Title of Project
Effects of an oral antioxidant cocktail on cardiovascular function and oxidative stress in cardiovascular disease patients

Project Description
Cardiovascular disease, endothelial function, and oxidative stress:
Cardiovascular disease (CVD) is considered to be the leading cause of death in the US, resulting in 1 death every 38 seconds on average [1, 2]. CVD is often partially due to several major risk factors such as elevated cholesterol levels, high blood pressure, obesity, smoking, and diabetes [3, 4]. The endothelial cells, which is the single cell lining inside of the lumen of our blood vessels, play a key role in modulating cardiovascular function. The endothelial cells release various vasoactive substances that regulate vascular tone, cell adhesion, and vascular smooth muscle cell proliferation[5]. Vascular endothelial dysfunction is consider to be a hallmark of CVD [6], namely because CVD risk factors are known to contribute to endothelial cell damage [7]. These damages can lead to atherosclerosis, or the build-up of plaque in the arteries, which contributes to various cardiovascular diseases [8]. Reactive oxygen species (ROS) production is thought to be a key player in endothelial dysfunction. When ROS is produced in excess, it can cause significant oxidative stress, and oxidative stress is defined as an imbalance between the rate of ROS production and clearance in the body by the endogenous antioxidant defense system [9-11]. Oxidative stress (OS) leads to damage in the vasculature by increasing vascular smooth muscle cell proliferation, often resulting in vascular remodeling [12] [13]. Increased OS is also believed to increase inflammation and reduce nitric oxide bioavailability, a potent vasodilator, which further impairs blood flow and ultimately contributes to the development of CVD[14]. Therefore, scavenging excessive ROS may help reduce the vascular damage that is thought to contribute to CVD.

Oral antioxidant intake and cardiovascular function:
When the endogenous antioxidant defense system cannot keep up with the demanding level of ROS clearance needed to maintain vascular homeostasis, such as what occurs in CVD [15], the utilization of an exogenous antioxidant may be needed to protect the vasculature. The impacts of individual antioxidants and antioxidant combinations (typically vitamin C and E) on CVD have been mixed, with some studies actually showing harmful effects with the intake of vitamin A and high doses of vitamin E [16]. However, the combination of vitamin C, vitamin E and alpha-lipoic (antioxidant cocktail) may be promising for improving vascular function in CVD, as this specific combination has significantly improved flow-mediated dilation, an indicator of endothelial function, in older individuals [17] as well as blood flow and muscle tissue oxygen saturation in chronic obstructive pulmonary disease [18]. It seems that the alpha lipoic acid component may be an important mediator for the beneficial effects of vitamin E and C, as studies examining vitamin C and E independently or in combination have shown limited success [16, 19], while studies examining alpha lipoic acid in combination with vitamin E and/or C have shown significant improvements in markers of cardiovascular function in both experimental [20, 21] and human models [17, 18, 22, 23]. This may be due to the ability of alpha lipoic acid to “recycle” vitamins C and E, in which alpha lipoic acid continues to reduce these vitamins after they have been oxidized, which could prolong their antioxidant properties [24]. The potential success of this antioxidant cocktail on combating the elevated OS and its associated impairment to endothelial function in CVD are not fully understood. The purpose of this project will be to examine the impacts of this antioxidant cocktail on endothelial function and levels of OS in CVD patients. If successful in improving measures of cardiovascular function, these findings may indicate a novel therapeutic treatment for improving vascular health in CVD.

Methodology
Study characteristics:
A total of 60 participants between the age of 35-85 [16] will be recruited (30 with CVD and 30 age-matched, healthy controls). Exclusion criteria for the CVD group will include: 1) any form of heart disease, including previous myocardial infarction, coronary artery disease, chronic heart failure, or any arrhythmias, 2) renal disease, 3) neurological or neuromuscular disorders, 4) pulmonary diseases, 5)
bleeding disorders, 6) any form of cancer, 7) any history of stroke, thrombosis or embolism, 8) any lower-body musculoskeletal injuries 9) currently pregnant, breast-feeding or trying to get pregnant, and/or 10) history of smoking in the last 6 months. Healthy controls must be 1) free from these and any other diseases, 2) be non-obese (no BMI ≥30 kg/m² and/or waist girth > 102 cm for men; > 88 cm for women), 3) must not have a blood pressure above 120/80 mmHg and 4) must not be taking any medications. All subjects will be asked to cease any vitamin, antioxidant, and/or NSAID use 7 days prior to data collection. This study will use a parallel study design with one 4-hour visit measuring height, weight body composition, hand grip strength, resting blood pressure, heart rate, total antioxidant capacity, endothelial function, blood flow, skeletal muscle tissue oxygenation, arterial stiffness, autonomic nervous system function, and walking capacity at baseline and 2 hours post antioxidant cocktail intake.

