THE EFFECT OF 6-WEEKS DAILY BLUEBERRY POWDER INGESTION ON PLASMA ANTIOXIDANT CAPACITY AND OXIDATIVE DAMAGE IN RELATIONSHIP TO SARCOPENIA

A Thesis
by
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Abstract

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Sarcopenia is a loss of muscle associated with reduced physical capacity. Muscle undergoes inflammation followed by rebuilding after weight lifting. This is known as the muscle regenerative stimulus (MRS). Blueberries contain anti-inflammatories and could be crucial in enhancing anti-inflammatory responses associated with weight training. Combining these two regimens may combat sarcopenia. To examine this combined mechanism, 21 participants over age 60 were randomized into blueberry (BB) (n=10) or placebo (PLA) (n=11) groups and given 6-wks of blueberry powder supplementation (38g/d) or a placebo. Individuals reported to the laboratory three times. Visit 1 consisted of a medical screen, diet instruction, anthropometric measurements, and blood draw. Visit 2, 6-wks afterwards, included anthropometric measurements, diet history, and blood samples. Visit 3, 24-hrs following the MRS, involved obtaining final blood samples. Blood was analyzed for ferric reducing ability of plasma (FRAP), a measure of antioxidant capacity, and F_2-isoprostanes, a measure of lipid peroxidation. No differences were observed between groups for age or anthropometric measures. FRAP and F_2-isoprostanes were not significantly

different between BB or PLA. Pre-BB vitamin C and selenium intakes were higher vs. PLA but did not affect FRAP. Blueberry powder, as given for 6-wks, does not alter FRAP or F_2-isoprostanes in an older population. Blueberry metabolites capable of exerting antioxidant effects may be short-lived. Future research should focus on acute supplementation effects.

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INTRODUCTION

Aging is a complex, multifaceted process associated with progressive physiological, molecular, and genetic changes that are responsible for an increased risk for morbidity and mortality.[1] These age-related changes, paralleled with an increased overall life expectancy, highlight the paramount need to identify and implement dietary and lifestyle interventions to preserve overall health and functional independence with increasing age.[2] Several hypotheses in the literature attempt to elucidate these deleterious age-related effects.[3-5] One such hypothesis focuses on the age-related loss of skeletal muscle mass, termed sarcopenia. Sarcopenia is a syndrome best characterized by a progressive loss of skeletal muscle. Skeletal muscle, or lean body mass, is accrued and maintained by the consumption and storage of protein from the diet. Skeletal muscle is also the primary storage site for protein; up to 60% of total body protein is contained within the body's lean mass. Since protein is the building block of lean mass, a decrease in lean mass brought on by sarcopenia leads to a decrease in protein storage, and a decrease in protein storage leads to a decreased ability to build and maintain muscle which exacerbates the condition throughout older adulthood. The condition occurs gradually with increasing age and is strongly tied to a decrease in physical activity, also associated with aging. After the age of 50, there is a progressive decrease in muscle mass at a rate of 1-2% per year. Similarly, muscle strength also decreases by roughly 3% yearly after the age of 60.[6]

One of the potential causes of muscle aging is a reduced ability for the muscle to regenerate due to an exaggerated response to inflammatory mediators, termed "muscle inflammation susceptibility".[7] Old muscle is less efficient at healing itself, compared to young muscle. Conboy and colleagues[8] showed this by observing muscle regeneration between young and old mice exposed to similar damage. Further, when exposing old mice to factors present in the serum of young mice, there was an increase in regenerative capacity after damage. The atrophy of aging muscle may also be caused in part by episodes of incomplete muscle repair throughout adulthood. Whether this is a normal part of aging is not known for sure; however, there is no debate that the muscle's regenerative capacity is compromised with age and following overt muscle damage.[8-10]

Additionally, the examination of cytokines in circulation indicates that the muscles of the elderly have increased and prolonged pro-inflammatory signaling that leads to interference with myogenesis and an overall decrease in muscle mass. The increase in inflammatory mediators may also lead to an increased accumulation of free radicals. Free radicals are a reactive chemical species with an odd number of electrons that seek to pair with another free electron.[11] Harman and colleagues[12] first postulated that free radicals may be in part responsible for the negative effects of aging. He proposed that the aging process and its degenerative consequences are directly related to free radical-induced damage to cells and the body's inability to counterbalance these

damages with endogenous antioxidant defenses. Harmon revised his theory in 1972 by identifying the mitochondria as the culprit behind the physiological process of aging. Higher consumption of oxygen results in higher levels of oxidative damage. Therefore, it could be inferred that mitochondria (consuming the most intracellular oxygen) have greater exposure to oxidative damage leading to a shorter lifespan.[13] With this revision in theory came several other studies affirming the mitochondria as a major component of cellular aging. As such, the once termed "Free Radical Theory of Aging" has since been renamed the "The Mitochondria Free Radical Theory of Aging".[14,15]

Reactive oxygen species (ROS) are one class of free radicals of particular concern. ROS are derived naturally from oxidative metabolism and have a greater reactivity than O_2. The cells of aerobic organisms constantly generate ROS by the addition of a single electron to O_2 molecules. This process subsequently brings about damage to biological macromolecules, particularly lipids, proteins, and nucleic acids. This interface between normal cellular structures and ROS can lead to irreparable damage to the cell and subsequent cell death.[16,17] The identification of free radicals as promoters of the aging process may imply that inhibition might limit the detrimental modifications exerted on the body. Ergo, if molecules with antioxidant capacities can counteract the oxidative damage, these compounds may also play a key role in preventing the onset of age-related health conditions.[18]

Antioxidants are substances able to reduce the rate at which oxidation occurs.[19,20] The main function of antioxidants is to maintain a state of balance by preventing the transformation of ROS and instead converting them to more stable molecules (H_2O and molecular O_2). One of the major sources of antioxidants is the

diet.[21] Increased antioxidant concentrations are inversely associated with isoprostanes, a marker of lipid peroxidation *in vivo*.[22] The type of antioxidant can have a bearing on effect as well as what molecules are affected. Hydrophobicity is one such characteristic that can dictate both the function and location of an antioxidant's action. For instance, fat soluble antioxidants such as vitamin E are more effective inhibiting the peroxidation of lipoproteins. Whereas, antioxidants that are water soluble in nature are particularly efficient in the aqueous phase. Also, interesting to note, antioxidants typically do not act independently. More often than not, the action is in synergy with similar compounds ingested from the diet or compounds produced endogenously.[23,24]

Assuming an important role for free radicals and inflammatory processes in muscle degeneration, an appropriate avenue to test the previously mentioned theory of muscle inflammation susceptibility would be to prevent the local muscle inflammation. Certain foods with high concentrations of antioxidants and anti-inflammatory compounds (anthocyanins) are ideal candidates to test this theory and have already been shown to be beneficial in reducing circulating inflammatory markers and improving muscle recovery following damage.[25,26] Bowtell and colleagues[27] examined the effect of cherry juice on the inflammatory profile of 10 well-trained male participants (age = 27.8 ± 1.6 yr). Participants completed two main trials separated by a two-week washout period and were given 30 mL twice per day of either Montmorency cherry juice concentrate or placebo. Participants were exposed to an intensive unilateral leg exercise by measuring creatine kinase, high sensitivity CRP, total nitrotyrosine, protein carbonyls (PC), total antioxidant capacity and maximum voluntary contraction and found that consumption improved recovery after intensive

exercise. Participants in the experimental group exhibited enhanced recovery of knee extensor maximum isometric strength accompanied by a reduction in serum PC indicative of reduced oxidative damage. These results align with those of Connolly et al.[28] who utilized a randomized, placebo- controlled, crossover design to examine the effect of twice daily ingestion of 12 fluid ounces (fl oz) tart cherry juice blend for eight days, on the symptoms of muscle damage in 14 male college students. Eccentric elbow flexion contractions were performed to obtain measures for isometric elbow flexion strength, pain, muscle tenderness, and relaxed elbow angle.

Results of the study indicated strength loss and pain to be significantly less in the cherry juice trial versus the placebo with no significant effect on relaxed elbow angle and muscle tenderness. Similar benefits of cherry juice were exhibited by Howatson et al.[29] who examined the efficacy of tart cherry juice in ameliorating recovery, reducing muscle damage, inflammation and oxidative stress in 20 recreational marathon runners.

Participants in the study were assigned to either an experimental or placebo group. Participants consumed two, 8 fl oz servings of a tart cherry blend or placebo five days prior to and for 48 hours following a marathon run. Markers of muscle damage (CK, LDH, muscle soreness and isometric strength), inflammation (IL-6, CRP and uric acid), total antioxidant status (TAS) and oxidative stress (thiobarbituric acid reactive species) were examined before and after the marathon race.

Results from the study demonstrated a significantly more rapid recovery time in the cherry group. None of the other damage indices were significantly different. These outcomes may have occurred due to the mitigation of oxidative damage by the high

phenolic capacity of the cherry juice. However, these studies did not directly determine inflammation status, localized muscle response or the potential benefit to aging muscle. Blueberries have a much higher anthocyanin concentration making them desirable to test for effectiveness in restoring the inflammatory profile of aged muscle and further improving the aged muscle regenerative response; thereby, slowing the rate of overall muscle loss associated with age.[30-33]

METHODOLOGY

Approval for this study was obtained from the Institutional Review Board of Appalachian State University. IRB# 15-0035

Inclusion and Exclusion Criteria

Participants recruited for this study consisted of men and women aged 60 years or older. Participants were cleared for physical activity through completion of a health history questionnaire. The results of the health history questionnaire allowed for exclusion of individuals with any disorder, musculoskeletal or other, that would preclude them from completing exercise tests. Individuals with any chronic end-stage disease expected to limit life expectancy to less than one year, induce anorexia, or restrict physical activity were excluded. These conditions include, but are not limited to, heart failure, end-state COPD, malignancy, renal failure, AIDS, or any disease leading to a chronic inflammatory state. Individuals with uncontrolled hypertension, unstable or exercise-induced angina pectoris or myocardial ischemia, uncontrolled diabetes mellitus or other medical conditions that would interfere with testing or increase one's risk of complications during exercise and/or mechanical load injury were excluded from participation. Individuals with seated resting systolic blood pressure >179 mmHg or a diastolic blood pressure >110 mmHg were further excluded. Individuals with a lidocaine allergy were excluded, as lidocaine was used as an anesthetic agent during the muscle biopsy. Individuals on prescription anti-coagulants or persons taking a daily

aspirin not willing to stop were also excluded. Additionally, those individuals with a BMI>30 as well as those receiving androgen, estrogen or anabolic therapy were excluded. Participants did not have a history of regular resistance training during the 6 months prior to recruitment and were not advised not to take prescription anti-inflammatory medications.

Recruitment

Several efforts were made in the recruitment of participants. A request was sent to the faculty and staff of Appalachian State University through the university listserv. Additionally, flyers (Appendix A) were placed at various community agencies in the town of Boone to advertise the study.

