Effect and Time Course of Acute Red Wine Consumption on Blood Antioxidant Capacity and Arterial Distensibility in Male Smokers

A Thesis
by
Rohan Elizabeth Krehbiel

Submitted to the Graduate School
Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

May 2011

Department of Nutrition and Health Care Management

Effect and Time Course of Acute Red Wine Consumption on Blood Antioxidant Capacity and Arterial Distensibility in Male Smokers

A Thesis
by
Rohan Elizabeth Krehbiel
May 2011

APPROVED BY:

Dr. Lisa McAnulty
Chairperson, Thesis Committee

Dr. Steve McAnulty
Member, Thesis Committee

Dr. Scott Collier
Member, Thesis Committee

Dr. Sarah Jordan
Chairperson, Department of Nutrition and Health Care Management

Dr. Edelma D. Huntley
Dean, Research and Graduate Studies

ABSTRACT

Effect and Time Course of Acute Red Wine Consumption on Blood Antioxidant Capacity and Arterial Distensibility in Male Smokers
(May 2011)

Rohan Elizabeth Krehbiel, B.S., University of Kentucky

M.S., Appalachian State University

Chairperson: Lisa McAnulty

INTRODUCTION: It is well known that smoking cigarettes leads to oxidative stress and is harmful to endothelial function. Research has previously demonstrated that antioxidants found in red wine can counteract the negative effects of reactive oxygen species caused by oxidative stress. The current study utilized a crossover design and investigated antioxidant capacity and vascular performance after smoking cigarettes and consuming wine. **METHODS:** Participants included eight male smokers between the ages of 21 and 35. These individuals attended an orientation session where baseline anthropometric measures were obtained one week prior to the beginning of the study. Participants then reported to the lab where they were randomly assigned to the wine or control group and the following week crossed over into the opposite group. The wine group consumed 300mL of muscadine red wine immediately following the baseline measures of arterial distensibility and blood samples. Smoking occurred 45 minutes and 2 hours and 45 minutes following wine consumption. Arterial distensibility measures and blood samples were again collected at these two time points. The control group followed the same procedure but did not consume

wine. Outcome variables included plasma antioxidant capacity (Ferric Reducing Ability of Plasma, FRAP; Oxygen Radical Absorbance Capacity, ORAC) and vascular measures (arterial distensibility). A 2 (treatments) x 3 (times) repeated measures analysis of variance was used to evaluate the main effects of treatment, time, and treatment time interaction. **RESULTS:** The results indicate that when given wine, the participants' blood antioxidant capacity increased in the FRAP assay (p=0.046). Additionally, the vascular measures showed a significant difference at time 3 between the wine and control groups (p=0.030). ORAC was not significantly different between groups or time periods. **CONCLUSION:** In conclusion, the results of the study suggest that drinking red wine while smoking cigarettes may increase antioxidant capacity but does not provide health benefits related to vascular performance.

ACKNOWLEDGMENTS

I would like to express my greatest gratitude to everyone involved in my education and writing of this document for their advice and support throughout the past two years. To Dr. Lisa McAnulty, thank you for all of your hours spent advising, your confidence in my abilities, and helping me through my graduate degree and internship. To Dr. Steve McAnulty and Dr. Scott Collier, thank you for agreeing to assist with this project, all of your time spent working with us, and answering our constant questions. To Chelsea Lyga and Kim Stikeleather Gordon, thank you for working with me on this project, answering random questions at random hours, and becoming friends. To my parents, Anne and Dick Krehbiel, thank you for all of your support and advice throughout my life. Last but not least, to my boyfriend, Joey Klare, thank you for your support and confidence during these past two years. You all helped to keep me sane, at least to some degree. All of you have helped me complete this thesis and my degree; you are all part of it. Your contributions to this paper and my life will never be forgotten.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Oxidative Stress

In the body, the balance between the formation of reactive oxygen species (ROS) and the reactions that turn the ROS into harmless end products is known as oxidative stress (1). Free radicals are ROS formed during the metabolism of oxygen or the metabolism of foreign compounds (1). Examples of foreign compounds consist of environmental factors, or exogenous agents, and include cigarette smoke, pollution, and exposure to ionizing and ultraviolet radiation (2). An endogenous factor that creates ROS occurs during the metabolism of oxygen, which the body consumes in large amounts during cardiovascular exercise. The formation of ROS is natural and occurs constantly. Consequences of oxidative stress include DNA damage or mutation, cell death by apoptosis or necrosis (3), lipid damage, and protein damage (2). Cardiovascular disease, neurodegenerative disease, cancer, and the aging process are also consequences of oxidative stress. The connection between these diseases and oxidative stress is the alteration of DNA, lipids, and protein damage (2). As an example, cigarette smoke exposure leads to the formation of ROS, which leads to oxidative stress, which leads to DNA damage, which can then lead to cardiovascular disease, emphysema, and/or cancer.

Acute Cigarette Smoking Effects and Vascular Performance

Approximately 45.9 million men and women smoke cigarettes across the United States (4). All of these people are at risk for many diseases because they smoke cigarettes, which change arterial function, creating multiple risk factors for cardiovascular disease (5). Previous research suggests the antioxidants in red wine could help to reduce the risk of these

diseases in cigarette smokers by combating the free radicals (6, 7). The free radicals found in cigarette smoke come from many different sources, and it is possible that exposure to these free radicals is one of the first steps in developing cancer, hypertension, atherosclerosis, and cardiovascular disease in general.

One of the endogenous free radicals, nitric oxide (NO), is produced in decreased amounts with both passive and active cigarette smoke exposure. NO acts as a communicator for cells in many processes in the body, including blood pressure regulation, neurotransmission, controlling the immune system, inhibiting platelet aggregation, and more (8, 9). It also acts as a relaxation factor for the endothelium (10). With a deficiency of NO, hypertension is induced (11). Rocchi et al. (12) found higher concentrations of NO in non-smokers as compared to smokers. A specific type of NO, endothelial NO, regulates physiological vasodilatation and inhibits platelet aggregation in order to better support blood flow and support an anti-atherogenic state in the endothelium (9, 12, 13). The availability of endothelial NO decreases when an excessive amount of ROS are present in the body. This decrease is due to the inactivation of NO caused by ROS by the formation of cytotoxic species (11). The decrease of endothelial NO causes a hyper-expression of vascular cell adhesion, an increase in homocysteine, and a reduction in vasodilator response, thereby marring vascular homeostasis. Rocchi et al. (12) reported that homocysteine was found in increased concentrations in the smokers, demonstrating an inverse relationship with NO in the blood. This inverse relationship causes damage to the endothelial lining. Homocysteine is an amino acid derivative that is a metabolite of another amino acid, methionine. When homocysteine is not broken down in the body, concentrations rise and increase the risk for

atherosclerosis (1). Tying all of these factors together, decreased NO, increased ROS, and increased homocysteine, result in a favorable setting for the development of atherosclerosis.

