young men and older men did not change in response to BH₄ acute oral consumption (Figures 1B & 6A-B; Figures 2B & 7A-B; Figure 3B). Young women also experienced no effect under the BH₄ condition (Figures 1B & 6C; Figures 2B & 7C; Figure 3B). Older women once again displayed a response to BH₄ supplementation with a decrease (p ≤ 0.05) in Ep, although there was no corresponding decrease in β or increase in relative diameter change as well (Figures 1B & 6D; Figures 2B & 7D; Figure 3B). Under the BH₄ condition, older women revealed a greater (p ≤ 0.05) PP than their young counterparts, like what was seen under the placebo condition (Table 2).
TABLE 1. Baseline characteristics of healthy young men, older men, young women, and older women

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Young Men (n = 6)</th>
<th>Older Men (n = 7)</th>
<th>Young Women (n = 7)</th>
<th>Older Women (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>22 ± 1</td>
<td>67 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23 ± 1</td>
<td>67 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>182.1 ± 2.1</td>
<td>176.4 ± 2.2</td>
<td>162.8 ± 1.8</td>
<td>158.6 ± 1.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.6 ± 4.1</td>
<td>82.2 ± 2.9</td>
<td>60.8 ± 4.4</td>
<td>64.4 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>19.0 ± 3.3</td>
<td>24.7 ± 1.9</td>
<td>27.3 ± 1.6</td>
<td>36.0 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2max&lt;/sub&gt; (ml/kg/min)</td>
<td>39.8 ± 3.4</td>
<td>30.5 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.8 ± 2.3</td>
<td>25.1 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>oxLDL (mg dL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.66 ± 0.05</td>
<td>0.72 ± 0.01</td>
<td>0.71 ± 0.02</td>
<td>0.72 ± 0.01</td>
</tr>
</tbody>
</table>

Values are mean ± SE. VO<sub>2max</sub>, maximal oxygen uptake; oxLDL, oxidized low-density lipoprotein. <sup>a</sup>Young Men vs. Older Men (p ≤ 0.05), <sup>b</sup>Young Women vs. Older Women (p ≤ 0.05).
**TABLE 2.** Resting blood pressure measurements of healthy young men, older men, young women, and older women prior to and concurrent with the collection of diameter measurements in the brachial artery and common femoral artery, respectively

<table>
<thead>
<tr>
<th></th>
<th>Young Men (n = 6)</th>
<th>Older Men (n = 7)</th>
<th>Young Women (n = 7)</th>
<th>Older Women (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prior to</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Brachial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>112 ± 2</td>
<td>110 ± 3</td>
<td>114 ± 3</td>
<td>118 ± 8</td>
</tr>
<tr>
<td>Placebo</td>
<td>BH₄</td>
<td>Placebo</td>
<td>BH₄</td>
<td>Placebo</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>52 ± 2</td>
<td>47 ± 3</td>
<td>53 ± 3</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>60 ± 3</td>
<td>63 ± 4</td>
<td>61 ± 6</td>
<td>71 ± 6ᵃ</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Concurrently</strong></td>
<td></td>
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<td></td>
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<tr>
<td><strong>with Femoral</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126 ± 2</td>
<td>124 ± 3</td>
<td>128 ± 7</td>
<td>134 ± 8</td>
</tr>
<tr>
<td>Placebo</td>
<td>BH₄</td>
<td>Placebo</td>
<td>BH₄</td>
<td>Placebo</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76 ± 2</td>
<td>74 ± 5</td>
<td>76 ± 2</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>51 ± 1</td>
<td>51 ± 2</td>
<td>52 ± 6</td>
<td>53 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58 ± 5ᵇ</td>
</tr>
</tbody>
</table>

Values are mean ± SE. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure. ᵃYoung Women, Placebo vs. BH₄ (p ≤ 0.05); ᵇYoung Women vs. Old Women, Placebo (p ≤ 0.05); ᶜYoung Women vs. Old Women, BH₄ supplement (p ≤ 0.05).
FIGURE 1. Pressure-strain elastic modulus $[E_p]$ of the brachial artery (A) and common femoral artery (B) of healthy young men, older men, young women, and older women at rest in response to placebo and BH$_4$ treatment.

Values are mean ± SE. * significant difference in BH$_4$ response within group ($p \leq 0.05$). + significant difference Young Women vs. Older Women, BH$_4$ supplement ($p \leq 0.05$).
FIGURE 2. Relative diameter change of the brachial artery (A) and common femoral artery (B) of healthy young men, older men, young women, and older women at rest in response to placebo and BH4 treatment.