Informed Consent

Informed consent (Appendix B) was presented to the potential participant during an information session, where one of the investigators detailed all aspects of the study including procedures, risks, benefits, anonymity and confidentiality, compensation ($225/participant), freedom to withdraw from the study at any time, approval of research and participant responsibilities.

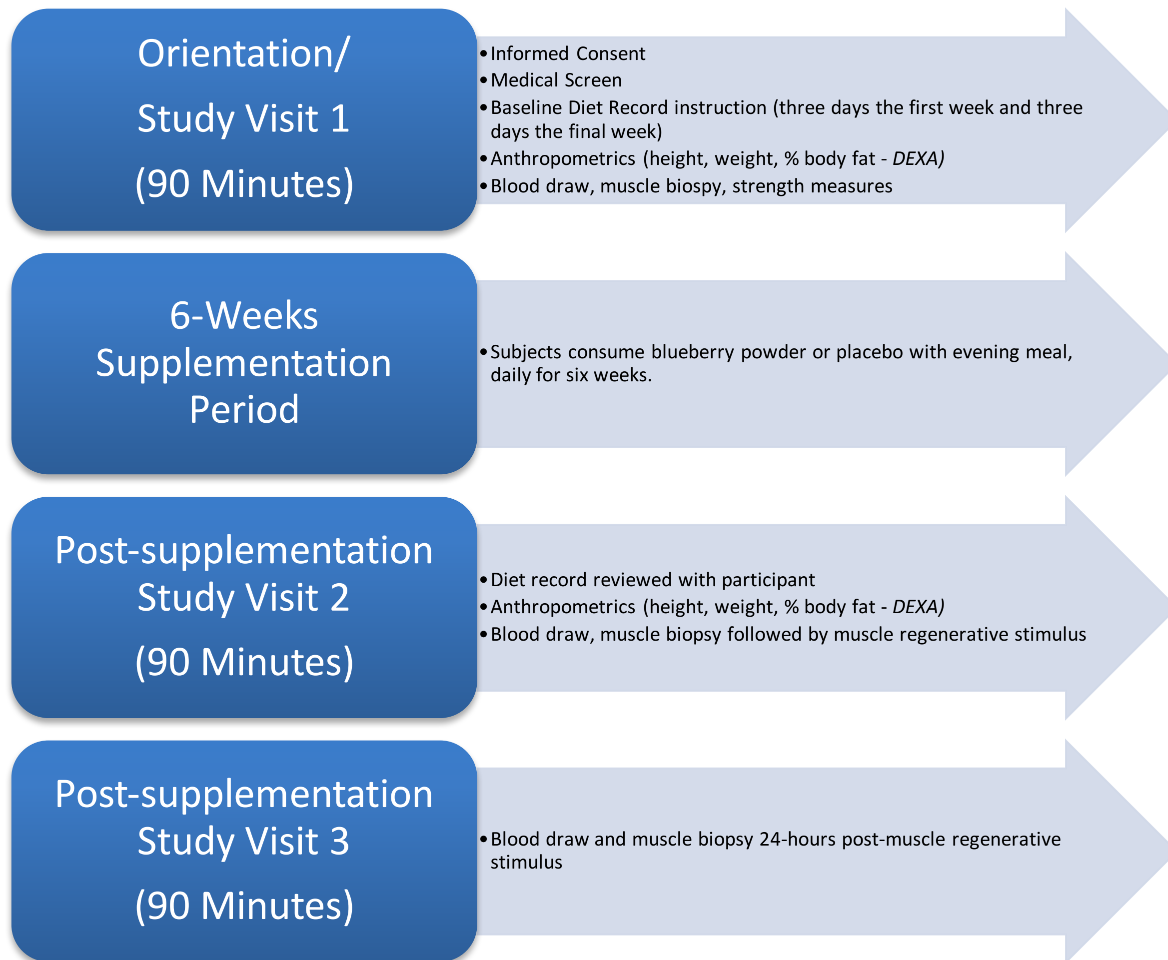
Randomization

Participants were randomized in a double blind fashion to the blueberry (n=10) or placebo group (n=11).

Participant Visits and Timeline

Visit 1 took take place 24-hours after the information session detailed in the above "Informed Consent" description. Visit 1 consisted of reading and signing the informed consent, medical screening (Appendix C), diet instruction and body composition assessment (height, weight, DEXA) immediately followed by blood draw, muscle biopsy and strength measurements. Upon completion of Visit 1, participants began 6-weeks of daily supplementation.

Participants reported back to the laboratory for Visit 2 six weeks later. During this visit, body composition measures were repeated, diet history information was collected and reviewed as needed with participants, and blood and muscle samples were obtained in a similar fashion to Visit 1. Following blood and muscle samples, participants underwent mechanically-induced muscle damage as a stimulus for regeneration (see section, Mechanically Induced Muscle Damage). Visit 3 occurred 24-hours after the muscle regenerative stimulus, when final blood and muscle samples were obtained.



Blueberry/Placebo Supplementation

The supplementation protocol consisted of participants ingesting a total of 38 grams per day of 100% freeze-dried blueberry powder (equivalent to 250g of whole blueberries) or the equivalent gram amounts of placebo packets daily for 6-weeks. Packets were apportioned into labeled week-by-week bags. Participants were blinded to the assignment. Packet contents were not discernable from one another. Participants were instructed to consume packets with 8-ounces of water with the evening meal. See Appendix D for detailed instructions concerning packet

consumption. Participants were instructed to return supplement packaging, empty or otherwise, to its respective weekly bag for return at the conclusion of the study. Upon return, packets were inventoried to determine compliance. Compliance was further measured via weekly e-mail/phone correspondence. Participants were also asked to answer questions based on their experiences during the supplementation period. Answers to these questions were returned and compiled at the conclusion of the study (Appendix D).

Body Composition Assessment

Measures of body composition were obtained by determining each participant's height in the Frankfurt position using a stadiometer[34] weight using a digital scale[35] and muscle/fat mass by a physician prescribed Dual Emission X-ray Absorptiometry (DEXA) scan[36] on each participant at Visits 1 and 2.

Diet Education/Analysis

Each participant was informed on how and when to accurately record dietary intake on a three-day diet record (Appendix F and G) during the first and final week of supplementation. On Visit 2, the returned diet records were reviewed with participant to ensure completeness and clarity. Data from diet records were entered into diet analysis software[37] and examined for total calories, carbohydrate, protein, fat, and select antioxidant micronutrients (vitamin A, vitamin E, vitamin C, copper, manganese, selenium, and zinc). Detailed printouts of nutrient intake were compared to written entries by another member of the research team to identify errors in entry and ensure

accurate data entry. Data from three-day diet records were analyzed to determine differences in dietary intake within and between groups at baseline and 6-weeks post-supplementation.

Strength Measurements**

*(** Denotes procedure or analysis carried out by Exercise Science researchers)*

Prior to experimental procedures, participants' knee extension 1 – repetition maximum was determined according to protocol designed by faculty within the Exercise Science Department according to a predetermined protocol. The resulting measure was used to calculate subsequent stimulus needed for mechanically-induced muscle damage.

Mechanically Induced Muscle Damage**

Participants executed a series of resistance exercises in order to force volitional fatigue. This was carried out under the supervision of researchers from the Exercise Science department according to a predetermined protocol.

Blood and Tissue Collection**

Muscle biopsies and blood draws were completed in the morning after an overnight fast. Blood samples were obtained from the antecubital vein by a qualified phlebotomist. Plasma was isolated from whole blood by centrifugation and stored at -80°C. Muscle samples were obtained under local anesthetic (1% lidocaine) from the vastus lateralis by percutaneous needle biopsy by a qualified professional, under the

supervision of a licensed physician. Participants were supplied with a handout detailing how to care for the site of the biopsy (Appendix H). Muscle samples were snap frozen in liquid nitrogen and stored at -80°C for analysis by Exercise Science researchers according to a predetermined protocol.

Sample Analysis/Assays

Total antioxidant capacity of the plasma was determined for each sample, from each visit, by measuring FRAP (ferric reducing ability of plasma) and oxidative damage assessed by measurement of 8-Isoprostane (Appendix I and J).

FRAP

Total plasma antioxidant potential was determined by the FRAP assay according to the methodology of Benzie and Strain [1]. The basis of this assay is that water soluble reducing agents (antioxidants) in the plasma will reduce ferric ions to ferrous ions, which then react with an added chromogen. Working FRAP solution was prepared daily and consisted of 300 mmol/L acetate buffer with the pH adjusted to 3.6 (3.1 g sodium acetate [Sigma, St. Louis, MO, USA] and 16 mL od 1N acetic acid [Sigma] per liter of buffer solution); 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) [Sigma] in 40 mmol hydrochloric acid (HCl) [Fisher Scientific, Pittsburgh, PA, USA]; 20 mmol iron trichloride hexhydrate (ITX) [Sigma] in doubly distilled deionized water. Working FRAP reagent was prepared as required by mixing 25 mL acetate buffer, 2.5 mL ITX solution, and 2.5 mL TPTZ solution. The working FRAP solution was placed in a water bath and warmed to 37° C. Then, 10 μL of blank, samples, and ascorbate STDs were

transferred by micropipette into designated well of 96 well plate. Three hundred μL of FRAP reagent was then added to all wells containing blank, samples, and STDs. The 96 well plate was then incubated for 4 minutes at 37° C before being read at 593 nm in a spectrophotometer (Genesys-5; Thermo Spectronic, Rochester, NY, USA). Samples and standards were analyzed in triplicate, and FRAP values were expressed as vitamin C equivalents as determined by linear regression from a vitamin C curve (0-1000 μmol).

F_2-Isoprostanes

The Express ELISA Kit (Cayman Chemical Company, Ann Arbor, MI) was used according to the manufacturer's instructions. The 8-IP assay was based on the principle of competitive binding between sample 8-IP, 8-IP acetyl cholinesterase (AChE) conjugate, and 8-IP tracer for a limited number of 8-IP-specific rabbit anti-serum binding sites. Fifty microliters of sample was added to each well, and 50 μL of 8-IP AChE tracer was added to all wells except total activity and blank wells. Fifty microliters of 8-IP enzyme-immunoassay antiserum was added to all wells except total activity, nonspecific binding, and blank wells. The plates were covered and incubated at 4°C for 18 h and then washed 5 times with buffer. Two hundred microliters of Ellman's reagent was added to each well, and 50 μL of tracer was added to total activity wells only. Plates were covered with plastic films for 90 min, and absorbance was read at 420 nm. The amount of 8-IP tracer bound was inversely proportional to the concentration of 8-IP in the sample.

Statistical Analysis

Statistical design was a 2 treatment x 3 times repeated measures ANOVA for main variables of FRAP and F2-isoprostanes using SPSS (IBM® SPSS© Version 22). Descriptive measures were compared using a paired *t-test.* All values are mean +SD.