Individuals who smoke cigarettes tend to have increased oxidative stress and decreased levels of antioxidants, mostly caused by the oxidants in cigarettes and a poorer diet than those individuals who do not smoke (14). Smoking also causes endothelial dysfunction, with both long-term and acute exposure, through the increase of oxidative stress and decrease of NO (6, 10). Endothelial dysfunction is best described as the impaired function of endothelial cells located in the arteries. This results in abnormalities in vasomotor control, the breakdown of fibrin and blood clots in the artery, and an inflammatory response (1). These abnormalities can lead to atherosclerosis and other forms of cardiovascular disease. Atherosclerosis occurs when the arteries experience hardening and narrowing. This does not allow enough blood flow through the artery and can eventually lead to blood clots. The hardening of arteries can stem from either acute or chronic inflammation of the artery, caused by endothelial dysfunction and other mechanisms (15). In simpler terms, smoking cigarettes leads to oxidative stress which leads to endothelial dysfunction. This leads to atherosclerosis and cardiovascular disease which greatly inhibits an individual's vascular performance. When the arteries cannot conduct the passage of enough blood to all the parts of the body, the body will not perform as well as it should.

Arterial distensibility is the ability of an artery to expand, allowing more blood to flow through. When the artery is more elastic, it absorbs energy during the systolic phase of the pulse flow. By absorbing this energy, the artery reduces the cardiac work during a given cardiac output. Pulse wave velocity is the best known measure of arterial distensibility, because the two are inversely related (16). Pulse wave velocity is how fast the pulse moves

along the vessel wall (17, 18). The quicker the pulse moves, the stiffer the artery; the slower the pulse moves, the more elastic the artery. When the artery takes the time to actually surround the pulse and help push it through, it flows through more slowly. If the artery is not moving around the pulse, then the pulse slides right through the artery, making the time between pulse waves quicker. The stiffer the artery, the greater the risk factor is for that individual to experience cardiovascular disease. The carotid-femoral pulse wave velocity is the measure used most often and is widely accepted as the measurement for aortic stiffness (5, 18, 19). Cigarette smoking has been known to increase the carotid-femoral pulse wave velocity measures, potentially causing this stiffness. Both long and short term cigarette smoking exhibit increased pulse wave velocity. This result is related to nicotine. Nicotine causes catecholamine concentrations to rise which stimulates the sympathetic ganglia, impairing NO generation, finally leading to endothelial dysfunction (5, 20).

Red Wine and Antioxidants

Antioxidants counteract oxidants and ROS. Red wine is commonly known to have high amounts of antioxidants. Interestingly, there are some wines that have high concentrations of polyphenols but show a lower antioxidant capacity, or vice versa. The red grapes used to make red wine contain polyphenols, and the concentrations of polyphenols can vary greatly from wine to wine depending on varying growth conditions for the grapes used (21). The principle polyphenols in red wine include the following: procyanidins, anthocyanins, quercetin, and resveratrol (22). Procyanidins are made up of oligomeric catechins that are covalently linked together. These compounds usually occur with monomeric catechins and epicatechins. Procyanidins affect the vascular system in a positive manner by increasing plasma antioxidant activity, decreasing platelet aggregation, and

increasing NO production (22). These three effects counteract the negative effects brought on by atherosclerosis and endothelial dysfunction.

Cao et al. (23) observed increased antioxidant capacity in elderly women after consuming red wine. The study assessed the antioxidant capacity of red wine, spinach, strawberries, and vitamin C. Three different antioxidant assays were utilized which were comprised of the Ferric Reducing Ability of Plasma (FRAP), Oxygen Radical Absorbance Capacity (ORAC), and Trolox Equivalent Antioxidant Capacity (TEAC). The ORAC and FRAP assays showed increased antioxidant capacity in urine and serum after consuming red wine, strawberries, spinach and vitamin C. The ORAC and FRAP antioxidant assays are explained in detail in the methods section.

Modun et al. (24) observed an increase in the FRAP concentration by dealcoholized red wine. This means that the increase was due only to the polyphenol content and not the alcohol. However, there is another possible explanation. When the liver metabolizes ethanol, it produces ROS. So by taking the ethanol out of the wine, the liver does not metabolize it and does not produce ROS. It is possible that there were less ROS to neutralize, thereby resulting in the increased FRAP values.

Another side effect of ROS generation is that it may reduce certain immune cell functions. However, when consuming regular red wine, it was found that the antioxidants may scavenge the ROS and defend the immune system against the activities of the ROS (25). Therefore, antioxidants might also support immune function. Antioxidants and polyphenols in wine have positive effects on the body in that there is protection from oxidative stress and consequent damage in many potentially different systems, specifically, the immune system and the cardiovascular system.

The antioxidants and polyphenols that provide health benefits in red wine come from the grapes used to make the wine. Muscadine grapes (*Vitis rotundifolia*) produce a sweet red wine with a significant amount of the phytochemical anthocyanin. Anthocyanins are an antioxidant and can prevent cancer and the tumors associated with cancer (26). Anthocyanins are the chemical responsible for the dark purple color of the muscadine grape and support a healthy blood pressure (27).

Red Wine and Vascular Performance

Moderate alcohol consumption, including red wine, has been touted as a beneficial addition to the diet and can provide protection from coronary heart disease as well as its risk factors. The key word is moderate. If copious amounts of alcohol, including red wine, are consumed, it can have detrimental effects on the body (24, 28-32). Red wine specifically is beneficial because of its antioxidant capacity, namely the polyphenol resveratrol. Resveratrol has been found to inhibit the oxidation of low-density lipoproteins, protect cells from collecting lipids, protect the cardiovascular system, and act as an anti-inflammatory agent. When protecting the cardiovascular system, resveratrol controls the endothelial cell growth, helping to prevent endothelial dysfunction (32). Polyphenols also provide vasorelaxing effects in human, rat, and rabbit arteries (29). Vasorelaxation is important because it decreases vascular pressure, which decreases the pulse wave velocity and promotes healthy vascular function. Even though the antioxidants in the red wine provided benefits, the alcohol content can have detrimental effects on vascular function. Zilkens et al. (30) found alcoholic beverages increased the blood pressure, most likely a consequence of the alcohol content rather than the antioxidant capacity.

Antioxidant Capacity Measures

When investigating free radical damage, it is important to have accurate assays available to assess the antioxidant capacity. The FRAP and ORAC assays have been used in many research studies to assess the antioxidant capacity of red wine and other substances (23, 24, 33-36). Antioxidant capacity can also be referred to as the reducing ability (FRAP) of a solution or the ability of a solution to protect against a radical generator (ORAC; 35). There are advantages and disadvantages to both assays. FRAP is quick and simple to perform and results are easily reproduced. It is also a relatively inexpensive assay to run (33, 36). However, Cao and Prior (36) demonstrated that it does not measure the antioxidant capacity of reducing glutathione, an antioxidant whose concentration is low in humans. This is because the reduction action of ferric (Fe^{III}) to ferrous (Fe^{II})does not always match the action of an antioxidant against a free radical (36). ORAC advantages include being standardized, using biologically relevant free radicals, and the integration of degree and time of antioxidant reaction. Using a standardized assay is important because it can be easily repeated and is easy to compare results from different laboratories. Disadvantages when assessing antioxidant capacity with ORAC can be the expensive equipment necessary, variability amongst data when different equipment is used, and the sensitivity of the assay itself (37).