Values are mean ± SE. * significant difference in BH4 response within group (p ≤ 0.05). + significant difference Young Women vs. Older Women, BH4 supplement (p ≤ 0.05).
FIGURE 3. Stiffness index [$\beta$] of the brachial artery (A) and common femoral artery (B) of healthy young men, older men, young women, and older women at rest in response to placebo and BH$_4$ treatment.

Values are mean ± SE. * significant difference in BH$_4$ response within group (p ≤ 0.05). + significant difference Young Women vs. Older Women, BH$_4$ supplement (p ≤ 0.05).
FIGURE 4. Individual pressure-strain elastic modulus \([E_p]\) values of the brachial artery in healthy young men (A), older men (B), young women (C), and older women (D) at rest in response to placebo and BH\(_4\) treatment.
FIGURE 5. Individual relative diameter changes of the brachial artery in healthy young men (A), older men (B), young women (C), and older women (D) at rest in response to placebo and BH₄ treatment.
**FIGURE 6.** Individual pressure-strain elastic modulus \([E_p]\) values of the common femoral artery in healthy young men (A), older men (B), young women (C), and older women (D) at rest in response to placebo and BH\(_4\) treatment.
FIGURE 7. Individual relative diameter changes of the common femoral artery in healthy young men (A), older men (B), young women (C), and older women (D) at rest in response to placebo and BH₄ treatment.
DISCUSSION

Age Group Comparisons in Men

During the placebo visit, $Ep$ and stiffness ($\beta$) estimates of the common femoral artery of older (compared to younger) men were not significantly different. The absence of any age differences in the elastic properties of this artery is consistent with most previous studies restricted to healthy male subjects across this age range.$^{19-22,50}$ The observation that the femoral artery does not stiffen with aging under quiet resting conditions in healthy men was reflected in the parameters that are used to calculate the $Ep$ and stiffness index ($\beta$). Relatively small distention of the men’s femoral arteries with each heart beat (~2.3% diameter change in both age groups) and the similarity in resting systolic, diastolic, and pulse pressures (126/76 vs. 128/76 mmHg in younger and older men, respectively) provide such evidence.

Acute BH$_4$ supplementation did not appear to influence elastic properties of the femoral artery in either group of men. This is not entirely unexpected since previous studies examining long-lasting increases in systemic vasodilation (i.e., via exercise training) have failed to demonstrate a significant increase in the distensibility of this artery in either younger$^{42-44}$ or older$^{51}$ men.

There were also no overt, statistically significant age group differences in the elastic properties of the brachial artery in these men during either condition (placebo or BH$_4$). However, close examination of the individual changes in brachial artery stiffness ($\beta$) (data not shown) and its determinants (relative diameter change, systolic, and diastolic pressures) across conditions (i.e., BH$_4$ relative to placebo) suggests a more consistent de-stiffening of the brachial artery in the older men (6 of 7) compared to the younger men.
(2 of 6). This could reflect a subtle vasodilatory effect of BH₄ in the older men that normalizes brachial stiffness (β) to that of the younger men, greater variability in brachial artery stiffness at baseline (placebo) in the younger men, or a combination of these effects.

In summary, age- and BH₄-specific effects were not observed in the common femoral artery in this sample group of men. Additionally, though the Ep showed no change in response to BH₄ in either age group of men, close inspection of individual stiffness (β) responses to BH₄ suggests it may have de-stiffening potential in the brachial artery. The artery-specific nature of these BH₄-induced effects in men should be examined further. Such effects, if supported through further research, could reflect greater age group differences and/or heterogeneity in habitual upper versus lower body physical activity patterns (i.e., a greater prevalence of upper body strength training in young compared to older men). For this reason, additional studies on this question should carefully screen and control for age differences in habitual upper and lower body physical activity.

**Age group comparisons in women**

Similar to men, no differences in Ep, β, or relative diameter change were observed in either the brachial or the common femoral arteries in younger and older women during the placebo visit. Such findings are both inconsistent and consistent with previous studies.⁶,¹⁶,¹⁷,¹⁹,²¹,²²,⁵² The different findings between studies investigating age and sex differences in muscular artery stiffening likely depend on a number of study-specific factors including the type/sensitivity of methods used, the health and dietary habits of
study subjects, and variation in subject sample size. Gender differences in central artery stiffening with aging, by contrast, are commonly observed despite these disparate methodological differences and subject characteristics between studies. As observed in past research, elevated pulse pressure, indicative of stiffer central circulation, was displayed more consistently in our older women in comparison to our younger women. In contrast, our older men exhibited very similar pulse pressure relative to their younger counterparts. Collectively, these findings suggest that aging has more consistent sex-dependent effects on the distensibility of central elastic arteries than it has on peripheral muscular arteries in healthy adults, likely due to the widespread vascular effects of estrogen and its loss after menopause.