RESULTS

General participant characteristics are shown in the Table 1. Age, height, weight, and body mass index were not different between groups. Plasma redox status was not affected by treatment, time, or treatment-time interactions as FRAP values (treatment, P = 0.065; time, P = 0.532; treatment-time, P = 0.428) did not exhibit significant differences between groups *(Figure 1)*. However, an increasing trend was observed for treatment (P = 0.065). Similarly, oxidative damage by measure of plasma levels of F_2-Isoprostanes did not differ between groups for treatment, time, or treatment-time interactions (treatment, P = 0.252; time, P = 0.482; treatment-time, P = 0.084) *(Figure 2)*. However, a trend was observed for treatment-time interaction (P = 0.084).
No significant differences were observed in diet (total calories, carbohydrate, protein, fat, vitamin A, vitamin E, vitamin C, copper, manganese, selenium, and zinc) between or within groups before or after treatment, with the exception of higher vitamin C and selenium levels pre-BB *(Raw data for participant diet records are presented in Appendix K)*. These higher intakes, however, did not affect measures of plasma redox.

Table 1. Participant Descriptives. Data are Mean ± SD.

	BB Pre	BB Post	PL Pre	PL Post	P
Age (y)	65 ± 5.32	—	66.75 ± 5.48	—	0.729
Height (m)	1.69 ± 0.09	—	1.64 ± 0.08	—	0.214
Weight (kg)	73.39 ± 10.47	73.41 ± 10.27	65.16 ± 12.94	65.57 ± 12.97	0.452
Body Mass Index (kg/m²)	25.60 ± 2.09	25.60 ± 3.18	24.11 ± 3.15	24.26 ± 3.18	0.374

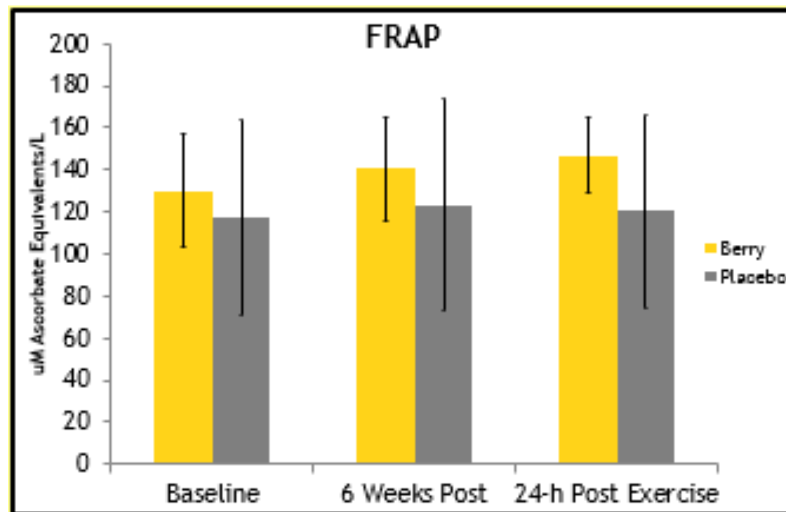


Figure 1. Ferric Reducing Ability of Plasma (FRAP). Data are Mean ± SD (treatment, P = 0.065; time, P = 0.532; treatment-time, P = 0.428)

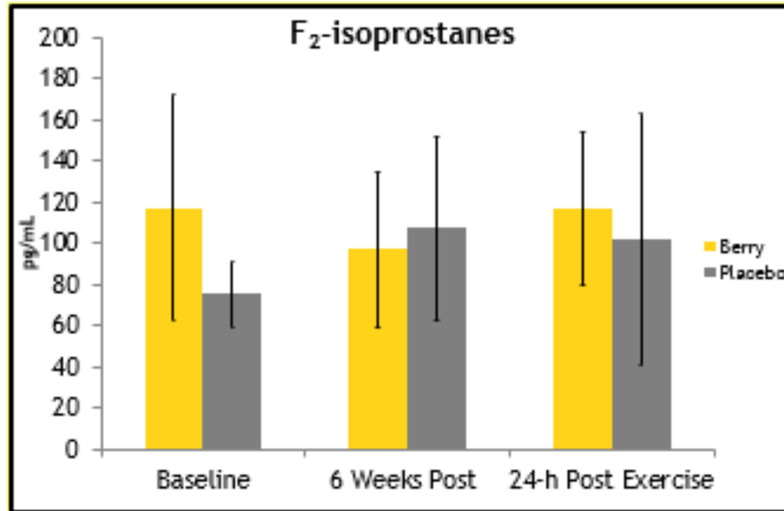


Figure 2. F_2-isoprostanes. Data are Mean ± SD.
(treatment, $P = 0.252$; time, $P = 0.482$; treatment-time, $P = 0.084$)

DISCUSSION

The findings of this study show that six weeks daily ingestion of freeze dried blueberry powder (equivalent to 250 g rehydrated berries) did not have a significant effect on antioxidant capacity or oxidative damage in aged skeletal muscle. A limitation to this study, similar to the majority of human intervention studies, was low statistical power as a result of low participant numbers. Another potential limitation of the study was the relative homogeneity of the participants. All participants of the study were white, non-Hispanic and predominantly female. In healthy muscle, amino acids and proteins are held in a relative balance between their breakdown and synthesis. In the muscle of the elderly, this balance is disturbed due to decreased myogenesis and an increased breakdown of myofibril and mitochondrial proteins. As mentioned earlier, it has been hypothesized that the onset and exacerbation of sarcopenia could be due in part to a low-grade chronic state of inflammation that increases linearly with age. In addition to improper and inadequate healing from muscle related injuries, part of the reasoning behind this could in part be due to a reduction in food intake that often accompanies increasing age.

Therefore, the idea of exogenous antioxidant supplementation in addition to the diet of older adults could prove to be a promising treatment in correcting dietary inadequacies and counteract the increased protein catabolism leading to skeletal muscle decline.[2] A study by Khan et al.[38] examined the effects of dietary intake of a blackcurrant juice drink, rich in vitamin C and polyphenols, on oxidative stress and

vascular function. In this double-blind, placebo-controlled, parallel group study, 66 healthy adults (mean age of 53 years) with low fruit and vegetable intake were randomly assigned to consume 250ml of placebo, low blackcurrant juice, or high blackcurrant juice drink four times a day for 6 weeks. Flow-mediated dilation (FMD), plasma concentrations of F_2-Isoprostane, and vitamin C were measured.

Results of the study indicated that the high and low blackcurrant groups FMD and vitamin C concentration increased significantly compared with the placebo group. F_2-Isoprostane concentrations were significantly lower in the high blackcurrant juice drink group, compared with the low blackcurrant juice drink and placebo group. Results of this study provided support that consumption of blackcurrant juice, high in vitamin C and polyphenols, can decrease oxidative stress and improve vascular health in individuals with habitually low dietary fruit and vegetable intake. Another study by McAnulty et al.[39] observed the effect of blueberry ingestion on natural killer cells, oxidative stress and inflammation on 25 well trained participants, before and after 2.5 hours of running. Participants in the study were divided into two groups, blueberry and control. The blueberry group was provided with and asked to consume 250g of whole blueberries per day for 6 weeks and a larger dose consumed one hour before running 2.5 hours at ~72% of maximum oxygen consumption. Blood and urine samples were obtained pre- and immediately post- exercise and then again one hour later. Blood was examined for F_2-isoprostanes, cortisol, cytokines, homocysteine, leukocytes, T-cell function, natural killer (NK), lymphocyte cell counts, and ferric reducing ability of plasma.

Results of this study showed increases in F_2-isoprostanes to be significantly less in the blueberry group compared to the control group. Further, plasma IL-10 and NK cell counts were significantly greater in blueberry vs. control. Other markers did not differ. The results of this study indicate that daily blueberry consumption for 6 weeks increases NK cell counts, and acute ingestion reduces oxidative stress and increases anti-inflammatory cytokines.

Lynn and colleagues[40] investigated the effect of a tart cherry juice supplement on arterial stiffness and inflammation in healthy adults aged 30-50 years. In this randomized control trial, participants consumed 30 ml of cherry concentrate diluted to a volume of 250 ml with water or the same volume of an energy matched control drink daily for six weeks. At each of the visits, pulse wave velocity, systolic blood pressure (SBP), diastolic blood pressure (DBP) and body weight were measured and a blood sample was obtained. Blood was examined for the determination of total cholesterol, high density lipoprotein (HDL) cholesterol, CRP and total antioxidant activity (ferric reducing ability of plasma; FRAP). The study concluded that there was no effect of the intervention on arterial stiffness, CRP, SBP, DBP, total cholesterol and HDL cholesterol. However, antioxidant capacity (FRAP) was significantly higher in the intervention group than the control group. The study therefore concluded that a tart cherry juice concentrate, rich in anthocyanins had no effect on arterial stiffness, CRP and risk markers for cardiovascular disease, but evokes an increase in antioxidant status in healthy adults.

Notwithstanding the overwhelming clinical significance of sarcopenia and great interest for antioxidant supplementation, the amount of clinical evidence available is

limited and somewhat acrimonious. This could in part be due to the fact that there is no clinical definition for sarcopenia. The majority of studies conducted, investigating physical performance and antioxidant supplementation, have focused on measures of muscular strength and protection from athletic injuries without exclusively examining sarcopenia and muscle loss in the geriatric population. For example, Jakeman and colleagues[41] observed vitamin C supplementation to have a protective effect against exercise-induced muscle damage. Resveratrol, found in berries, grapes, and peanuts is a polyphenol that has been shown to attenuate oxidative stress in skeletal muscle by expression of antioxidant enzymes.[42] Another study by Ryan and colleagues[43] showed that after more than four weeks of training, vitamin C and E supplementation was able to reduce muscular levels of oxidative stress in the muscles of repetitively loaded older mice; however, no increase in muscle mass or maximal force production was observed. Conversely, there are several studies that have shown the potential negative effects of antioxidant supplementation. One study by Strobel et al.[44] showed that after 14 weeks of vitamin E and α-lipoic acid supplementation, post-treatment antioxidant levels were actually decreased from baseline levels. Several other studies showed there to be no effect at all. Theodorou et al[45] found no significant effect on muscle performance after supplementing with ascorbic acid and tocopherol. Similarly, a study that examined mitigating signs of premature aging (muscle wasting, joint ossification, corneal inflammation, cataracts) in BMAL1 knockout mice with N-acetyl-L-cysteine, resulted in a slightly extended lifespan but had no significant effect on sarcopenia.[46]

There are several potential reasons for the inconsistencies in the results of these studies attempting to shine light on whether antioxidant supplementation can have a

beneficial effect on age-related muscle wasting. On the one hand, it is possible that sarcopenia and oxidative damage are entirely independent of each other. It is also important to note that when testing interventions in the geriatric population, it is very difficult to obtain a truly homogeneous pool of participants. Due to the general novelty of this field of study, there is a dearth of experiments with similar methodologies for use of comparison. Variations in the type and dose of antioxidants administered as well as the timing of supplementation and methods used to measure antioxidant biomarkers *in vivo* are all exceptionally inconsistent in the literature.