Red wine and smoking effects

The numerous antioxidants found in red wine provide many protective effects to the body. This includes protecting against the ROS produced in the body when a person smokes a cigarette. Two separate studies found that by consuming red wine at the same time as smoking, the deleterious effects to endothelial function from the smoke were reversed by the

red wine (6, 7). While both of these studies investigated acute consumption of smoke and red wine on endothelial function, neither examined arterial distensibility. Papamichael et al. (7) had 16 participants for their crossover study lasting three days. Each participant drank 250 mL of Grand Reserve red wine or Grand Reserve dealcoholized red wine and smoked one cigarette. Flow mediated dilatation was used to assess endothelial dysfunction, and was measured after fasting and repeated at 15, 30, 60, and 90 minutes post-wine consumption and post-smoking. Results of this study included that flow mediated dilatation decreased after smoking one cigarette but did not change when the participant also drank wine or dealcoholized wine. Karatzi et al. (6) conducted a very similar study to Papamichael et al. (7). They also utilized a crossover design. The group was comprised of 20 participants who experienced three study days consisting of: smoking one cigarette, smoking one cigarette and drinking 250 mL of red wine, and smoking one cigarette and drinking 250 mL of dealcoholized red wine. Flow mediated dilatation was also used in this study, being measured at fasting, 30, 60, and 90 minutes after the completion of each consumption. Results from this study also concluded that after smoking one cigarette flow mediated dilatation decreased but did not change significantly after consuming wine or dealcoholized wine along with the cigarette. Also, Karatzi et al. (6) considered the main limitations in their own study to be that it did not measure antioxidant capacity or oxidative stress. Thus, results of these two studies did not fully elucidate the relationship between smoking and acute red wine consumption.

Therefore, we sought to investigate the effects of red wine on measures of both arterial distensibility and antioxidant capacity. Additionally, collecting blood samples at several time points post wine ingestion provided information about when the antioxidants are

at a peak in the blood. Antioxidant capacity was measured using FRAP and ORAC, and changes to the vasculature were directly measured by examining changes in arterial distensibility. By measuring both antioxidant capacity and arterial distensibility, a better understanding of the interaction between smoking and red wine consumption was determined in each subject. The hypothesis of this study is that red wine consumption ameliorates the deleterious effects of smoking in an acute manner. The results of this study are important to further our understanding of the effects of red wine and polyphenols on the body's vascular system.

METHODOLOGY

Subjects

This study was approved by the Institutional Review Board at Appalachian State University. A copy of the Institutional Review Board documents is located in Appendix B. Male smokers between the ages of 21 and 35 were recruited as subjects to participate in the study. Fliers and public service announcements were used to recruit the subjects. A copy of the recruitment flier is located in Appendix A. For inclusion in the study, subjects were required to have a history of smoking at least one cigarette per day. Subjects were excluded on the basis of gender, age, and if chronic medication was used. No exclusions were made on the basis of race or color.

Research Design

One week prior to beginning the study, subjects attended an orientation and screening session in University Hall. Informed consent, body composition, height, weight, and health information were also obtained at this session. A copy of the health questionnaire used is located in Appendix C. Body composition was assessed using Bioelectrical Impedance. A scale from Tanita was utilized, model TBF 300a (Arlington Heights, IL). Subjects were asked to refrain from consuming large-dose vitamin/mineral supplements, herbs, and medications during the study's entirety. The subjects were instructed to avoid alcohol and over the counter medications 24-hours before the study began each week, as well as caffeine and nicotine each morning on the day of the study. Subjects were instructed on and kept a three day food record prior to each test.

Testing Sessions

The subjects were randomized into two groups, wine (n = 6) and control (n = 2). Upon arrival at the laboratory, weight and body composition were obtained again. The subjects sat quietly for 10 minutes before participating in a pre-wine consumption blood test and arterial distensibility test. Fourteen mL of blood were taken each time blood was drawn. After the initial tests of the morning, subjects drank 300mL of Duplin red wine while sitting or did nothing if they were in the control group. Forty-five minutes after finishing their wine, subjects moved to a designated smoking area and were given five minutes to smoke one unfiltered, Camel cigarette. When the subjects returned to the laboratory at approximately1 hour following wine consumption, post consumption arterial distensibility tests and blood draws were conducted. The smoking protocol mentioned previously was repeated at approximately 2 hours and 45 minutes post consumption. At 3 hours post consumption, the last blood draw and arterial distensibility test of the day were performed. Following these final tests, snacks and water were provided for consumption, and the three hour time period following alcohol consumption allowed blood alcohol levels to return to normal, thereby enabling the subjects to safely leave the laboratory. To measure the blood alcohol levels in the subjects, each subject was tested with a breathalyzer 15 minutes after drinking the wine, at 1 hour, and at 3 hours. A portable breath tester from Lifeloc Technologies (Wheat Ridge, Colorado) was used, model FC10. This 3 hour time period was repeated, after a one-week wash out period, when the subjects crossed over into the opposite group.

Arterial Distensibility

Arterial stiffness is measured by pulse wave velocity. Measuring pulse wave velocity is known to be noninvasive, safe, and easy to conduct in any setting while also requiring average training before beginning (16, 18, 19). To measure the pulse wave velocity, the distance between the two sites being measured is divided by how many seconds it takes for the pulse to travel in the artery (16-18). In this study, the measurements between sites were obtained using a tape measure while the subject was in the supine position. The first measurement was from the left carotid to left femoral arteries and the second was from the left carotid to the left dorsalis-pedis arteries. The SphygmoCor CPV instrument (Itasca, IL) was utilized. The SphygmoCor CPV allows the user to measure pulse wave velocity between two arterial locations. An electrocardiogram was also utilized to monitor the subjects while pulse wave velocity was being taken. In addition to the pulse wave velocity measures, radial augmentation index was also measured. A minimum of 12 pulse contours were recorded and analyzed by the computer.

Blood Collection

Each subject had blood drawn three times during each session, making a total of six draws for the entire study. Blood samples were taken pre-wine consumption, 1 hour post-wine consumption, and 3 hours post-wine consumption. At 14 mL for each draw, the subjects provided a total of 42 mL on each day of the study. This amount is approximately 400 mL less than what is typically donated at a blood bank, making it a safe amount to collect from the subjects on each day.

Blood Analysis

FRAP and ORAC were used to analyze all blood samples provided by each subject.

FRAP

Reducing refers to when a reactive species, or oxidant, is reduced by the reductant, or antioxidant. In FRAP, a Fe^{III} compound is reduced to the Fe^{II} form (33). This reduction will take place when a reductant, or antioxidant, is present in the substance. In the *in vitro*, or test tube reaction medium, this reduction results in a blue chromogen. Therefore, the intensity of the blue color correlates to the amount of water soluble antioxidants present in the sample (33). For this study, a working FRAP reagent was composed of acetate buffer, TPTZ (2,4,6 – tripyridyl-s-triazine) solution, and an iron chloride plus water solution. Standards and samples were analyzed by adding the FRAP reagent and measuring absorbance. The standard was an ascorbate solution made of ascorbic acid and distilled water, and the samples were the plasma collected from each participant. Each sample and standard was measured twice to ensure the quality of readings (33). Directions to complete the FRAP assay are located in Appendix E.