Younger women experienced no effect on stiffness in the peripheral arteries under the BH₄ condition. On the other hand, Ep values did decrease in both the brachial and common femoral arteries in older women in response to BH₄ supplementation. These findings support the idea of an age dependent effect on peripheral muscular arteries in women in response to acute BH₄ consumption.

The mechanisms responsible for the improved peripheral arterial distensibility in older women may be linked to local alterations in vessel wall structural and/or alteration in vascular tone created under the BH₄ condition. Estrogen has been shown to both increase the elastin/collagen ratio and maintain vascular endothelial function, with pre-menopausal women displaying a greater endothelium-dependent dilation than men and post-menopausal women. Though BH₄ supplementation is just beginning to be used as a substance intervention in human studies, BH₄ supplementation has already been shown to improve vascular endothelial function in ovariectomized rats. Furthermore,
while Moreau et al. (2007) has reported that oxidative stress contributes to chronic leg vasoconstriction in postmenopausal women and our lab has reported that hormone therapy preserves smooth muscle structure and dilation in the arterial vasculature in the leg of older women, estrogen therapy has been shown to replenish vascular BH₄ levels and reduce oxidative stress in those ovariectomized subjects.

Subsequently, even though an increase in stiffness (β) and an attenuated relative diameter change was recorded in the brachial artery, no other structural determinants changed in response to BH₄ for the common femoral artery (although group x treatment relation was trending towards significance for both factors). These results support the notion that aging may have less of a vascular endothelial effect on the arteries in the leg as compared with those in the arm.

**Study Limitations**

Key limitations of the present study include 1) small sample sizes per group, 2) postural differences during brachial (fully supine) versus femoral (semi-recumbent) testing, and 3) temporal (during the brachial testing) and regional (during femoral testing) disparities of the arterial diameter versus pressure measurements used to calculate elastic properties. Additionally, there was considerable variation in the absolute values of the elastic properties for both arteries, a common occurrence in studies using these variables. It is also unclear whether the oral dose of BH₄ we used (10mg/kg body weight) was sufficient to significantly (and uniformly) increase BH₄ concentrations and bioavailable nitric oxide in all four subject groups. These limitations and uncertainties are balanced by the fact that we used a placebo controlled (and experimenter blinded) study
treatment design in which each subject served as his or her own control. Thus, the major new finding that older women exhibited BH$_4$-induced reductions in stiffness indices in two difference muscular arteries is valid and should be viewed as a robust physiological finding.
CONCLUSIONS AND SIGNIFICANCE

Consistent with the findings of many previous cross-sectional studies, we observed no age group differences in either brachial or femoral artery elastic properties. The absence of any age differences in both sexes and in more than one peripheral site (arm and leg), supports the general consensus in the literature that peripheral muscular arteries do not stiffen to a significant degree with advancing age in healthy, normotensive adults. The novel finding of the present study is that peripheral artery distensibility can be acutely increased in normotensive older women due to a BH₄-induced effect, which likely reflected improved systemic endothelial vasodilator function in these subjects. The variable effects of acute BH₄ supplementation on peripheral artery stiffness in older men is unexplained, but once again illustrates the sex-dependent nature of the arterial aging process in humans.

The results of this study support the continued use of BH₄ supplementation in clinical research trials. Further research should examine the peripheral (and central) circulatory effects of both acute and chronic BH₄ supplementation in older adults of both sexes. Such information could aid in the development of sex-specific therapies for cardiovascular aging and disease prevention.
REFERENCES


9. Rasmey MW, Jones CJH. Large arteries are more than passive conduits. *Br Heart J* 1994;72:3-4.


APPENDIX – INFORMED CONSENT FORM

Inform consent Form for Biomedical Research
The Pennsylvania State University

Title of Study: Influence of age and sex on baroreflex control of leg vascular conductance at rest and during exercise

Investigators:
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Julie A. Miedlar jam525@psu.edu
Hannah M. Woytowicz hmw142@psu.edu

Why am I being invited to take part in this research?
You are a man or woman between the ages of 21-30 or 60-75 years that is sedentary or recreationally active. You do not have a history of cardiovascular, metabolic, or neurological disease nor are you pregnant. Lastly, you are not taking medications that could affect cardiovascular function or interfere with tetrahydrobiopterin (BH4) supplementation.

Who is doing the study?
This research is being conducted by Matthew A. Barlow, Ph.D., a postdoctoral fellow at Noll Laboratory that is training under the direction of David N. Proctor, Ph.D., an Associate Professor of Kinesiology and Medicine at Pennsylvania State University. Other members of Dr. Proctor’s research team will be assisting with the study.