CONCLUSION

In conclusion, ingestion of freeze dried blueberry powder (equivalent to 250 g rehydrated berries) did not have a significant effect on antioxidant capacity or oxidative damage on aged skeletal muscle. Trends were observed for plasma redox status, treatment (P = 0.065) and F_2-Isoprostanes, treatment-time (P = 0.084) lending to the idea that perhaps future studies should examine more closely the acute effects of antioxidant supplementation with blueberries on plasma redox and oxidative damage. Therefore, at this time, we reject our hypothesis that blueberry consumption has a statistically significant benefit on antioxidant capacity and inflammation in relation to sarcopenia in older adults. Theoretically, as the concentration of antioxidants in the blood rises, an inverse effect on F_2-Isoprostanes would be observed, therefore offering a protective effect against oxidative damage. However, the results of this study proved to be inconsistent with this theory.

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APPENDIX A

Recruitment Flyer

Looking for Research Subjects

Researchers with Appalachian State University's College of Health Sciences are looking for moderately active men and women **over 60 years old** to participate in a study to help determine ways to improve muscle health.
Participants will be required to attend 1 visit on campus followed by a 6-week long blueberry supplementation intervention and 2 subsequent visits.
Participants will gain valuable information about their health and be compensated **$225** (upon completion of the study) for their time and effort.
If you are interested in learning more about this research study, please contact Edward Merritt, Ph.D. by e-mail at merrittek@appstate.edu.

Edward Merritt, Ph.D. merrittek@appstate.edu	Edward Merritt, Ph.D. merrittek@appstate.edu	Edward Merritt, Ph.D. merrittek@appstate.edu	Edward Merritt, Ph.D. merrittek@appstate.edu	Edward Merritt, Ph.D. merrittek@appstate.edu

APPENDIX B

Informed Consent

Consent to Participate in Research
Information to Consider About this Research

The influence of blueberry consumption on the muscular health of older adults.

Principal Investigator: Edward Merritt, Ph.D.
Department: Health and Exercise Science
Contact Information: ASU Box 32071 Boone, NC 28608
merrittek@appstate.edu
205-262-7986
This research is funded by: U.S. Highbush Blueberry Council

What is the purpose of this research?

The purpose of this research study is to determine how daily blueberry consumption affects the muscular health of older adults.

Why am I being invited to take part in this research?

You are being invited to participate in this research study because you are over 60 years old, a non-smoker, not participating in any regular exercise program, and are considered a healthy weight and not actively trying to gain or lose weight, but are otherwise considered healthy.

Are there reasons I should not take part in this research?

If you have a history of cardiovascular, pulmonary, metabolic, or neuromuscular diseases you should not participate in this study. If you have had a lower body musculoskeletal injury in the previous 6 months or have an inflammatory disorder such as rheumatoid or osteoarthritis, you should not participate in this study. If you are currently undergoing any type of hormone therapy, taking prescription anti-inflammatories or anti-coagulants, you should not participate in this study. Additionally, you cannot participate in this study if you are allergic to the local anesthetic, lidocaine or if you have an intolerance or allergy to blueberries.

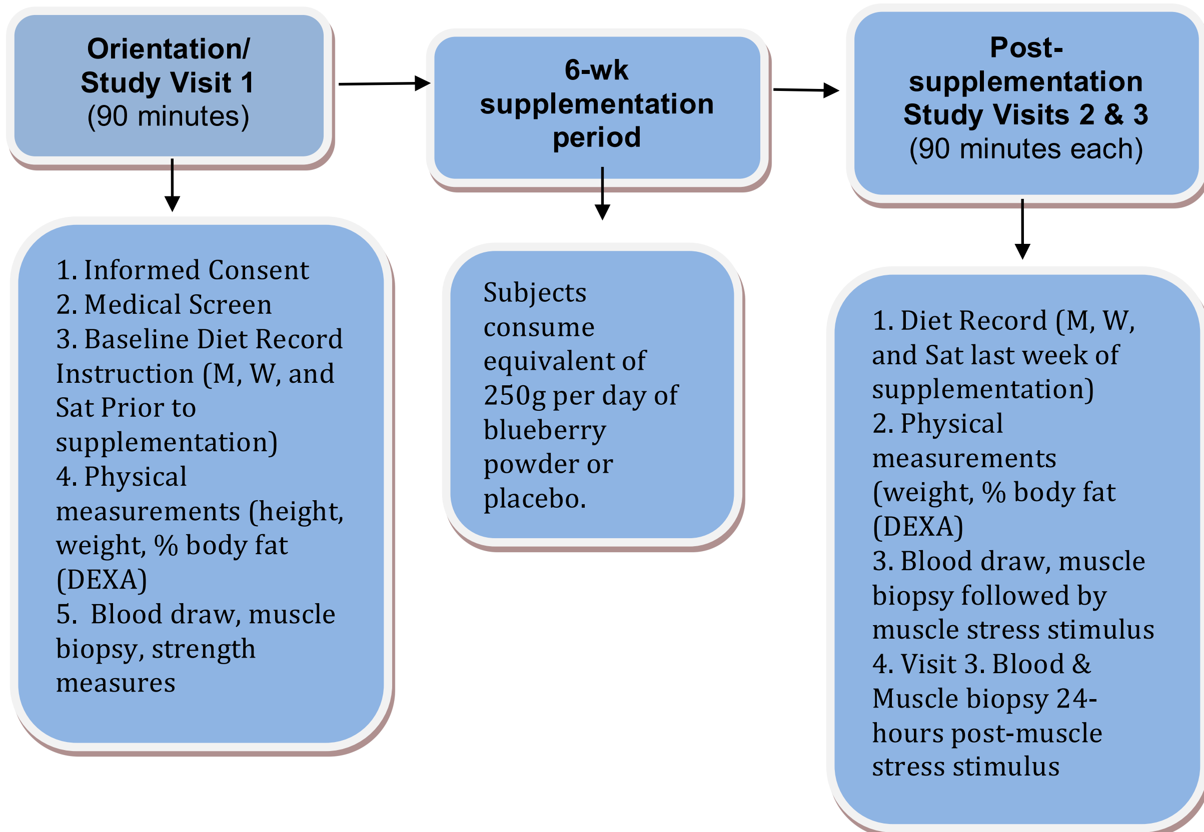
What will I be asked to do?

If you agree to participate you will need to complete one information session and three study visits over the course of the next 6+ weeks. Each study visit will require you to be fasted (no food or drink other than water for 12 hours prior to the study visit) and will take approximately 90 minutes. The total amount of time you will be asked to volunteer for this study is five hours over the course of one 6-week period, although this estimate does not include the time it will take you to mix and consume your supplement (twice daily for 6 weeks) or complete your diet record (~ 10 minutes/day for 3 days during the first and last week of your study enrollment).

You are now partaking in the orientation and information session, which consists of thoroughly reading this form and asking any questions you have about the research study.

During your Study Visit 1, at least 24 hours after the information session, you will complete a health history questionnaire. Your height and weight will be recorded and resting heart rate and blood pressure will be measured. If you are still eligible to participate, you will then have a dual energy x-ray absorptiometry (DEXA) scan to determine your body composition. You will have to lie perfectly still on the machine for approximately five minutes. Immediately following the DEXA scan, you will perform a maximal strength test, followed by a blood draw and a muscle biopsy (described below). If you take aspirin on a regular basis, we ask that you do not take it 48 hours before or after the muscle biopsies, because it can lead to excessive bleeding and bruising (One biopsy will be done during each study visit).You will be randomly assigned to the blueberry supplement or placebo supplement group and begin 6-weeks of daily supplementation following Visit 1. We will provide you with a supply of the supplement or placebo at this visit. You will also be given instructions on how to keep a 3-day diet record and asked to do so during the first and last week of supplementation. You agree not to initiate a formal exercise program and maintain your normal diet over the course of the 6-weeks.
You will report back to the lab after the 6 weeks of supplementation for Visit 2. During this visit body composition measures, diet history, and blood and muscle samples will be obtained before you undergo a muscle stress stimulus (described below). Visit 3 will occur 24-hours after the muscle stress stimulus. The final blood and muscle samples will be obtained at this time.

Subject Timeline:



Strength Measurements (Study Visit 1)

Prior to supplementation and other experimental procedures, your knee extension 1-repetition maximum strength will be determined. After a 5 minute warm-up on the treadmill or stationary bike, your maximum voluntary contraction knee extension force will be measured using a Cybex knee extension machine. The resulting force output will be used to calculate the subsequent stimulus needed for mechanically-induced muscle damage.

Blueberry/Placebo Supplementation

Depending on your randomly assigned group, you will be ingesting either 100% blueberry powder made from freeze-dried, crushed whole blueberries (two, 18 gram servings of powder mixed with water per day, which is equivalent to 1.7 cups, ~145 whole blueberries, or placebo powder (a powder composed of carbohydrates and natural coloring with the same amount of calories as the blueberry powder) mixed with water daily for 6 weeks. The freeze-dried blueberries and placebo powder will be supplied by Mercer Foods and the U.S. Highbush Blueberry Council. You will not know whether or not you have received the blueberry or placebo powder. A 6-week supply in individual packets (~18 grams of powder) will be provided to you during your first study visit. You will mix the packet with 1-2 cups of water and consume one packet in the morning and one in the evening. If you forget to take the packet, there is no need to double your dose for the next time, just continue as normal. You will return any unused packets at the end of the 6-week period.

Mechanically-Induced Muscle Damage (Study Visit 2)

You will perform knee extensions against weight equal to 60% of your previously determined 1-repetition maximum. You will perform 8-12 of these exercises per set for a total of 9 sets with one minute rest between each set.

Tissue Collection (Study Visits 1, 2, 3)

Muscle biopsies and blood draws will be performed 3 total times (Study Visits 1, 2, and 3) in the morning, at least 12 hours after the last meal, prior to supplementation, following 6 weeks of supplementation and prior to induced muscle damage, and 24 hours post-muscle damage. For blood sampling, we will withdraw six teaspoons of blood from a vein near the crease of your elbow. For muscle sampling, we will numb the area with a local anesthetic (lidocaine), and we will remove a muscle sample (approximately the size of a pencil eraser) from your thigh using a needle designed for this purpose. You are free to bring a snack to each study visit in case you would like to eat after the blood draw and muscle sample collection. At the end of each visit, you will be briefed on how to care for the muscle biopsy site and also given a care sheet with information on how to care for the site and who to contact should you have any questions.
All research activities will take place in Appalachian State University's University Hall and Holmes Convocation Center in the Department of Health, Leisure, and Exercise Science's Neuromuscular and Exercise Science Laboratories. You will be provided with a temporary parking pass on the days of your study visits.