ORAC

The ORAC assay was also used in this study to analyze the antioxidant capacity in all blood samples. ORAC is an inhibition method (34). When a free radical is generated during the assay, the inhibition of its action is measured. The amount of inhibition that occurs shows the antioxidant capacity in the sample. AAPH [2,2′-Azobis(2-amidinopropane) dihydrochloride] is used to make the free radicals, and fluorescein is used as the protein to which the free radicals will cause damage. ORAC measures the ability of an antioxidant to extinguish the free radicals in the sample (36). The standard curve was made using a vitamin E analogue, trolox. The results of the plate samples were measured as trolox equivalents (35). A plate reader, Spectra Max Gemini XPS (Molecular Devices, Inc., Sunnyvale, CA),

was used to read the results of ORAC. In the ORAC plate, there was one well containing only fluorescein. This well acted as an adjuster for the plate reader. The remaining wells contained phosphate buffer, samples, and trolox standards, arranged in a specific order to obtain the correct results from the plate reader. After fluorescein was added to the plate, it was incubated which allows the free radicals to cause damage. When antioxidants were present in the sample, the damage to the fluorescence was reduced or inhibited, and allow for the measurement of the antioxidant capacity (35). Directions to complete the ORAC assay are located in Appendix F.

Statistical Analysis

A 2 (treatment) x 3 (time) repeated measures analysis of variance was used to evaluate the main effects of treatment, time, and treatment time interaction changes. A Bonferroni post-hoc analysis was performed to examine point to point differences. All statistical analyses were performed using SPSS version 17 (IBM, Armonk, NY).

RESULTS

Subjects

Eight male subjects with an average age of 23.88 years completed the study in its entirety. Subject characteristics are presented in Table 1. Appendix D contains the participant descriptive raw data.

Table 1. Participant Descriptive Information

	Baseline	**Treatment**	**Control**	***p* Value**
Age (yr)	23.88 ± 4.45	-	-	-
Height (in)	69.13 ± 2.64	-	-	-
Weight (lbs)	175.25 ± 52.47	173.38 ± 51.42	173.50 ± 51.89	0.870
BMI (kg/m^2)	25.64 ± 6.55	25.37 ± 6.42	25.38 ± 6.49	0.906
Smoking (days/month)	22.75 ± 10.24	-	-	-

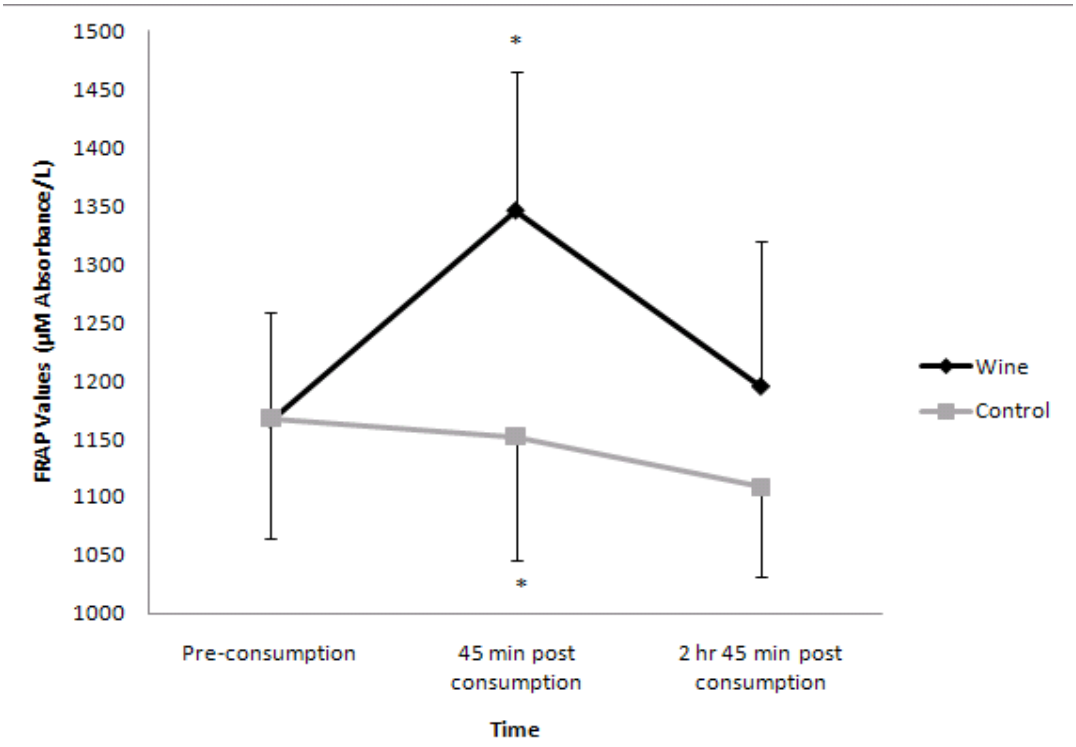
Values are means ± standard deviation; yr = year, in = inches, lbs = pounds, kg/m^2 = bodyweight in kilograms/ height in meters squared.

FRAP

The results are presented in Figure 1 and the raw data is in Appendix G. There were significant effects of acute red wine consumption on increasing the overall plasma antioxidant reducing potential based on the FRAP assay versus Controls ($p = 0.046$). There were no significant effects on FRAP related to the time course of acute red wine consumption, although there was a 17% difference between the wine and control groups at

time 2 and an 8% difference at time 3. In Figure 1, the FRAP values for both the wine group and control group started at the same points but by time point 2 they had separated. The wine group FRAP values increased at time point 2, representing an increase in the subjects' antioxidant capacity. However, by time point 3 the effect seemed to have worn off based on the fact that the FRAP values decreased. The control group's FRAP values actually decreased slightly at each time point.

Figure 1. Ferric Reducing Ability of Plasma (FRAP)