What is the purpose of this study?
Aging is associated with an increase in leg vasoconstriction both at rest and during exercise. This causes an increase in pressure within the vessel that is an important contributor to the development of vascular disorders such as hypertension. The purpose of this study is to better understand the reason why older humans have high levels of leg vasoconstriction by externally applying pressure to blood pressure receptors in your neck that play a role in controlling leg vasoconstriction levels.

Where is the study going to take place and how long will it last?
This research study will take place in Noll Laboratory and the General Clinical Research Center (GCRC) at Penn State University. Your part of this study will involve 1 screening visit, 1 body composition/treadmill test/familiarization visit, and 2 experimental study visits. The entire study will be completed within approximately 4-6 weeks.
WHAT WILL I BE ASKED TO DO?
A member of the research team will fully explain each procedure that applies to your participation. The study and its procedures are outlined below:

• Visit 1 - Screening Visit. Your height and weight will be measured using standard medical equipment along with vital signs (heart rate and blood pressure) at the GCRC. Medical staff at the GCRC will give you a physical exam and will monitor your resting ECG for the presence of arrhythmias. Venous blood samples will be collected to measure lipid profile, blood chemistry tests, and plasma levels of oxidative stress, BH₄, and Nitric Oxide. If these screening procedures uncover a previously unknown condition(s) you will be advised to contact your personal physician. (1 hour)

______  (your initials)

Visit 2 – DEXA/Treadmill Test/Familiarization Visit. If you are a young woman, a urine sample will be used in a pregnancy test prior to any testing (another test will be required if your involvement in this study exceeds 30 days). You will be asked to visit the GCRC where you will lie on a padded table for assessment of your body composition using DEXA technology. After this test, you will have ECG electrodes attached to your chest so that heart rate and cardiac rhythm can be monitored during a graded treadmill test. For this test, you will be asked to walk and/or run on a motorized treadmill as the incline on the treadmill is increased every 2-3 minutes until you cannot continue. Inspired and expired gases (oxygen uptake) will be measured at your mouth using a mouthpiece, while heart rate, blood pressure, and perceived effort will be monitored throughout the exercise test. (1 hour)

______  (your initials)

Lastly, you will be familiarized with the knee-kick machine and neck suction device used during study visits 3 and 4. Briefly, you will be assisted into a reclined padded chair where your left foot will be strapped into a boot attached to a kicking machine. You will be instructed to extend and relax your upper leg as you kick against no resistance at a specific contraction frequency. Once you are comfortable performing this type of exercise, two head-phone looking devices will be placed on your neck for neck suction. We will apply neck suction at different pressures so you can become familiar with the sensation. After the familiarization, you will return to the GCRC where your vital signs (blood pressure and heart rate) will be monitored before checking out. Additionally, the GCRC staff will provide you with either the antioxidant drug or placebo for the next visit (randomized). (30 minutes)

______  (your initials)

Visit 3 – Study Visit. You will be asked to consume the provided single dose of BH₄ or placebo 3 hours prior to arriving at Noll laboratory. Each drug (BH₄ or placebo) will be mixed in an eight oz. glass of juice (orange, apple, cranberry, etc). When you check in a GCRC nurse will draw (collect) a 6 ml (0.4 tbl) blood sample. If you are a young woman, a urine pregnancy test will be provided by the GCRC staff for you to use in the GCRC before consuming either the BH₄ or placebo 3 hours prior to your exercise visit. If the pregnancy test reading is negative, you will then take the BH₄/placebo and come to the scheduled appointment 3 hours later for blood draw and experiment. However, if a positive reading on the pregnancy test is present, you will not take the BH₄/cellulose placebo and immediately discuss the results with the GCRC staff. Finally, we ask that you schedule an appointment with your physician (Ob/Gyn) to confirm the positive test.
All groups need to bring the unsealed drug vial (empty) back to the GCRC to confirm complete consumption.

Next you will be asked to lie flat on a bed for vascular health measurements. Then a blood pressure cuff will be placed around your forearm and will be inflated for 5 minutes at a pressure that stops blood flow through your forearm. We will place a Doppler probe (plastic device) on the surface of your skin just above your elbow to measure artery size and the speed of blood in this artery before and after the cuff is deflated. Following these measurements, you will be asked to rest in a reclined position for 10-15 minutes before neck suction is applied to acutely stimulate cardiovascular receptors in your neck. Following 10 minutes of recovery, you will be asked to perform knee kick exercise at three workloads (8 minutes each) as neck suction is applied for short periods during exercise. In addition, blood pressure, heart rate, quadriceps muscle oxygenation, and leg blood flow will be measured while you exercise. After exercise is complete, a blood pressure cuff will be placed around your upper leg and inflated for 3 minutes so that blood flow in your leg is stopped and we can measure the lowest level of oxygenation in your quadriceps muscle. Finally, you will return to the GCRC where your vital signs (blood pressure and heart rate) will be monitored before checking out. Additionally, the GCRC staff will provide you with either the antioxidant drug or placebo for the next visit (randomized). (3.0 hours)