What are possible harms or discomforts that I might experience during the research?

You will be carefully screened at the beginning of this study to determine if you can participate safely, but should you choose to participate in this research study there are some risks and discomforts associated with the procedures.

- Maximum strength tests are considered safe for apparently healthy individuals, but there are some minimal risks associated with maximal strength testing. You might experience dizziness and lightheadedness during or for a short time after exercise. These symptoms should subside within 2-3 minutes. To minimize these risks, you will be monitored by research personnel at all times before, during, and until all symptoms have resolved after the test. You might also feel fatigued immediately after testing and might experience muscle soreness in the days following the maximal testing. These symptoms should resolve without any treatment.

- The risks associated with a DEXA scan include exposure to small amounts of radiation. DEXA scanning utilizes radiation to obtain an image of your body. Everyone receives a small amount of unavoidable radiation from the environment each year. Some of this radiation comes from space and some from naturally-occurring forms of radioactive water and minerals. The DEXA scan technique gives your body the equivalent of about 4 extra days' worth of this natural radiation. The radiation dose we have discussed is what you will receive from this study only and does not include any exposure you may have received or will receive from other tests. If you are pregnant or trying to get pregnant, you should not participate in a DEXA scan.

- The risks of collecting a blood sample (via venipuncture) from include the possibility of local discomfort (slight pinch when the needle enters their skin), minor bruising or bleeding at the site (10%), possible temporary lightheadedness, infection (<0.01%), or development of a blood clot (<0.01%). The amount of blood being withdrawn is about 6 teaspoons and will not influence the ability to participate in normal daily activities. A trained and experienced individual will perform the technique and the blood will be collected in a hygienic setting with sterile materials and biohazard protection measures to minimize these risks.

- There are some risks associated with the skeletal muscle biopsy technique. These risks may include slight discomfort with pressure or "tugging" sensations; however, most of the discomfort will subside 1-2 days after the technique. The use of a local anesthetic (numbing agent) will help minimize these risks. You may experience some mild discomfort (burning or stinging) when the local anesthetic is injected (before the area becomes numb). There is an extremely small risk that you will have an allergic reaction to the local anesthetic. If you have a history of allergies to local anesthetics then you

cannot participate in the study. Other risks associated with the muscle biopsy technique include bleeding at the biopsy site, temporary lightheadedness, infection (approximately 1 in 2,000), and bruising of the area (approximately 1 in 4,000).Using sterile materials/techniques and applying a cold compress and pressure to the biopsy site will minimize these risks. The muscle biopsy incision site will be closed by a steri-strip or stitches. If stitches are used, you will need to return to the lab to have your stitches removed. You will be given a checklist of symptoms to watch for and asked to report the occurrence of any of the symptoms immediately to the research staff. You will be asked to seek medical attention if you have any of the symptoms on the list. All muscle biopsy procedures will be performed under sterile conditions in a hygienic setting with biohazard protections. In the rare case of exposure of blood or tissue to research personnel, testing will be conducted for HIV and hepatitis (a positive HIV or hepatitis test will be reported to you).

- The risks associated with the muscle stress stimulus are similar to the risks of maximal muscle strength testing. The muscle stress stimulus consists of multiple repetitions (8-12) and multiple sets (9) of a standard isotonic, knee extension exercises. You might experience dizziness and lightheadedness during or for a short time after exercise. These symptoms should subside within 2-3 minutes. To minimize these risks, you will be monitored by research personnel at all times before, during, and until all symptoms have resolved after the test. You might also feel fatigued during and immediately after testing and will likely experience muscle soreness in the days following the protocol. These symptoms should resolve without any intervention.

- Dietary supplements contain a variety of ingredients, such as vitamins, minerals, amino acids, and herbs or other botanicals. To use dietary supplements safely, read and follow the label instructions, and recognize that "natural" does not always mean "safe". Some dietary supplements may interact with medications or pose risks if you have medical problems or are going to have surgery. Most dietary supplements have not been tested in pregnant women, nursing mothers, or children. The U.S. Food and Drug Administration (FDA) regulates dietary supplements, but the regulations for dietary supplements are different and less strict than those for prescription or over-the-counter drugs. Tell all your health care providers about any complementary health approaches you use. Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

What are possible benefits of this research?

By participating in this research, you might personally benefit by learning about your body composition. The information gained from this study might also lead to recommendations to improve the muscular health of older adults.

Will I be paid for taking part in the research?

You will receive $225 for completing all phases of this research study. Should you have to withdraw before completion of the study, your compensation will be prorated and you will be paid $75 per study visit completed.

What if I get sick or hurt while participating in this research study?

- If you need emergency care while you are at the research site, it will be provided to you. If you get hurt or sick when you are not at the research site, you should call your doctor or call 911 in an emergency. If your illness or injury could be related to the research, tell the doctors or emergency room staff about the research study, the name of the Principal Investigator and study physician, and provide a copy of this consent form if possible. Call the principal investigator, Edward Merritt, at (828)-262-7986, and the study physician, Dr. Cate Trate, at (828) 264-7760. They need to know that you are hurt or ill.

- There are procedures in place to help attend to your injuries or provide care for you. Costs associated with this care will be billed in the ordinary manner, to you or your insurance company. However, insurance companies, Medicare, and Medicaid might not pay bills that are related to research costs. You should check with your insurance about this and talk to the Principal Investigator if you have concerns.

How will you keep my private information confidential?

- Your information will be combined with information from other people taking part in the study. When we write up the study to share it with other researchers, we will write about the combined information. You will not be identified in any published or presented materials. To ensure that your information is kept confidential, identification numbers but not names will be used on all documents. All data entry and analysis will be conducted with statistical programs using identification. Your files will be stored in Dr. Merritt's office under lock and key and identifiable information will be deleted after one year.

After completion of this research study remaining serum and muscle samples collected during the experiment as described above will be stored, with your permission for use in future research studies on muscle physiology. All samples will be coded and only research staff will have access to the code identifiers which will be stored securely. Your serum and muscle samples, but no identifying information, might be shared with other research laboratories and investigators studying muscle physiology. If your samples are used for future research, you will not be contacted

for additional consent. Research results from your samples will be published in medical and scientific journals, but your identity will not be revealed. Your samples will not be used for commercial gain. There are no additional foreseeable risks or benefits if your samples are stored and used in future research studies. If you do not wish your samples to be used, following the completion of this study, they will be destroyed.

Please initial your choice(s) below:

____ I agree to allow my samples to be preserved for future research on muscle physiology.

____ I do not agree to allow my samples to be preserved for future research on muscle physiology.

Whom can I contact if I have a question?

The people conducting this study will be available to answer any questions concerning this research, now or in the future. You may contact the Principal Investigator, Edward Merritt, at (828) 296-7986. You may contact the study physician, Cate Trate, at (828) 264-7760. If you have questions about your rights as someone taking part in research, contact the Appalachian Institutional Review Board Administrator at 828-262-2692 (days), through email at irb@appstate.edu or at Appalachian State University, Office of Research and Sponsored Programs, IRB Administrator, Boone, NC 28608.

Do I have to participate?

No, your participation in this research is completely voluntary. If you choose not to volunteer, there is no penalty or consequence. If you decide to take part in the study you can still decide at any time that you no longer want to participate. You will not lose any benefits or rights you would normally have if you do not participate in the study.

This research project has been approved on 8/28/2013 by the Institutional Review Board (IRB) at Appalachian State University. This approval will expire on 4/15/2014 unless the IRB renews the approval of this research.

I have decided I want to take part in this research. What should I do now?

If you have read this form, had the opportunity to ask questions about the research and received satisfactory answers, and want to participate, then sign the consent form and keep a copy for your records.

____

Participant's Name (PRINT) Signature Date

Photography and Video Recording Authorization

With your permission, still pictures (photos) and/or video recordings taken during the study may be used in research presentations of the research findings. Please indicate whether or not you agree to having photos or videos used in research presentations by reviewing the authorization below and signing if you agree.

Authorization

I hereby release, discharge and agree to save harmless Appalachian State University, its successors, assigns, officers, employees or agents, any person(s) or corporation(s) for whom it might be acting, and any firm publishing and/or distributing any photograph or video footage produced as part of this research, in whole or in part, as a finished product, from and against any liability as a result of any distortion, blurring, alteration, visual or auditory illusion, or use in composite form, either intentionally or otherwise, that may occur or be produced in the recording, processing, reproduction, publication or distribution of any photograph, videotape, or interview, even should the same subject me to ridicule, scandal, reproach, scorn or indignity. I hereby agree that the photographs and video footage may be used under the conditions stated herein without blurring my identifying characteristics.

____

Participant's Name (PRINT) Signature Date

APPENDIX C

Heath and Medical History Questionnaire

Subject ID: ________________________ Interviewer's Name:___________________

Date (mm/dd/yy):_______________ Birthdate ___________

HEALTH AND MEDICAL HISTORY QUESTIONNAIRE

BACKGROUND

1. What is your highest level of education?

__Elementary __Jr High School __High School __College __Post College

2. What is your ethnic background?

__Hispanic or Latino (Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish origin)

__Not Hispanic or Latino

3. What is your race? __White (Europe, the Middle East, or North Africa) __ African American __ Asian __ Native Hawaiian/Pacific Islander __ American Indian/Alaska Native

OVERALL HEALTH

4. How would you rate your present health condition?
__Poor __Fair __Good __Excellent

5. Typically, how many days/year are you sick enough to stay in bed?___________

WEIGHT HISTORY

6. Has your weight changed more than 10 lbs in the last 12 months? __Yes __No

If yes, why:

__change in diet __change in physical activity __illness __depression/stress

__other

7. Do you have a history of an eating disorder, such as anorexia or bulimia?__No __Yes

8. Have you ever smoked?

__Never __Not now, but more than 12 months ago __Not now, but within the past 12 months

__Yes, currently smoking

MEDICAL HISTORY

9. Please check which of the following conditions you have had or now have. Also check medical conditions in your family (father, mother, brother(s), or sister(s)). Check as many as apply

Personal	Family	Medical History
☐	☐	Coronary heart disease, heart attack
☐	☐	Surgery
☐	☐	Angina
☐	☐	High blood pressure
☐	☐	Peripheral vascular disease
☐	☐	Phlebitis or emboli
☐	☐	Other heart problems (specify:__________________)
☐	☐	Lung cancer
☐	☐	Breast cancer
☐	☐	Prostate cancer
☐	☐	Colorectal cancer
☐	☐	Skin cancer
☐	☐	Other cancer (specify:_________________)
☐	☐	Stroke
☐	☐	Chronic obstructive pulmonary disease (emphysema)
☐	☐	Pneumonia
☐	☐	Asthma
☐	☐	Bronchitis
☐	☐	Diabetes mellitus
☐	☐	Thyroid problems
☐	☐	Kidney disease
☐	☐	Liver disease (cirrhosis of the liver)
☐	☐	Hepatitis (A,B,C,D, or E)
☐	☐	Gallstones/gallbladder disease
☐	☐	Osteoporosis
☐	☐	Arthritis
☐	☐	Gout

☐ ☐ Anemia (low iron)

☐ ☐ Stomach/duodenal ulcer

☐ ☐ Rectal growth or bleeding

☐ ☐ Cataracts

☐ ☐ Glaucoma

☐ ☐ Depression

☐ ☐ Substance abuse problems (alcohol, drugs etc)

10. Please indicate the approximate number of alcoholic beverages **per every two weeks**. (Beer: one drink = one 12-ounce beer; Liquor: One drink = 1.5 ounces of liquor; Wine: One drink = 5 ounces)

☐ 0 Drinks

☐ 1-2 Drinks

☐ 3 or more Drinks

11. Please check any of the following **medications** (prescription and/or over the counter) you currently take regularly. Also give the name of the medication.