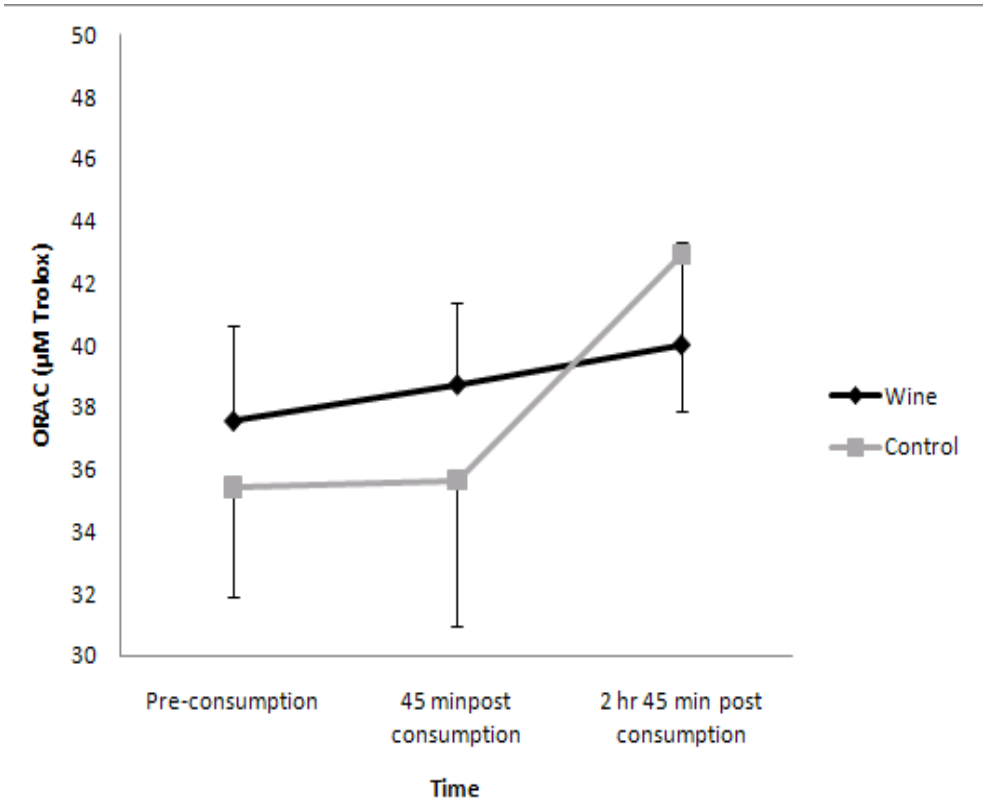
Values are mean ± standard deviation; * Notes significant difference between groups; (*n* = 8); hr = hour, min = minutes.

ORAC

The ORAC assay did not indicate any significant differences between treatments (Figure 2). Raw data for the ORAC assay is located in Appendix H. Three possibilities exist, one being that smoking alone was not enough to create a sufficient level of oxidative

stress to alter the ORAC assay, or that the wine does not provide enough change in antioxidant capacity, or both.

Figure 2. Oxygen Radical Absorbance Capacity (ORAC)



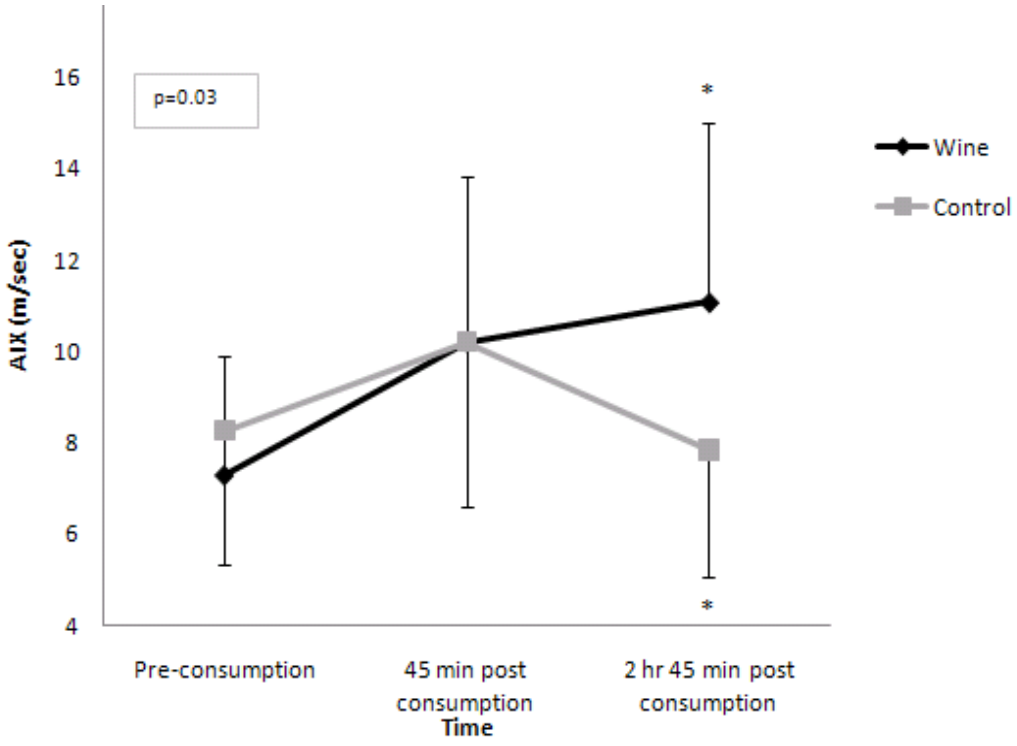
Values are mean ± Standard Deviation; (n = 8); hr = hour, min = minutes

Augmentation Index

Appendix I contains the raw data for the augmentation index results. The vascular tests completed during the study showed that wine consumption while smoking has a negative effect on arterial distensibility. Figure 3 demonstrates this observation with the wine group's augmentation index at time 3 increasing significantly (p = 0.030). A higher augmentation index signifies stiffer arteries. Wine negates the smoking effect on the aorta but not immediately. The wine group increased above the control group showing that wine

consumption combined with smoking is worse for arteries than smoking alone. Therefore, the research suggests that drinking red wine while smoking has no protective effects on the vascular system and may cause greater harm than smoking alone.

Figure 3. Augmentation Index (AIX)



Values are mean ± Standard Deviation; * notes sig difference between groups; (n = 8); hr = hour, min = minutes.

DISCUSSION

With significant results in FRAP and the arterial distensibility, the present study has agreed with previous studies that red wine increases antioxidant concentrations in the plasma (6, 7, 23, 28). In the FRAP results, the decrease of antioxidant capacity in the control group was unexpected. This decrease could be from the subjects' exposure to cigarette smoke at time points 2 and 3. Since the subjects did not have the antioxidants in their blood from the wine, the ROS from the cigarette smoke could have depleted some of the antioxidants which contribute to FRAP. With decreased concentrations of antioxidants in the body, FRAP values became lower. Our results indicate that drinking red wine while smoking appears to have no protective effects on the vascular system and may actually cause greater harm than smoking alone. The alcohol in the wine is potentially the cause of the harm to the vascular system.

According to Zilkens et al. (30), the alcohol in alcoholic beverages, including red wine, causes a rise in blood pressure. The study looked at healthy normotensive men who drank beer, red wine, dealcoholized red wine, and partook in abstinence as the control. When comparing the blood pressures of their subjects from the dealcoholized red wine and control time periods, no significant difference was experienced. This result led the researchers to conclude that the antioxidants in the red wine may not provide protection from the blood pressure elevating effects of alcohol. Another study conducted by Papamichael et al. (7) found opposite results from the current study; consumption of red wine, regular or dealcoholized, concurrent with smoking ended up decreasing the harmful effects of the cigarette smoke on the vascular system. It has been found that moderate alcohol

consumption in conjunction with healthy lifestyle behaviors reduces the risk of a myocardial infarction (31). This study looked at only lifestyle behaviors, using no measurements such as pulse wave velocity. The results of the current study show the exact opposite effect of alcohol on the vasculature; it causes harm to the endothelial lining, increasing the risk of cardiovascular disease and potentially a myocardial infarction.