______ (your initials)

Visit 4 – Study Visit. You will be asked to consume the provided single dose of BH₄ or placebo 3 hours prior to arriving at Noll laboratory. Each drug (BH₄ or placebo) will be mixed in an eight oz. glass of juice (orange, apple, cranberry, etc). When you check in a GCRC nurse will draw (collect) a 6 ml (0.4 tbl) blood sample. If you are a young woman, a urine pregnancy test will be provided by the GCRC staff for you to use in the GCRC before consuming either the BH₄ or placebo 3 hours prior to your exercise. If the pregnancy test reading is negative, you will then take the BH₄/placebo and come to the scheduled appointment 3 hours later for blood draw and experiment. However, if a positive reading on the pregnancy test is present, you will not take the BH₄/cellulose placebo and immediately discuss the results with the GCRC staff. Finally, we ask that you schedule an appointment with your physician (Ob/Gyn) to confirm the positive test. All groups need to bring the unsealed drug vial (empty) back to the GCRC to confirm complete consumption. (3.0 hours)

______ (your initials)

Visit 5-Neural Activity (Optional) – Study Visit. If you choose to participate in our optional sub-study you will be asked to remain in a seated position while a trained microneurographer will map the course of the peroneal nerve (located near your knee) by stimulating through the skin with a pencil-shaped electrode. Then we will insert two tiny, sterile, needle electrodes through the skin positioning one into the nerve to record the electrical activity at that site. Once we have a good signal of electrical activity we will instrument you with the neck suction device used in visits 2-4. We will apply neck suction at different pressures in order to verify the changes in neck pressure and nerve activity. In addition, blood pressure, heart rate and respiratory rhythm will be measured during the neck suction protocol.

______ (your initials)

The following measurements and procedures are involved in this study (visits 2-4):

- **Venipuncture.** The risks involved with taking blood include some local pain and bruising where the blood is taken. Well-trained and experienced GCRC medical staff
will take your blood. Blood sampling can also cause light-headedness and dizziness. If this occurs, having you lie flat with your feet raised will alleviate the symptoms. As with any procedure involving taking blood, infection is possible. All precautions will be taken to avoid infection. There is a rare risk of developing a clot or swelling of the vein and surrounding tissue from the blood draw.

______ (your initials)

• **Dual Energy X-ray Absorptiometry (DEXA).** You will lie flat on a padded table without moving for approximately 10 minutes while the DEXA machine scans the length of your body.
  ______ (your initials)

• **Oxygen Uptake Measurement.** At rest and during treadmill exercise, oxygen uptake (VO$_2$) will be measured using a mouthpiece, nose clip, and a machine that analyzes your expired air for oxygen and carbon dioxide. Measurements will be made at rest while you are seated and intermittently during the exercise test.
  ______ (your initials)

• **Vascular Health Measurements.** You will lay flat on a bed with blood pressure cuffs on your ankles and arms. Plastic cube-shaped sensors will be placed on your chest, wrists, neck, and upper thigh to measure how fast each pulse of blood travels through your blood vessels. The information collected from this procedure will allow us to estimate the stiffness of the arteries in your upper and lower body as well as the adequacy of blood flow in your leg arteries.

Following this test, a blood pressure cuff will be placed around your forearm and will be inflated for 5 minutes at a pressure that stops blood flow through your forearm. We will place a Doppler probe (plastic device) on the surface of your skin just above your elbow to measure artery size and the speed of blood in this artery before and after the cuff is deflated.

______ (your initials)

• **Neck Suction.** You will be fitted with a neck collar that will encircle the anterior part of your neck for the application of neck suction. Suction will be given in 5 second pulses while you hold your breath at the end expiration for 10 to 15 seconds. You will not have to hold your breath while we apply neck suction during exercise. A minimum of 30 seconds of recovery will be given between neck suction trials to allow blood pressure and heart rate to return to pre-stimulus values.
  ______ (your initials)

• **Knee Extension Exercise.** In a reclined seated position, you will be asked to perform 8 minutes of single-leg knee extension exercise at a mild, moderate, and heavy workload. Each workload will be separated by ~20 minutes to allow the return of cardiovascular variables to baseline levels. During exercise, your left foot will be tightly secured to the kicking machine while you kick at a rate of 30 kicks per minute.
  ______ (your initials)