	Medication	**Name of Medication**
☐	Heart Medicine	______________________
☐	Blood Pressure Medicine	______________________
☐	Blood cholesterol Medicine	______________________
☐	Hormones	______________________
☐	Birth Control pills	______________________
☐	Medicine for breathing/lungs	______________________
☐	Insulin	______________________
☐	Other medicine for diabetes	______________________
☐	Arthritis Medicine	______________________
☐	Medicine for depression	______________________
☐	Medicine for anxiety	______________________
☐	Thyroid Medicine	______________________
☐	Medicine for Ulcers	______________________
☐	Pain killer Medicine	______________________

- ☐ Allergy Medicine ______________________
- ☐ HIV/AIDS Medicine ______________________
- ☐ Hepatitis Medicine ______________________
- ☐ Other (please specifiy) ______________________

Supplement Use

12. Are you presently using or have you used within the last 12 months the following supplements at least three times/week:

Type of Dietary Supplement Note: If calcium and vitamin D are taken as one supplement, separate into two categories under "single vitamin" and "single mineral". If a supplement contains more than 3 components, enter as either "multivitamin", "multimineral", or "multivitamin/mineral".	Provide Brand Name or Type (i.e., vitamin E, calcium, iron, etc.) Add important comments	Use			Dosage/Tab (single substances) & Units Cups for herbal teas Spoons or scoops for some
		No. Tabs per time point	No. Times: Per day or Per week	Last Used (mm/yyyy)	
Multivitamin					NA
Multimineral					NA
Multivitamin/mineral					NA
Single vitamin(s)					
Single mineral(s)					
Herbal dietary supplement(s)					
Herbal tea*		NA			
Other over-the-counter supplement(s)					
Fiber Supplement (i.e., Metamucil, Fibercon)					

Physical Fitness, Physical Activity/Exercise

13. In general, compared to other persons your age, rate how physically fit you are:

1 □ 2 □ 3 □ 4 □ 5 □ 6 □ 7 □ 8 □ 9 □ 10 □

Not at all Physically active	Somewhat physically active	Extremely physically fit

14. Outside of your normal work or daily responsibilities, how often do you engage in exercise that at least moderately

increases your breathing and heart rate, and makes you sweat, for at least 20 minutes (such as brisk walking, cycling, swimming, jogging, aerobic dance, stair climbing, rowing, basketball, racquetball, vigorous yard work, etc.)

□ 5 or more times per week □ 3 to 4 times per week □ 1 to 2 times per week

□ Less than 1 time per week □ Seldom or never

15. How much hard physical work is required on your job?

□ A great deal □ A moderate amount □ None

16. How long have you exercised or played sports regularly?

□ I do not exercise regularly □ less than 1 year □ 1 to 2 years

□ 2-5 years □ 5-10 years □ more than 10 years

APPENDIX D

Supplement Instructions

Packet Instructions:

- Consume packets each evening with dinner.
- Mix contents of **two** packets with one cup (8-oz) of water. Once consumed, use additional water to consume any residual powder.
- Avoid consuming supplement with dairy products (milk & yogurt).
- Return packets to their respective weekly zip-lock bags and bring bags back to lab at 6-week follow-up visit.

Please answer the following questions and return this sheet at 6-week follow-up:

1. Which supplement do you think you were consuming? (Placebo or Blueberry)

Did you notice any side-effects during the 6-week supplementation period?

APPENDIX E

Participant Contact Information

Contact Information:

Participant Number: ______

Phone: ______________________ E-mail: ______________________

Preferred method of contact? (circle one) PHONE /E-MAIL

Anthropometric Information:

Baseline Ht: ________

Baseline Wt: _______ Visit Two Wt: _______ Visit Three Wt: _______

APPENDIX F

Three-Day Diet Record – Food Recording Form

Food Recording Form

Subject number_______ Date ____________
(mm/dd/yyyy)

Directions for Using the Food Diary

1. Please record ALL food, beverage, and supplements eaten/ingested for the three days that are assigned for you. To reduce error, please follow these directions:

   a. Keep your food diary current (list foods immediately after they are eaten).

   b. Whenever possible, MEASURE the volume consumed by using cups and tablespoons. Record amounts in household measures---ounces, tablespoons, cups, and slices.

   c. Be specific when describing the foot item eaten, and the method that was used to prepare the food. Remember to include condiments, sugar, oils, butter, and other visible fats For example:

Apple, raw, fresh, with peel	*1 medium*
Bread, whole wheat, fresh	*2 slices*
Margarine, soft from tub	*1 tablespoon*
Cereal, corn flakes	*1.5 cups*
Sugar, white	*1 teaspoon*
Milk, non fat	*2 cups*
Fish, salmon, baked	*10 ounces*

   d. This food record will be analyzed with a computerized dietary analysis program, so please provide sufficient detail.

Week One Day One

Time Food/Beverage Consumed	**Food Item and Method of Preparation**	**Amount Eaten** (in cups, tablespoons, ounces, etc.)

Week One Day Two

Time Food/Beverage Consumed	Food Item and Method of Preparation	Amount Eaten (in cups, tablespoons, ounces, etc.)

Week One Day Three

Time Food/Beverage Consumed	Food Item and Method of Preparation	Amount Eaten (in cups, tablespoons, ounces, etc.)

Week Six Day One

Time Food/Beverage Consumed	Food Item and Method of Preparation	Amount Eaten (in cups, tablespoons, ounces, etc.)

Week Six Day Two

Time Food/Beverage Consumed	Food Item and Method of Preparation	Amount Eaten (in cups, tablespoons, ounces, etc.)

Week Six Day Three

Time Food/Beverage Consumed	Food Item and Method of Preparation	Amount Eaten (in cups, tablespoons, ounces, etc.)

APPENDIX G

Portion Size Diagram

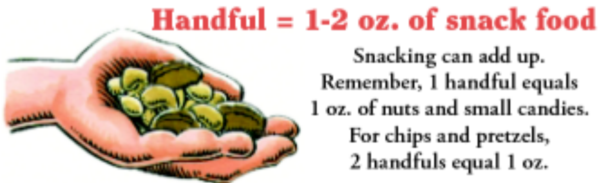
The secret to serving size is in your hand.

A fist or cupped hand = 1 cup

1 cup = 1½-2 servings of fruit juice
1 oz. of cold cereal
2 oz. of cooked cereal, rice or pasta
8 oz. of milk or yogurt

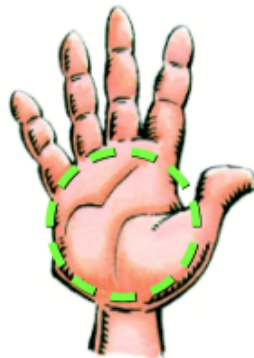
A thumb = 1 oz. of cheese

Consuming low-fat cheese helps you meet the required servings from the milk, yogurt and cheese group. 1½ oz. of low-fat cheese counts as 8 oz. of milk or yogurt.



Handful = 1-2 oz. of snack food

Snacking can add up. Remember, 1 handful equals 1 oz. of nuts and small candies. For chips and pretzels, 2 handfuls equal 1 oz.



Palm = 3 oz. of meat

Choose lean poultry, fish, shellfish and beef. One palm size portion equals 3 oz. for an adult and 1½-2 oz. for a child under 5.

Thumb tip = 1 teaspoon

Keep high-fat foods, such as peanut butter and mayonnaise, at a minimum. One teaspoon is equal to the end of your thumb, from the knuckle up. Three teaspoons equals 1 tablespoon.

1 tennis ball = $^1/_2$ cup of fruit and vegetables

Healthy diets include a variety of colorful fruits and vegetables every day.

Because hand sizes vary, compare your fist size to an actual measuring cup.

Iowa WIC Program - Iowa Department of Public Health - 2007
Adapted from North Carolina Nutrition Network

APPENDIX H

Biopsy Care Instructions

Instructions for Care of the Muscle Biopsy Area

Here are some instructions for you to take care of the muscle biopsy area so that it heals well and the chance of infection is minimized. We've also included what is normal and what is not normal as part of the healing process.

General Instructions

- Keep the pressure wrap on for at least 8 hours following the biopsy.
- Keep the steri-strip(s) and Band-Aids on for as long as possible (until they fall off ~4 days).
- Trim the loose ends of the steri-strip as needed.
- Replace steri-strip(s) if it completely comes off before 4 days.
- It is OK to shower, but you will want to avoid taking a bath or using a hot tub or swimming pool for at least 4 days. Change Band-Aids after showering to keep the incision dry.
- If you have had stitches, arrange to have them removed about 5-7 days after the biopsy.
- Icing the biopsy area is suggested for immediately after and the days following.
- You are encouraged to walk and renew normal activity within 1-2 days. There is no need to 'baby it'.

Do Not:

- Perform intense, "all-out" exercise for 1-2 days.
- Get in a river, lake, pool, or hot tub for at least 4 days after the biopsy.
- Consume any pain-relief medications without checking with us first.

Normal Reactions following Muscle Biopsy:

- Localized stiffness, soreness, and bruising (light to moderate).
- There may be soreness and slight weakness in the biopsied leg that is noticeable when you go down stairs. You will want to go slowly, lead with the opposite leg, and use the handrail the day of the biopsy.

Reactions <u>Not</u> Normal Following Muscle Biopsy:

- Intense, excruciating pain in the leg or in the area of the biopsy
- Bleeding which does not stop
- Intense redness in the area of the biopsy
- Heat in the area of the biopsy
- Presence of pus
- Fever
- Hives or other signs consistent with allergic reaction (i.e. difficulty breathing, swelling)

IF YOU EXPERIENCE ANY OF THE ABNORMAL REACTIONS ABOVE, CONTACT US IMMEDIATELY - REGARDLESS OF THE TIME OF DAY. IN CASE OF AN EMERGENCY, DO NOT HESITATE TO GO TO THE EMERGENCY ROOM OR CALL 911.