Physiologically, smoking has an effect on the vasculature. This could explain why the control group has a higher endpoint in the augmentation index than the wine group. Augmentation index represents the stiffness of the artery. It occurs when the incident pulse wave and reflected pulse wave meet, and the ratio of the two is expressed as a percentage (18, 38). Stiffness of arteries can be a risk factor attached to cardiovascular disease, making this measurement helpful in prognosis (18).

Limitations

Part of the present study's focus was the time course of the effects of wine consumption. Unfortunately, there were circumstances beyond the researchers' control that might have delayed the time course by a few minutes. These included: not being able to draw blood right away, not finding a pulse with the arterial distensibility machine, and subjects taking longer than the five minutes allotted to smoke a cigarette.

The limited number of subjects participating in the study presented several limitations. The power of interaction was not high enough to detect a small effect of FRAP between the two groups. If there had been more participants, the power of interaction could have been higher as well as potentially providing more significant results.

It is possible that ORAC did not show any effects because the study protocol did not provide enough oxidative stress through smoking to impact the ORAC assay. ORAC reflects the composite and cumulative antioxidant capacity of the blood (34).

CONCLUSION

There were significant protective effects of acute red wine consumption compared to the control on the overall plasma antioxidant reducing potential, based on the FRAP assay. Although FRAP values increased, no significant effects were found related to the time course of acute red wine consumption. Significant protective effects on arterial distensibility were found in acute red wine consumption compared to the smoking only treatment. Over time, the protective effects of acute red wine consumption diminished and had no effect on arterial distensibility. This study has shown that drinking red wine while smoking has no protective effects on the vascular system and may cause greater harm than smoking alone. Future studies will need to be performed including more subjects with more indices included, such as the renin angiotensin aldosterone system. Blocking the renin angiotensin aldosterone system can be a pulse wave velocity modifier, which might change the results presented above (19). Also to be considered would be including a group to consume dealcoholized red wine to compare results to measure the affect of the alcohol on the arterial distensibility and antioxidant capacity.

REFERENCES

1. Mahan LK, Escott-Stump S. *Krause's Food and Nutrition Therapy, 12th ed.* Philadelphia, PA: Saunders-Elsevier; 2008.
2. Salmon TB, Evert BA, Song B, Doetsch PW. Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic Acids Res.* 2004;32(12):3712-23.
3. Kusano C, Ferrari B. Total Antioxidant Capacity: a biomarker in biomedical and nutritional studies. *J Cell Mol Biol.* 2008;7(1):1-15.
4. American Heart Association. Cigarette Smoking Statistics. http://www.americanheart.org/presenter.jhtml?identifier=4559. Updated January 1, 2011. Accessed March 2, 2011.
5. Kubozono T, Miyata M, Ueyama K, et al. Acute and Chronic effects of Smoking on Arterial Stiffness. *Circ J.* 2010; 75(3):698-702
6. Karatzi K, Papamichael C, Karatzis E, et al. Acute smoking induces endothelial dysfunction in healthy smokers. Is this reversible by red wine's antioxidant constituents? *J Am Coll Nutr.* 2007;26(1):10-5.
7. Papamichael C, Karatzis E, Karatzi K, et al. Red wine's antioxidants counteract acute endothelial dysfunction caused by cigarette smoking in healthy nonsmokers. *Am Heart J.* 2004;147(2): E5.
8. Eiserich J, Butler J, Van Der Vliet A, Cross CE, Halliwell B. Nitric Oxide Rapidly Scavenges Tyrosine and Tryptophan Radicals. *Biochem J.* 1995;310(Pt 3):745-9.
9. Kingwell B. Nitric Oxide-Mediated Metabolic Regulation During Exercise: Effects of Training in Health and Cardiovascular Disease. *FASEB J.* 2000;14(12):1685-96.

10. Barberà JA, Peinado V, Santos S, Ramirez J, Roca J, Rodriguez-Roisin R. Reduced Expression of Endothelial Nitric Oxide Synthase in Pulmonary Arteries of Smokers. *Am J Respir Crit Care Med.* 2001;164(4):709-13.

11. Barton C, Ni Z Vaziri ND. Enhanced Nitric Oxide Inactivation in Aoritc Coarctation-Induced Hypertension. *Kidney Int.* 2001;60(3):1083-7.

12. Rocchi E, Bursi F, Ventura P, et al. Anti- and Pro-Oxidant Factors and Endothelial Dysfunction in Chronic Cigarette Smokers with Coronary Heart Disease. *Eur J Intern Med.* 2007;18(4): 314-20.

13. Förstermann U, Boissel JP, Kleinert H. Expressional Control of the 'Constitutive' Isoforms of Nitric Oxide Synthase (NOS I and NOS III). *FASEB J.* 1998;12(10):773-90.

14. Jain A, Agrawal BK, Varma M, Jadhav AA. Antioxidant status and smoking habits: relationship with diet. *Singapore Med J.* 2009;50(6): 624-7.

15. Laurent S, Boutouyrie P. Recent Advances in Arterial Stiffness and Wave Reflection in Human Hypertension. *Hypertension.* 2007;49(6):1202-6.

16. Asmar R, Benetos A, Topouchian J, et al. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. *Hypertension.* 1995;26(3): 485-90.

17. Yildiz M. Arterial distensibility in chronic inflammatory rheumatic disorders. *Open Cardiovasc Med J.* 2010;4:83-8.

18. Tomiyama H, Yamashina A. Non-Invasive Vascular Function Tests: Their Pathophysiological Background and Clinical Application. *Circ J.* 2010;74(1):24-33.

19. Mitchell G, Hwang SJ, Vasan, RS, et al. Arterial Stiffness and Cardiovascular Events: The Framingham Heart Study. *Circulation.* 2010;121(4):505-11.

20. Mahmud A, Feely J. Effect of Smoking on Arterial Stiffness and Pulse Pressure Amplification. *Hypertension.* 2003;41(1):183-7.

21. Di Majo D, La Guardia M, Giammanco S, La Neve L, Giammanco M. The antioxidant capacity of red wine in relationship with its polyphenolic constituents. *Food Chem.* 2008;111(1):45-49.

22. Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr.* 2005;81(1 Suppl): 243S-255S.

23. Cao G, Russell R, Lischner N, Prior RL. Serum Antioxidant Capacity Is Increased by Consumption of Strawberries, Spinach, Red Wine or Vitamin C in Elderly Women. *J Nutr.* 1998;128(12):2383-90.

24. Modun D, Music I, Vukovic J, et al. The Increase in Human Plasma Antioxidant Capacity After Red Wine Consumption is Due to Both Plasma Urate and Wine Polyphenols. *Atherosclerosis.* 2008;197(1):250-6.

25. Watzl B, Bub A, Briviba K, Rechkemmer G. Acute intake of moderate amounts of red wine or alcohol has no effect on the immune system of healthy men. *Eur J Nutr.* 2002;41(6):264-70.

26. Hudson T, Hartle DK, Hursting SD, et al. Inhibition of Prostate Cancer Growth by Muscadine Grape Skin Extract and Resveratrol through Distinct Mechanisms. *Cancer Res.* 2007;67(17):8396-405.