• **Blood Flow Measurement.** The blood flow entering your thigh will be measured using a Doppler ultrasound machine that produces sound waves to measure blood vessel size and the speed of your blood. This machine will be used when you are at rest and during exercise.
  ______ (your initials)
• **Heart Rate and Blood Pressure.** Heart rate will be measured by placing sticky electrodes on your chest and reading the electrocardiogram (ECG) signal. Blood pressure will be measured with an automated machine that requires the placement of a blood pressure cuff on your upper arm (bicep) and a small cuff on your wrist that will periodically inflate on its own to measure pulse pressure. ______ (your initials)

• **Quadriceps Muscle Oxygenation.** Tissue oxygenation during exercise will be measured by placing sticky electrodes on the quadriceps muscle. A near infrared machine will measure levels of oxygen in the tissue by sending an infrared light into the muscle. ______ (your initials)

• **Placebo (Microcrystalline Cellulose).** Microcrystalline Cellulose is a common non-active ingredient used in pharmaceutical prescriptions and placebo pills. ______ (your initials)

• **Oral BH₄.** Your body naturally makes BH₄ and it is found in your cells. Your body primarily uses BH₄ to make vasodilator substances, but BH₄ can also protect your body’s cells from oxidative injury by shutting down waste products that produce reactive oxygen molecules in cells. You will be asked to take a single dose (10 mg/kg of body weight) of BH₄ three hours before your last study visit by dissolving the BH₄ tablet in 8 ounces of water or orange juice. If you weigh 70 kg (157 lbs), you would have to take 700 mg of BH₄. A handout will be given to you with information about BH₄ that will include instruction on how to store and take BH₄ along with possible side effects. ______ (your initials)

• **Neural Activity (Microneurography) (Visit 5 Only):** We will record activity (nerve signals) in the peroneal nerve (located near your knee). First, we will map the course of the nerve by stimulating through the skin with a pencil-shaped electrode. When we electrically stimulate the nerve, you will notice involuntary twitching or tingling sensations in the lower region of your leg. The twitching or tingling sensations will disappear when the stimulation is stopped. Once we have located the nerve, we will insert two tiny, sterile, needle electrodes through the skin. This is done without local anesthesia since the needle electrodes are so small they do not produce appreciable pain when inserted. One needle electrode is inserted just under the skin a short distance away from the nerve. The other needle will be positioned to contact your nerve. When the needle enters the nerve you will once again notice involuntary twitching or tingling. We will position the needle electrode to cause twitches without tingling sensations, and move it to obtain a good recording of your neural activity. These adjustments may take up to 1 hour. When we have obtained a good recording, we will begin the experiment. You will probably be unaware of the needle electrode once we stop adjusting its position (your leg may feel like it is falling asleep). It is important that you keep your leg very still to maintain good recording throughout the experiment, which will last approximately 1 hour. After the experiment, the needle electrodes will be removed by simply pulling them out of the skin. Since they are very small, there is no need to numb your skin before the needles are inserted or removed. ______ (your initials)

**WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?**
It is not possible to identify all potential risks associated with these research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

- **Venipuncture.** The risk associated with blood sample collection obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, dizziness and nausea, and bruising. Thrombosis, embolism, and infections are very rare but are also potential risks. ______ (your initials)

- **DEXA Scan.** The Dual Energy X-ray Absorptiometry (DEXA) bone density procedure exposes an individual to a small amount of radiation where the X-ray beam crosses the body. The radiation exposure is not necessary for your medical care and is for research purposes only. This protocol calls for a total body scan that may be repeated several times over the course of this protocol. The dose for one total body scan is equivalent to a whole body radiation dose of about 1.5 millirem.

  A millirem is a unit of whole body radiation dose. For comparison purposes, the average person in the United States receives a radiation exposure of 300 millirem per year from natural background sources, such as from sun, outer space, and from radioactive materials that are found naturally in the earth’s air and soil. 1.5 millirem is less than you would receive from 2 days of natural background radiation. ______ (your initials)

- **Blood Pressure/Vascular Health Measurements.** There is a risk of temporary discomfort at the sites where blood pressure cuffs are inflated. The discomfort might be greater the longer the cuffs are inflated. In addition, you may feel a tingling sensation in the finger or toes while the cuff is inflated; however, this feeling goes away quickly after the cuff is deflated. ______ (your initials)

- **Doppler Ultrasound.** There is a minimal risk that the ultrasound probe will irritate your skin. ______ (your initials)

- **Heart Rate.** There is a minimal risk that an allergic reaction could occur from the adhesive on the ECG electrodes. ______ (your initials)

- **Neck Suction.** There is a minimal risk of neck discomfort, dizziness, and fainting from neck suction. There is also a risk of plaque rupture in people with carotid artery atherosclerosis ______ (your initials)

- **Maximal Graded Treadmill Test.** There is discomfort associated with exercise testing to maximum effort, including temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience lightheadedness, chest discomfort, cramping in the legs, irregular heartbeats, and irregular blood pressures during this test. The risk of life-threatening problems (such as a heart attack) is very rare (1 in 2500 tests). Other potential risks, including fainting, nausea, muscle strain, muscle soreness, or falling, will be minimized by proper warm-up, familiarization procedures, and cool-down. GCRC staff will closely watch you throughout exercise and recovery. ______ (your initials)
• **Knee Kick Exercise.** There is a slight risk of thigh muscle strain and muscle soreness resulting from this exercise. Muscle soreness may be present the following day.  