**Antioxidant intake:**
The oral AOC supplement ( Lucky Vitamin, Conshohocken, PA) will be administered in a split-dose during the visit to optimize absorption and efficacy [18]. Immediately after baseline testing, the first dose of the AOC will be administered. 30 minutes after this, the second dose will be administered. The first dose will contain 300 mg alpha-lipoic acid, 500 mg vitamin C and 200 IU vitamin E. The second dose will contain 300 mg a-lipoic acid, 500 mg vitamin C, and 400 IU vitamin E [18, 22, 23].

**Oxidative stress and total antioxidant capacity:**
Blood samples will be taken from an antecubital vein using a vacutainer and needle before and after antioxidant intake. Blood samples will be stored for later analysis at -80°C and will be analyzed for oxidative stress and total antioxidant capacity levels with commercially available ELISA kits.

**Endothelial function measurement:**
Flow mediated dilation (FMD) will be used to assess endothelial function. A Doppler ultrasound system (Terason uSmart 3300, Burlington, MA) is used to measure the resting diameter of the brachial artery. A rapid inflation cuff (Hokanson, Bellevue, WA) is placed on the forearm and inflated to partially occlude the brachial artery downstream. The cuff is deflated after 5 min, and the ultrasound system is used to record the endothelium-dependent vasodilatory response of the brachial artery. FMD has been shown to be a safe, non-invasive assessment of systemic endothelial function [25], and has been shown to be highly predictive of CVD, even when plaque is not present [26, 27]. An improved vasodilatory response is highly correlated with enhanced nitric oxide production [28], which is what we expect from this study.

**Leg blood flow measurement:**
Femoral and popliteal artery blood flow, characterized by changes of intraluminal diameter of artery and blood flow volume and velocity, will be measured in the dominant leg at baseline and two-hours after antioxidant cocktail intake. Arterial diameter changes and blood flow will be obtained using Doppler ultrasound Participants will lie in a supine position on a padded table. Measurements of the femoral artery will be taken 2-3 cm above its bifurcation into the superficial branches. Popliteal artery blood flow will be taken from the popliteal fossa proximal to the bifurcation into the posterior tibial artery. Arterial diameter and mean blood flow (volume and velocity) will be determined by the ultrasound system during passive leg movement technique, a technique that has been shown to cause an endothelium-dependent hyperemic response [29], which is indicative of NO bioavailability and endothelial function.

**Oxygen saturation and utilization:**
Tissue oxygenation will be measured non-invasively using near-infrared spectroscopy (Artinis PortaMon, Einsteinweg, The Netherlands). An oxygen sensor adhered to the subject’s skin over the gastrocnemius muscle. Testing will occur before and after antioxidant cocktail intake. This device will allow continuous monitoring of tissue oxygenation, representative of blood perfusion and mitochondrial oxygen utilization.

**Arterial stiffness measurement:**
Arterial stiffness will be assessed using carotid-to-radial, carotid-to-distal, and carotid-to-femoral pulse-wave velocity analysis (Complior Analyse, Alam Medical, Saint-Quentin-Fallavier, France and SphygmoCor XCEL, AtCor Medical, New South Wales, AU) before and after antioxidant intake. Participants will lie in a supine position on a padded examination table. Pressure sensor tonometers will be placed on the skin over the right carotid artery, right radial artery, and right posterior tibial artery. The software will then calculate pulse wave velocity and augmentation index, which represent arterial
stiffness. Carotid-to-femoral arterial stiffness will be assessed with applanation tonometry and a blood pressure cuff on the leg and a sensor on the neck.