27. Xia EQ, Deng GF, Guo YJ, Li HB. Biological Activities of Polyphenols from Grapes. *Int J Mol Sci.* 2010;11(2):622-46.

28. Hines L Rimm E. Moderate Alcohol Consumption and Coronary Heart Disease: a Review. *Postgrad Med J.* 2001;77(914):747-52.

29. Cuevas A, Guasch V, Castillo O, et al. A High-Fat Diet Induces and Red Wine Counteracts Endotehlial Dysfunction in Human Volunteers. *Lipids.* 2000;35(2):143-8.

30. Zilkens R, Burke V, Hodgson JM, Barden A, Beilin LJ, Puddey IB. Red Wine and Beer Elevate Blood Pressure in Normotensive Men. *Hypertension.* 2005;45(5):874-9.

31. Mukamal KJ, Chiuve SE, Rimm EB. Alcohol Consumption and Risk for Coronary Heart Disease in Men with Healthy Lifestyles. *Arch Intern Med.* 2006;166(19): 2145-50.

32. Penumathsa S, Maulik N. Resveratrol: a Promising Agent in Promoting Cardioprotection Against Coronary Heart Disease. *Can J Physiol Pharmacol.* 2009;87(4):275-86.

33. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem*. 1996; 239(1):70-6.

34. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J Agric Food Chem.* 2002;50(16):4437-44.

35. Perez-Jimenez J, Sauro-Calixto F. Effect of solvent and certain food constituents on different antioxidant capacity assays. *Food Research International.* 2006;39(7):791-800.

36. Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin Chem.* 1998;44(6 Pt 1):1309-15.

37. Zulueta A, Esteve M, Frigola A. ORAC and TEAC assays comparisons to measure the antioxidant capacity of food products. *Food Chem*. 2009;114(1): 310-6.

38. Segers P, Kips J, Trachet B, et al. Limitations and pitfalls of non-invasive measurement of arterial pressure wave reflections and pulse wave velocity. *Artery Res.* 2009;3(2):79-88.

APPENDIX A

Recruitment Flier of the Wine Study

May/June 2010

Male Smokers Needed for Red Wine Antioxidant Study

Need an extra $30?

We are looking for male smokers ages 21-35 who would be willing to drink red wine, smoke and give blood samples.

- This is a study to investigate the effect of acute red wine consumption on blood antioxidant and blood vessel performance

Your obligations:

- Attend an orientation and 2 morning test sessions
- Allow professional phlebotomist to collect a total of 6 blood samples
- Allow technician to perform a safe, painless and non-invasive blood vessel performance test

You will be provided with:

- $30.00 (upon completion of study)
- Results of all lab tests including assessment of body composition

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

Wine Study
Dr. Lisa McAnulty
262-2630
mcanultyl@appstate.edu
Chelsea Lyga
lygacm@appstate.edu

APPENDIX B

Institutional Review Board Documents

APPALACHIAN STATE UNIVERSITY

Informed Consent for Participants in

Research Projects Involving Human Subjects

Title of Project: Effect and time course of acute red wine consumption on blood antioxidant and vascular performance in male smokers

Primary Investigator: Lisa McAnulty, Ph.D., R.D.

Co-investigators: Scott Collier, Ph.D.

Steven R. McAnulty, Ph.D.

Gregory Anoufriev, M.D.

Tim Radak, Dr.PH., R.D.

Purpose of this Research/Project

The purpose of this study is to examine the blood antioxidant and vascular performance (blood vessel) effects as well as the time course (1 hour and 3 hours after consumption) of consumption of 300 ml (~10 oz) of red wine from North Carolina in 15 subjects. This study will be the first study to incorporate an investigation of the ability of red wine consumption to stop or reduce the damaging effects of cigarette smoking as measured by state of the art blood vessel performance measures and changes in blood antioxidants over time. Given the large amount of damaging blood vessel

effects and blood pressure changes associated with cigarette smoking, this project is highly relevant and will have implications for a sizable section of the population. Additionally, given the positive outcomes anticipated, this project could lend support to the North Carolina wine industry as well as support possible development of natural compounds which might protect the blood vessels from damage.

BRIEF REVIEW

Smoking

Smoking causes damage to the blood vessels by injuring cells and increasing blood pressure, but the exact mechanism still needs to be found. The relationships between smoking and cardiovascular disease most likely result from multiple mechanisms that interact to contribute to blockage and vessel cell damage. Because of the complexity of the interactions between nicotine and other components of smoke with the blood vessel cells, the complete mechanisms by which smoking contributes to cardiovascular disease are still not clear. Interestingly, previous research found that healthy subjects that smoked one cigarette and drank 250 ml (1 cup) of red wine maintained good blood pressure versus smoking one cigarette without wine. However, blood antioxidants were not examined. It is possible that the damage caused by smoking could be attributed to increased oxygen damage and that antioxidants found in red wine could stop or reduce the bad effects on the blood vessel cells.

Blood Antioxidant Capacity and Red Wine Polyphenolic Compounds

Certain compounds in red wine can act as antioxidants. This has led to the belief that red wine consumption provides beneficial effects compared to other alcoholic beverages. However, studies assessing the effects of red wine consumption on plasma antioxidants and oxygen damage in humans have not given final results.

There is evidence that at least part of the process responsible for several of the major diseases known to contribute to mortality in the United States (for example, heart disease, cancer, diabetes, and Alzheimers) is related to oxygen damage and inflammation. Oxygen damage is derived from the formation of compounds known as reactive oxygen species (ROS). Research has shown supplementation with naturally occurring wine compounds to be safer than traditional antioxidants (such as vitamins E and C) and have many positive effects. The action of these wine chemicals involves antioxidant activities and the killing of cancer cells. Functional foods and natural compounds have great promise to improve health and prevent aging-related chronic diseases. Many of the natural antioxidant compounds found in red wine have been found to possess anti-inflammatory, anticancer, and antioxidant activities.

II. Procedures

Orientation and Baseline Testing

The purpose of this study is to examine the blood antioxidant and blood vessel

performance effects as well as the time course (1 hour and 3 hours post consumption) of acute consumption of 300 ml (~10 oz) of red wine from North Carolina within 10 minutes. One to two weeks prior to beginning the study, subjects will report to 168B University Hall at 4:00 p.m.for orientation, screening, obtaining informed consent, body composition, height and weight. Body composition (% body fat) will be determined using Bioelectrical Impedance (BIA). Subjects will be instructed not to consume alcohol or other over the counter medications or supplements 24 hours prior to reporting and to avoid caffeine and nicotine use the morning of reporting to the lab until study conclusion. Each subject will keep a 3-day food record prior to the test and be encouraged to maintain normal dietary patterns prior to the study. During orientation, a dietitian will instruct the subjects on how to adhere to the dietary restrictions and how to record intake in a food record.