  ______ (your initials)

• **Quadriceps Muscle Oxygenation.** There are no known risks associated with use of the infrared machine. Discomforts for the near infrared adjustment procedure include slight bruising from the blood pressure cuff used to block blood flow as well as a moderate rise in blood pressure and minor rise in heart rate during cuff occlusion. Also, you may experience discomfort and numbness in the occluded leg. These sensations go away quickly after the cuff is released and blood flow is restored.  

  ______ (your initials)

• **Placebo (Microcrystalline Cellulose).** Microcrystalline Cellulose is a common non-active ingredient used in pharmaceutical prescriptions and placebo pills. Researchers have not observed any digestive absorption or toxic effects of cellulose consumption in human or animal. No gastro-intestinal disturbances are expected.  

  ______ (your initials)

• **Oral BH₄.** Tetrahydrobiopterin (BH₄) is a substance found in the body of healthy individuals and is not expected to cause any complications. In the United States, physicians prescribe a similar BH₄ pill called “Kuvan” to treat Phenylketonuria (PKU), a condition where a liver enzyme is missing. In a clinical trial investigating this drug in PKU patients, researchers did not observe any severe allergic reactions, but rather the most serious bad reactions reported included stomach problems, spinal cord injury, infection, testicular cancer, and urinary tract infection. However, these serious problems may not have been related to taking BH₄ since the number of bad reactions in PKU patients using BH₄ was similar to that reported by patients taking a placebo pill. Mild to moderate allergic reactions could include redness, itching, rash, and/or swelling. In addition, 4% of PKU patients were found to have a mild to moderate fall in the number of white blood cells while using BH₄. The most common problems (>4% of PKU patients) were headache, dizziness, diarrhea, vomiting, stomach pain, runny or stuffy nose, cough, sore throat, and nausea. You will be given a handout that will include a list of these and other possible side effects and what to do if you think that you are having problems.  

  ______ (your initials)

• **Neural Activity (Microneurography) (Visit 5 Only):** There have been no significant medical complications resulting from this procedure. About 7% of subjects experience some aching at the recording site or “pins and needles” sensations below the recording site for a few days after the procedure. Some people report their lower leg to be slightly weaker for a few days (similar to sensations you might feel after jogging), probably because of the muscle twitches they experienced. To minimize chances of any problems, you should not rub the site or perform heavy leg activity for at least 24 hours after the experiment. Additionally, to minimize the risk of infection, needle electrodes are sterilized and your skin will be cleansed with alcohol prior to insertion and after removal of the needle electrodes.  

  ______ (your initials)
WILL I BENEFIT FROM TAKING PART IN THIS STUDY?
There are no direct benefits for you in participating in this study.

DO I HAVE TO TAKE PART IN THE STUDY?
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits, clinical care, or treatment to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE?
There is no cost to you for participating except that associated with your transportation to our research facilities at Noll Laboratory.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other participants taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. 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.). In the event of any scientific publication resulting from the research, no personally identifiable information will be disclosed.

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records which will be given a code number. Your name and the associated code number will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. The Penn State University Office of Research Protections, the Penn State Institutional Review Board, and the Office of Human Research Protections in the U.S. Department of Health and Human Services may review records related to this research study.

CAN MY TAKING PART IN THE STUDY END EARLY?
Your participation in the study could end in the rare event of muscle strain or cramps that prevents you from exercising for the required time. In addition, we may not seek your participation if you miss an excessive number of study visits.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THE STUDY?
Yes. Participants will be paid a total of $60 for their participation in the entire study (Visits 1-4). If for some reason you do not complete the study, you will be paid for the visits you did complete with the exception of the screening visit ($0 for visit 1, $20 each for study visits 2-4). Participants will be paid a total of $60 for their participation in the optional study visit 5 (Nerve Activity). Total payments within one calendar year that exceed $600 will require the University to report these payments to the IRB annually. This may require you to claim the compensation that you receive for participation in this study as taxable income.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH?
Please be aware that 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 Pennsylvania State University for injury resulting from negligence of the University or its investigators.