**Autonomic nervous system measurement:**
Autonomic nervous system function will be assessed using heart rate variability (HRV). Participants will wear a heart rate monitor (Suunto Smart Sensor, Suunto, Vantaa, Finland) and will lay in a supine position on a padded tilt table for 5 minutes. After the five-minute period, heart rate will be recorded for 2 minutes. The subject will then be put at a 70° angle on the tilt table for 20 minutes and heart rate will be recorded for 2 minutes. HRV will be assessed via Kubios HRV Software. This is a commonly used protocol for testing HRV and has been shown to be reliable and valid in age-matched individuals [30, 31].

**Walking capacity measurement:**
A treadmill six-minute walk test will be used to measure walking capacity before and antioxidant cocktail intake. The 6-minute walk test has been shown to be reliable and valid [32].

**Statistical analysis:**
Shapiro-Wilk’s test will be used to determine data normality. A 2x2 ANOVA with repeated measures [group (healthy and CVD) x time (before and after antioxidant cocktail intake)] will be used to compare the differences between pre- and post-antioxidant cocktail intake within and between groups. Alpha will be set at 0.05. If significance is found, a Tukey’s test will be used for post-hoc comparisons.

**Project timeline**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Start Date</th>
<th>End Date</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRB preparation/approval</td>
<td>11/2019</td>
<td>11/2019</td>
<td>Compliance approval sent to ORCA by 5/1/2020</td>
</tr>
<tr>
<td>Recruit participants</td>
<td>04/2020</td>
<td>06/2020</td>
<td>Recruit participants w/ posters at UNO/UNMC</td>
</tr>
<tr>
<td>Purchase supplies</td>
<td>05/2020</td>
<td>06/2020</td>
<td>Supplies for the project will be purchased</td>
</tr>
<tr>
<td>Data collection/analysis</td>
<td>06/2020</td>
<td>09/2020</td>
<td>Data collections/analyses will be performed</td>
</tr>
<tr>
<td>Drafting writing materials</td>
<td>09/2020</td>
<td>11/2020</td>
<td>Manuscript/abstract/poster drafting of results</td>
</tr>
<tr>
<td>Conference abstracts</td>
<td>11/2020</td>
<td>11/2020</td>
<td>Project abstract will be submitted for approval</td>
</tr>
<tr>
<td>Present at RCAF</td>
<td>03/2021</td>
<td>03/2021</td>
<td>Present at the RCAF at UNO</td>
</tr>
<tr>
<td>Conference presentations</td>
<td>05/2021</td>
<td>05/2021</td>
<td>Present at 2021 ACSM National Conference</td>
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**Student and mentor roles:**
I will write and submit the IRB application and Dr. Park will help me with this process. I will recruit subjects using fliers on UNO and UNMC campuses and will handle the purchasing of supplies for the proposed project. The Vascular Research Lab will perform the data collections, which I will lead under the mentorship of Dr. Park. He will provide me with help and feedback on my techniques. I will conduct data analysis and interpretation with Dr. Park’s help. I will draft the abstract, manuscript, and the poster for RCAF and the ACSM Annual meeting. Dr. Park will review all of the materials I produce and will provide feedback. He will also help me with my poster presentation practice prior to RCAF and ACSM.

**Previous internal funding:**
I successfully completed a GRACA project during my master’s at UNO. The project was titled *Dietary Nitrate Supplementation and Thermoregulation During Exercise*, which resulted in a presentation at the 2019 RCAF and a master’s thesis. The results showed benefits of nitrate on vascular function and exercise capacity in peripheral artery disease (PAD). This proposal differs from my previous project by using a different subject population and intervention (antioxidant cocktail). This intervention is being investigated as a different therapeutic treatment for those with less severe forms of cardiovascular diseases, in hopes of preventing further severe disease manifestation, such as PAD.
### Budget Justification:

<table>
<thead>
<tr>
<th>Item</th>
<th>Price</th>
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<tbody>
<tr>
<td>Blood draw supplies</td>
<td>$500</td>
</tr>
<tr>
<td>Antioxidant cocktail and plastic serving cups</td>
<td>$250</td>
</tr>
<tr>
<td>EKG electrodes</td>
<td>$300</td>
</tr>
<tr>
<td>Antioxidant capacity assay kit</td>
<td>$230</td>
</tr>
<tr>
<td>Superoxide dismutase assay kit</td>
<td>$490</td>
</tr>
<tr>
<td>Gloves (different sizes)</td>
<td>$350</td>
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<tr>
<td>Sterilization equipment</td>
<td>$300</td>
</tr>
<tr>
<td>Ultrasound gel</td>
<td>$200</td>
</tr>
<tr>
<td>Participant stipend (60 participants $25/visit x 1 visit ea.)</td>
<td>$1,500</td>
</tr>
<tr>
<td>Rapid inflation cuffs (x2; different sizes)</td>
<td>$80</td>
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<tr>
<td>3-lead cable and 3-lead wire set for EKG</td>
<td>$300</td>
</tr>
<tr>
<td>Kubios HRV Software update</td>
<td>$500</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>$5,000</strong></td>
</tr>
</tbody>
</table>

I am requesting $5,000 in total for the completion of this research project. The Vascular Research Lab is where the research will take place, so most of the necessary equipment will be provided to me free of charge (i.e. ultrasound, rapid inflation cuff system, etc.). I have requested $500 for blood draw supplies (2 blood draws per subject) and $250 for purchasing the antioxidant cocktail and plastic serving cups. EKG electrodes ($300), gloves ($350), sterilization equipment ($300), and ultrasound gel ($200) are necessary for our FMD measurement using the ultrasound, EKG monitor, and cuff inflation system we have in the lab. We need a 3-lead cable and wire set for the trigger EKG system ($300) and rapid inflation cuffs of different sizes ($80) for the FMD measurement. We will also update our Kubios software for analysis of autonomic function ($500). The antioxidant capacity assay kit ($230) and superoxide dismutase assay kit ($490) will be used to analyze oxidative stress and antioxidant capacity in the blood.
References
Graduate Research and Creative Activity (GRACA)
Letter of Mentor support for Liz Pekas
October 14, 2019

GRACA Review Committee:

It is with great pleasure that I write this recommendation letter for the GRACA proposal titled *Effects of an oral antioxidant cocktail on cardiovascular function and oxidative stress in cardiovascular disease patients*. By the end of this letter, you will understand my admiration for Liz and her motivation, work ethic, and knowledge. She has helped carry out several projects previously, each with great success and presentation. Liz is a first-year PhD student in the School of H&K and has been working in the Vascular Research Lab since January 2018.

Liz is uniquely qualified to carry out the proposed GRACA project. Since joining my lab in early 2018, Liz has been extremely productive and has produced 5 co-authored publications, 3 manuscripts in-press, and has given several poster presentations locally and nationally. She has received previous fundings for her work, including a GRACA during her master’s and a NASA Fellowship this year. Liz is a driven young scientist, and GRACA support for this project will provide the proper support for supplies necessary to perform the study.

We have discussed several aspects of the project prior to this proposal, including the study design, specific aims, and hypotheses. Liz has gained experience with project development, implementation, data collection, and data analysis under my mentorship. With her high level of commitment and admirable experience early in her career, this project will allow for a greater level experience and leadership development for her to progress to the next level in the field.

I believe that Liz is becoming an excellent young researcher, and it is evident that she has clear career goals. However, she will require guidance on several project aspects, including study initiation, subject recruitment, experimental techniques, data analysis, and abstract/manuscript drafting for publication. We will meet on a weekly basis (at our scheduled weekly lab meetings) and as-needed about progress of the project. I am committed to provide the mentorship for Liz and the resources necessary, such as equipment in the Vascular Research Lab. Although we have the majority of the equipment necessary, this funding is necessary for other supplies to carry out this study. This project is a crucial step for developing her early professional research career. I am confident that Liz is well prepared for this project and will produce a final outcome of a published peer-reviewed journal article and presentation at the Research and Creative Activity Fair in 2021.

Best Regards,

[Signature]

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