Kevin Zwetsloot, Ph.D.	wk 828-262-7281	cell 828-406-3862
Ed Merritt, Ph.D.	wk 828-262-7986	cell 512-779-8039
Cate Trate, M.D.	wk 828-264-7760	cell 828-268-1755

APPENDIX I

FRAP Protocol

FERRIC REDUCING ABILITY OF PLASMA (FRAP)

Modified for 96 Well Plate

1. Collect and snap freeze plasma. Store at -80 until analysis.

Assay procedures: **Note: Remove and discard top layer of all solid chemicals before using**

1. Make up **300 mmol/L acetate buffer** (adjust to pH 3.6) ***Light Sensitive***
    a. weigh **3.1** grams sodium acetate trihydrate (*On dry chem. Shelf*)
    b. add **16 ml** acetic acid (1N) (Molarity = Normality/Valence) (*In Corrosives cabinet)*
    c. bring to final volume **of one liter** with DI water

2. Make up **10 mmol/L TPTZ** (2,4,6 - tripyridyl-s-triazine) (store in refrigerator) (MW=312.3) in 40 mmol HCl (*In Corrosives cabinet*) (adjust to make **30 mL**)

    a. make up 40 mmol/l HCl (Molarity = Normality/Valence) Examples: (**330 uL** HCl (12N) + **99.670 mL** DDI water (in hood)

    b. weigh out **187.38 mg** TPTZ (*In Fridge*)

    c. add TPTZ to **60 mL** HCl, final concentration = 10 mmol and mix until dissolved.

3. Make up **20 mmol/L iron trichloride hexhydrate** (ITX) (MW=270.29) in DDI water (Make 100 mL) (**0.54g ITX + 100 mL DI**) (*ITX stored on Dry Chem Shelf*)

4. Make up working **FRAP solution** (300 mL total volume) (Note: solution should be bright orange color, if color is dark purple or purple tinted, your iron hexhydrate was most likely pre-oxidized.)

    a. measure out **200 ml** acetate buffer
    b. add **20 ml** TPTZ solution
    c. add **20 ml** ITX solution

This is good to run multiple plates with multiple samples on it. Also note, it is a 1:1:1: ratio so you can use 100mL:10mL:10mL respectively)

5. **STD Curve**. Make up **100, 200, 400, 600, 800, and 1000 uM/L** solutions of ascorbate/trolox in DI water. For ascorbate, measure **44 mg ascorbate + 250 mL** DI to make 1000 uM ascorbate.

Concentration (ascorbate)	Amount	DI H_2O
1000 uM	5.0 mL	0 mL
800 uM	4.0 mL	1 mL
600 uM	3.0 mL	2 mL
400 uM	2.0 mL	3 mL
200 uM	1.0 mL	4 mL
100 uM	0.5 mL	4.5 mL
0 uM	0.0 mL	5 mL

APPENDIX J

F_2-Isoprostane Protocol

8-Isoprostane Express ELISA Kit

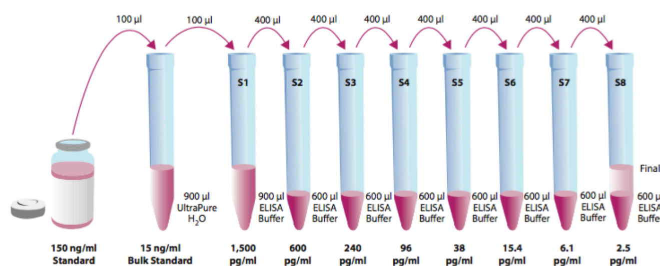
Cayman's 8-Isoprostane Express ELISA Kit is a competitive assay that can be used for quantification of 8-isoprostane in plasma, urine, and other sample matrices. The assay has a range from 2.5-1,500 pg/ml and a sensitivity (80% B/B_0) of approximately 10 pg/ml.

8-Isoprostane Express ELISA Standard

- Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the 8-Isoprostane Express ELISA Standard (Item No. 416364) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 15 ng/ml.

NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer, culture medium should be used in place of ELISA Buffer for dilution of the standard curve.

- To prepare the standard for use in ELISA: obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 600 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (15 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the rst point on the standard curve, will be 1,500 pg/ml. Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.



8-Isoprostane Express AChE Tracer

- Reconstitute the 8-Isoprostane Express AChE Tracer as follows:
  - **100 dtn 8-Isoprostane Express AChE Tracer (96-well kit; Item No. 416360):** Reconstitute with 6 ml ELISA Buffer.
    OR 500 dtn 8-Isoprostane Express AChE Tracer (480-well kit; Item No. 416360): Reconstitute with 30 ml ELISA Buffer.
    Store the reconstituted 8-Isoprostane Express AChE Tracer at 4°C *(do not freeze!)* and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

8-Isoprostane Express ELISA Antiserum

- Reconstitute the 8-Isoprostane Express ELISA Antiserum as follows:
    - **100 dtn 8-Isoprostane Express ELISA Antiserum (96-well kit; Item No. 416362):** Reconstitute with 6 ml ELISA Buffer.
    OR 500 dtn 8-Isoprostane Express ELISA Antiserum (480-well kit; Item No. 416362): Reconstitute with 30 ml ELISA Buffer.
    Store the reconstituted 8-Isoprostane Express ELISA Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of antiserum has been included to account for any incidental losses.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 4°C. Be sure the packet is sealed with the desiccant inside.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non- specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Performing the Assay

Addition of the Reagents:

1. ELISA Buffer

Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B_0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl ELISA Buffer to NSB wells).

2. 8-Isoprostane Express ELISA Standard

Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. Samples

Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. 8-Isoprostane Express AChE Tracer

Add 50 µl to each well *except* the TA and the Blk wells.

5. 8-Isoprostane Express ELISA Antiserum

Add 50 µl to each well *except* the TA, the NSB, and the Blk wells.

Incubation of the Plate

Cover each plate with plastic film and incubate for two hours at room temperature on an orbital shaker.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):
    - 100 dtn vial Ellman's Reagent: Reconstitute with 20 ml of UltraPure water. OR
    - 250 dtn vial Ellman's Reagent: Reconstitute with 50 ml of UltraPure water.
2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark at room temperature. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.

2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*

3. Read the plate at a wavelength between 405-420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells is in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, wash the plate, add fresh Ellman's Reagent and let it develop again.

APPENDIX – K

Collected Data

Participant 1-11 Descriptive and Compliance Data

Subject	Returned Packets (out of 84)	# Consumed (out of 14/week)						Placebo/ Blueberry Guess	Side Effects?	Age	Weight (kg) Pre	Weight (kg) Post	Height (in)	A/B
		1	*2*	*3*	*4*	*5*	*6*							
1	84	14	14	14	14	14	14	BB	BP decrease	75	80.20	79.80	65.50	**B**
2	84	**14	14	14	14	14	9	Placebo	laxitive	63	73.50	75.50	64.25	**B**
3	84	14	14	14	14	14	12	placebo	blue teeth	62	70.70	72.30	70.25	**A**
4	79	14	**14	13	14	14	12	No idea/leaning toward BB	None	69	53.40	54.00	63.00	**B**
5	**0	**12	**14	**14	**14	**14	**14	Placebo	None	62	50.91	51.55	60.40	**B**
6	82	**14	**14	14	14	14	14	BB	None	64	70.91	71.82	64.75	**A**
7	84	14	14	14	14	14	14	Placebo	None	66	94.55	95.09	72.50	**B**
8	**82	12	14	14	14	14	14	BB	none	70	66.82	67.73	64.75	**A**
9	84	14	14	14	14	14	14	no idea	freq bathroom	76	73.64	74.09	63.00	**A**
10	84	14	14	14	14	14	14	BB	freq bathroom	71	87.64	86.40	71.25	**A**
11	84	14	14	14	14	14	14	BB	freq bathroom	60	67.27	66.40	65.75	**A**

Participant 12-23 Descriptive and Compliance Data

12	84	14	14	14	14	14	14	BB	none	67	59.40	59.00	65.75	**B**
13	84	14	14	14	14	14	14	BB	"acid & taste" strange dreams	66	59.80	60.30	63.25	**B**
14														**A**
15	84	14	14	14	14	14	14	Placebo	Possible consiptation	60	54.00	55.30	63.75	**B**
16	84	14	14	14	14	14	14	BB	Dark stools	61	88.10	87.40	70.25	**A**
17	**0							BB	Loose bowels, occational mild diarrhea	61	85.80	86.00	69.50	**A**
18	84	14	14	14	14	14	14	BB	none	67	65.70	65.00	64.25	**A**
19	84	14	14	14	14	14	14	placebo	none	78	70.10	71.30	63.00	**B**
20	84	14	14	14	14	14	12	no idea	none	67	59.70	59.00	61.75	**B**
21	82	14	14	14	14	14	12	BB	none	60	70.10	69.40	66.25	**B**
22	84	14	14	14	14	14	14	BB	loose bowels, occational mild diarrhea	63	57.30	57.00	61.75	**A**
23	84	14	14	14	14	14	14	placebo	none	68	56.30	56.60	64.50	**B**

Participant 1-11 Diet Data

Subject	Diet Pre													Diet Post												
	kcals	*CHO (g)*	*Pro (g)*	*Fat (g)*	*Vit A (IU)*	*Vit A (RAE)*	*β-Car (mcg)*	*Vit E*	*Vit C*	*Cu*	*Zn*	*Se*	*Mn*	*kcals*	*CHO (g)*	*Pro (g)*	*Fat (g)*	*Vit A (IU)*	*Vit A (RAE)*	*β-Car (mcg)*	*Vit E*	*Vit C*	*Cu*	*Zn*	*Se*	*Mn*
1																										
2	1575.75	194.34	74.13	48.09	3545.87	164.83	1308.05	1.01	40.07	0.41	2.06	37.16	1.57	2002.99	254.18	92.05	67.68	2317.01	259.45	151.45	3.74	54.35	0.39	4.26	56.60	0.85
3	1871.08	233.03	95.37	61.66	31941.10	1637.44	16421.68	17.53	189.86	0.85	5.58	101.23	2.45	2165.14	208.08	83.29	89.71	29587.05	1381.82	15618.39	12.68	137.74	0.59	4.15	43.32	2.05
4	1193.56	128.00	56.53	55.60	18919.43	969.42	9589.68	1.91	51.81	0.42	6.75	50.96	0.65	1652.58	157.92	58.22	88.03	11626.76	682.21	5507.45	4.20	59.30	0.48	5.33	59.93	1.38
5	1608.47	228.00	49.57	43.23	19677.86	1011.79	9753.63	2.87	71.63	0.79	5.21	32.65	2.00	1796.49	183.34	65.28	80.42	14228.40	706.18	6872.33	3.09	111.60	0.62	3.85	34.01	0.88
6	1629.27	169.71	82.58	62.54	10603.74	450.52	3944.10	3.11	169.44	0.74	7.66	64.66	1.25	2154.82	192.54	117.06	83.08	22055.04	1124.20	10362.28	5.27	142.66	0.59	2.66	45.65	2.18
7	1906.52	145.98	93.39	106.71	1849.34	256.64	207.04	5.80	35.10	0.71	5.45	28.72	1.98	1957.87	129.38	124.39	87.45	7441.11	44.42	3210.95	9.30	45.15	0.87	12.59	115.09	2.00
8	1715.54	186.33	95.84	44.54	13887.16	789.84	7513.88	5.81	139.08	0.90	10.92	74.24	2.23	1705.00	190.36	83.83	54.96	3306.43	242.66	1321.75	2.22	37.07	0.63	6.94	54.40	0.82
9	1285.53	154.36	57.68	51.17	9903.20	584.81	3592.33	1.63	115.11	0.40	3.87	30.21	1.24	956.88	136.57	33.28	32.72	414.70	20.59	44.41	1.03	16.98	0.22	1.40	18.09	0.64
10	1915.46	224.03	70.47	85.71	1816.10	231.16	47.42	3.51	58.28	0.21	2.88	45.24	0.78	1558.46	136.21	55.68	90.12	2768.04	384.23	540.36	6.07	44.27	0.45	3.51	57.14	0.79
11	2090.77	245.57	58.98	*113.31*	*12221.96*	*590.07*	5360.32	14.18	121.00	1.04	3.92	26.07	1.82	2025.26	174.56	59.66	126.09	10977.92	567.10	4265.53	8.68	69.05	0.92	4.56	17.09	1.81