WHAT IF I HAVE QUESTIONS?
Please contact Matthew Barlow (Principal Investigator) at 865-1235 or Sandy Smithmyer (Study Coordinator) at 863-3182 with questions, complaints, or concerns about the study. You can also call this number 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 Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about the research procedures. Questions about research procedures can be answered by the research team.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 4 pages.

____________________________________________
Participant Signature
Date

____________________________________________
Printed name of person agreeing to take part in the study

____________________________________________
Name of person providing information to participant
Date
ACADEMIC VITA of Carly Diana Comer

Carly Diana Comer
1834 London Road• Abington, PA 19001
Email: cdc5105@psu.edu

EDUCATION
The Pennsylvania State University, Schreyer Honors College, University Park, PA
Eberly College of Science
Bachelor of Science in Biology, Vertebrate Physiology option, August 2011
Minor in Business and the Liberal Arts
Interdisciplinary Honors in Kinesiology and Biology
Thesis Title: *Age- and Sex-Specific Effects of Tetrahydrobiopterin Supplementation on Peripheral Artery Stiffness*
Thesis Supervisor: David N. Proctor, Ph.D.

RELEVANT EXPERIENCE
Penn State Vascular Aging & Exercise Lab, Noll Laboratory, University Park, PA
Research Assistant September 2009 – July 2011
• Investigated and completed senior honors thesis on sex differences in peripheral muscular artery stiffness with advancing age and in response to acute BH₄ supplementation
• Analyzed and interpreted data about peripheral arterial distensibility in both healthy women and women with risk factors at different stages of menopause
• Assisted in the recruitment of subjects and execution of clinical research studies

Penn State Molecular Population Genetics & Genomics Lab, University Park, PA
Research Assistant September – December 2008
• Retrieved and stained the complete *Drosophila* genome through the dissection of larvae and extraction of their salivary glands

Penn State Eberly College of Science Biology Department, University Park, PA
Teaching Assistant, Medical Embryology January – May 2011
• Attended class lectures, held weekly evening office hours for students, assisted with quiz design, and proctored midterm exams and final

Mount Nittany Medical Center, State College, PA
Emergency Department & Surgical Center Volunteer May 2010 – January 2011
• Provided comfort amenities and support to patients and their families
• Transported patients to CT, MRI, X-Ray, and Ultrasound tests, makes up litters, and stocks linens
• Assisted post-operative out patients to their cars (specific for Surgical Center)

Children’s Hospital of Philadelphia, Philadelphia, PA
Pediatric Orthopaedic Observership June 2009 & August 2010
• Shadowed pediatric orthopaedic surgeon, Richard Davidson, M.D., during patient appointments and in the OR
**Premier Orthopaedics & Sports Medicine**, Upland, PA  
*Penn State Externship Program*  
May 2008

- Shadowed a physician of sports medicine during patient appointments & small joint procedures and an orthopaedic surgeon in the OR

**LEADERSHIP AND EXTRACURRICULAR INVOLVEMENT**

**Penn State Alumni Association**

*Penn State Lion Ambassadors*
- Served as liaison between the University and its prospective and current students, alumni, and community
- Promoted pride and tradition of the University through campus events & projects
- *Be A Part From The Start 2010* Co-Chair
  - Planned, marketed, and executed *Be A Part From The Start 2010*, an event designed to welcome the new freshmen class to Penn State

**Penn State IFC/Panhellenic Dance Marathon (THON)**

*THON 2011 Morale Committee Member*
- Provided physical and emotional support for the dancers THON weekend and the THON community throughout the entire year

*THON 2010 Hospitality Committee Member*
- Kept the dancers and families fed and hydrated THON weekend

**Phi Gamma Nu Professional Business Fraternity**, Delta Theta Chapter

*Organization member*
- Participated in events and programs framed around one or more of the fraternity’s three pillars- professional development, philanthropy, and social
- Fall 2009 New Member Vice President

**Phi Beta Kappa Lambda Student Organization**

*Founding Vice President (May 2010 – May 2011)*
- Assisted in the development of PBK student executive board and organization
- Planned and implemented events for Phi Beta Kappa honor society members

**AWARDS AND HONORS**

- Student Marshal, Eberly College of Science, Summer 2011 Commencement
- Evan Pugh Scholar Senior Award (2011)
- Schreyer Honors College Academic Excellence Scholarship, Fall 2007-Spring 2011
- Schreyer Honors College Ambassador Travel Grant, London Study Tour Winter 2010
- Phi Beta Kappa, Lambda Chapter of Penn State University, inducted Spring 2010
- Golden Key International Honors Society, inducted Spring 2009
- Dean’s List, all semesters
- The United States Congressional Award, Gold Medal (2007)