Participant 12-23 Diet Data

12	1391.90	162.87	56.68	54.89	3288.15	289.53	256.38	7.65	73.20	0.53	4.28	53.48	1.55	1596.25	177.83	55.93	60.99	1657.75	187.66	426.12	3.03	40.16	0.30	2.79	27.33	1.37
13	3124.78	154.50	64.34	149.57	11298.91	567.72	6504.55	1.22	89.37	1.95	6.88	25.68	4.51	1965.48	166.16	63.14	124.59	29269.64	1549.20	15405.63	3.89	166.96	1.27	4.99	59.24	2.31
14																										
15	1676.90	194.21	64.68	71.83	3283.22	145.86	940.68	15.48	123.40	0.72	6.59	19.12	1.52	1308.97	170.51	56.28	49.13	12430.67	538.50	6166.51	10.68	242.87	1.63	3.10	58.25	1.66
16	3220.64	338.60	138.09	107.23	3577.08	274.21	1216.13	6.86	208.87	1.91	12.11	139.85	4.37	2895.97	244.37	136.34	100.71	2565.60	405.16	735.94	6.76	130.32	0.75	12.76	123.98	1.70
17	1860.75	235.54	53.35	50.32	2789.46	359.97	332.57	6.88	100.55	0.32	2.73	10.69	0.58	1621.48	234.24	56.85	57.19	4414.07	201.20	1688.67	1.13	71.37	0.65	5.43	22.80	2.81
18	1247.45	173.25	59.69	41.36	14995.57	853.87	7676.61	9.40	297.51	1.30	4.80	67.60	2.22	1423.31	178.58	70.06	55.21	8321.59	507.49	3877.35	3.50	134.17	0.61	4.97	50.99	1.51
19	1625.43	250.49	51.41	51.34	3534.05	353.89	744.16	14.67	115.73	0.62	8.13	10.97	2.27	1407.39	218.06	48.44	38.71	4446.89	392.28	1975.11	8.09	82.93	0.39	5.23	7.21	1.30
20	1172.00	109.91	45.18	44.57	7046.88	397.87	3045.24	2.29	25.32	0.25	2.28	21.29	0.34	962.74	72.86	47.96	45.92	4093.85	153.30	1569.04	1.63	55.18	0.24	2.10	17.49	0.54
21	1548.73	169.95	68.64	69.63	3159.52	513.24	334.39	3.11	80.51	4.38	0.27	34.88	0.44	1664.51	187.12	100.54	56.7	22097.14	885.79	9936.95	4.1	168.98	0.87	7.19	91.47	1.35
22	1566.98	167.85	66.65	58.46	12641.09	795.09	6100.42	2.56	44.20	0.39	2.12	43.65	0.60	1351.52	122.42	75.23	59.55	4702.78	288.89	2056.08	3.84	57.54	0.30	4.46	55.68	1.21
23	1167.98	185.44	36.12	34.53	2964.70	189.49	1321.42	3.83	115.80	0.27	2.15	27.15	0.97	1335.35	155.22	41.00	49.66	7041.63	360.08	2541.25	1.87	82.86	0.34	2.40	42.18	0.70

FRAP – Raw Data

Participant #	Study Visits												
	A				B				C				
	Mean	SD	CV	***ABS***	Mean	SD	CV	***ABS***	Mean	SD	CV	***ABS***	
1	0.267	0.011	4.237	***52.700***					0.310	0.003	1.038	***95.367***	y = .0011x + 0.2143
2	0.331	0.004	1.070	***116.200***	0.315	0.012	3.836	***101.033***					R2 = 0.9924
3	0.297	0.016	5.238	***82.700***	0.344	0.010	2.856	***129.367***	0.339	0.024	7.089	***124.367***	
4	0.337	0.029	8.733	***122.367***	0.298	0.016	5.467	***103.583***	0.293	0.005	1.753	***99.556***	
5	0.437	0.004	0.971	***219.833***	0.414	0.020	4.793	***200.667***	0.413	0.017	4.030	***199.833***	
6	0.302	0.025	8.342	***107.333***	0.341	0.020	5.985	***139.833***	0.367	0.007	1.927	***161.500***	
7	0.376	0.007	1.862	***169.000***	0.382	0.001	0.302	***174.000***	0.363	0.015	4.096	***158.167***	
8	0.359	0.014	3.909	***154.833***	0.331	0.027	8.010	***131.500***	0.353	0.007	1.858	***149.833***	
9	0.355	0.014	3.974	***151.500***	0.397	0.021	5.235	***186.500***	0.396	0.028	7.087	***185.667***	
10	0.336	0.007	2.104	***135.667***	0.362	0.007	1.953	***157.333***	0.337	0.016	4.643	***136.500***	y = .0012x + 0.1732
11	0.360	0.015	4.032	***155.667***	0.355	0.018	4.967	***151.500***	0.342	0.008	2.393	***140.667***	R2 = 0.997
12	0.316	0.024	7.615	***101.700***	0.293	0.016	5.309	***78.700***	0.281	0.011	4.026	***66.700***	
13	0.339	0.017	5.083	***138.167***	0.360	0.013	3.536	***155.667***	0.351	0.001	0.165	***148.167***	
14													
15	0.326	0.003	0.985	***127.333***	0.399	0.016	3.899	***188.167***	0.387	0.002	0.549	***178.167***	
16	0.333	0.001	0.425	***133.167***	0.333	0.023	6.762	***133.167***	0.331	0.015	4.649	***131.500***	
17	0.328	0.015	4.438	***129.000***	0.341	0.019	5.603	***139.833***	0.350	0.012	3.429	***147.333***	
18	0.363	0.025	6.787	***158.167***	0.346	0.028	7.983	***144.000***	0.364	0.017	4.677	***159.000***	
19	0.339	0.009	2.728	***124.367***	0.307	0.008	2.764	***92.700***	0.304	0.017	5.435	***89.700***	
20	0.320	0.025	7.806	***106.033***	0.308	0.029	9.365	***93.700***	0.323	0.012	3.570	***108.700***	
21	0.267	0.018	6.627	***52.367***	0.261	0.022	8.495	***46.367***	0.272	0.027	9.937	***58.033***	y = .0011x + 0.2143
22	0.308	0.014	4.545	***93.700***	0.304	0.022	7.124	***89.700***	0.347	0.011	3.274	***133.033***	R2 = 0.9924
23	0.293	0.027	9.145	***78.367***	0.311	0.022	7.091	***96.367***	0.312	0.031	9.802	***98.033***	

F_2-Isoprostane – Raw Data

Plate 1	Participant #/Visit		**pg/mL**		StdDev	%CV
	3A		88.650			
	3B		67.445			
	3C		99.635			
	5A		50.015			
	5B		44.855			
	5C		27.525			
	6A		132.260			
	6B		177.345			
	6C		197.394			
	7A		74.447			
	7B		151.483			
	7D		73.007			
	8A		68.533			
	8B		62.593			
	8C		111.443			
	9A		228.220			
	9B		74.447			
	9C		132.770			
	10A		173.043			
	10B		115.443			
	CBStD		120.437			
Plate 2			**pg/mL**		StdDev	%CV
	10C		95.480		31.051	32.521
	11A		55.825		11.758	21.063
	11B		74.502		19.291	25.893
	11C		149.047		37.312	25.033
	12A		66.195		6.808	10.284
	12B		59.841		9.731	16.262
	12C		61.839		9.280	15.007
	13A		57.204		2.417	4.225
	13B		64.734		17.895	27.644
	13C		51.347		4.192	8.165
	15A		91.432		7.205	7.880
	15B		176.070		52.583	29.865
	15C		227.967		281.869	123.645
	16A		103.023		19.504	18.931

	16B		66.015		17.866	27.064
	16C		128.369		43.843	34.153
	17A		63.042		2.865	4.544
	17B		97.963		48.812	49.826
	17C		86.654		23.783	27.446
	18A		148.341		45.702	30.809
	CBStD		60.373		8.108	13.429
Plate 3			**pg/mL**		StdDev	%CV
	18B		95.640		8.838	9.241
	18C		68.584		26.009	37.923
	19A		77.016		31.550	40.965
	19B		109.868		28.879	26.285
	19C		139.723		46.503	33.282
	20A		96.018		17.523	18.250
	20B		110.388		20.301	18.391
	20C		130.983		50.124	38.267
	21A		74.415		23.071	31.003
	21B		108.584		33.517	30.867
	21C		74.631		15.592	20.893
	22A		114.521		18.429	16.092
	22B		142.217		26.201	18.423
	22C		101.748		13.272	13.044
	23A		91.208		10.305	11.298
	23B		141.046		15.290	10.841
	23C		130.128		31.211	23.985
	CBStD		98.433		2.342	2.379

Vita

Christian E. Behrens Jr. was born in Miami Springs, Florida. He was the first child of Christian E. Behrens and Elizabeth A. Behrens. He has one younger sister. He attended high school at Cypress Bay High School, Weston, FL and graduated in June 2007. The following August he moved to Boone, NC at the age of 18 to attend Appalachian State University where he received two Bachelor of Science degrees in Exercise Science and Nutrition and Foods in August of 2014. In the fall of 2014, he entered into Appalachian State University's Master of Science in Nutrition and Dietetic Internship. This degree was awarded in August of 2016. Upon successfully passing the Registration Examination for Dietitians, credentialed by the CDR of the Academy of Nutrition and Dietetics, Mr. Behrens will be recognized as a Registered Dietitian. In the fall of 2016, Mr. Behrens will continue his academic career as he begins doctoral studies at the University of Alabama at Birmingham.