First Laboratory Test

After the orientation visit, subjects will report back to the lab in a fasted state at 8:00 am for the first experimental trial into which the subject was randomized. Subjects will be instructed not to consume alcohol or other over the counter medications or supplements 24 hours prior to reporting and to avoid caffeine and nicotine use the morning of reporting to the lab. Subjects will have weight and BIA obtained and then sit quietly for 10 minutes before having a pre-wine consumption blood vessel performance test and blood draw (1 tablespoon or 14 ml blood total). Subjects will then consume 10 oz (300 ml) of Duplin red wine within 10 minutes while sitting in the laboratory. Exactly 45 min later, subjects will move to a smoking area outside the building and smoke one cigarette within a five minute period. Subjects will then have a post blood vessel performance test repeated and post-consumption blood draw (1 tablespoon or 14 ml blood total). To examine the time course of blood vessel and blood effects, subjects will remain in the lab and 2-h and 45 min later repeat the previously described smoking protocol before having a final blood vessel test and blood draw approximately 3-h post wine consumption or control. Before subjects depart the lab after each visit, snacks and fluids will be available for subjects to consume after the 3-h testing. Also, before being allowed to leave the study site, a breathalyzer test will be performed on all subjects to confirm that blood alcohol concentrations are 0.0%.

Washout Period and Second Laboratory Test

All subjects will then not do anything in the lab for a period of 1-wk (washout) and then crossover (go into) into the opposing treatment and repeat the above described sequence of events as described in the first laboratory test. The second test will be with or without wine depending upon the initial or first laboratory test in which the subject participated. Before subjects depart the lab after each visit, snacks and fluids will be available for subjects to consume after the 3-h testing.

Blood Sampling

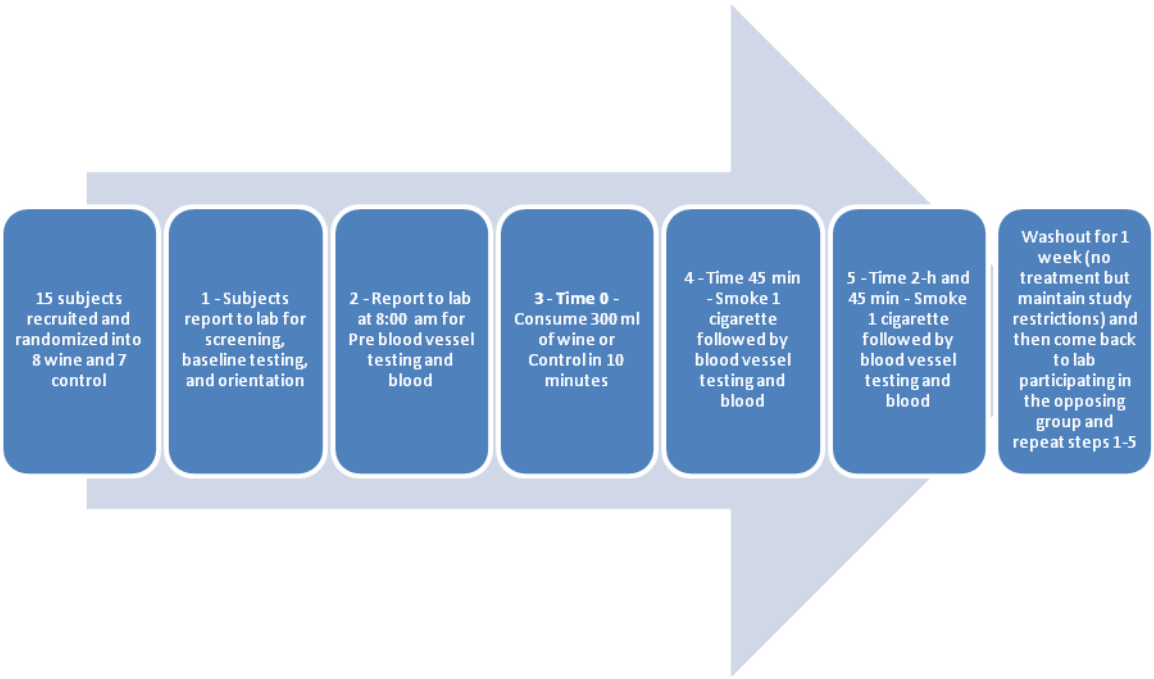
Blood samples will be drawn six times total during the entire experiment which includes Pre-consumption, 1-h Post wine consumption, and 3-h Post wine consumption and Pre-control, 1-h Post control, and 3-h Post control. The following tubes of blood will be drawn during each sampling time: one x 7 ml (1 tsp) tube to examine plasma antioxidant capacity and one x 7 ml (1 tsp) tube for another aspect of antioxidant capacity. This is a

total of 84 ml (about 6 tablespoons) of blood for the entire experiment. The risks related to blood sampling will be minimized by having trained technicians perform the procedures. Nonetheless, there is a small risk of infection or bruising. The procedures are identical to those successfully used many times in our laboratory. The local rescue squad is 1 mile from our lab, with a documented response time of 2-3 minutes. All subjects will be provided instructions for care as well as contact phone numbers for the investigators involved in the study. Universal precautions will be used throughout all blood collections. This refers to a "mindset" or "attitude" taken by the researchers that assumes all blood or body tissues are potentially infectious. In the case of exposure of an experimenter by your blood or tissue, that blood will be analyzed for HIV and hepatitis (a positive HIV or hepatitis test will be reported to the subject).

Vascular Performance Testing

Changes in blood flow and blood vessel function will also be measured pre acute wine consumption, 1-h post acute consumption, and 3-h post acute consumption using the Sphygmacor Cardiovascular Management System Arterial Pulse Wave Velocity and Aortic Blood Pressure Waveforms (PWV, ABPW respectively). All data will be stored and analyzed off-line after completion of testing. Changes in blood flow and blood vessel function measured using the Sphygmacor Cardiovascular Management System Arterial Pulse Wave Velocity and Aortic Blood Pressure Waveforms assessment of blood flow is completely safe, painless, and non-invasive. A small pen-like probe is placed over your carotid artery (side of your neck), over the femoral artery (top of your leg) and ankle artery. A transducer (like a microphone) uses ultrasound waves (sound waves which bounce off the blood in the blood vessel) to measure the speed and direction of blood flow through an artery. No physical discomfort should be experienced during this test. Your privacy will be upheld with great care during the assessment of the femoral artery, as this is best located near the pubic area. There are no known risks associated with the Doppler ultrasound used in this technique.

Study Timeline:



III. Risks

The risks related to blood sampling will be minimized by having trained technicians perform the procedures. Nonetheless, there is a small risk of infection or bruising. The procedures are identical to those successfully used in a previous study. The local rescue squad is 1 mile from our lab, with a documented response time of 2-3 minutes. All subjects will be provided instructions for care as well as contact phone numbers for the local hospital and the principal investigator. Universal precautions will be used throughout all blood collections. This refers to a "mindset" or "attitude" taken by the researchers that assumes all blood or body tissues are potentially infectious. In the case of exposure of an experimenter by your blood or tissue, that blood will be analyzed for HIV and hepatitis (a positive HIV or hepatitis test will be reported to the subject).

The measurement of your body composition via BIA will expose you to a small dose of electricity. The amount of alcohol consumed in this study is considered modest and has been safely used in a prior research project (unpublished results). However unlikely, we will ask subjects to report any adverse symptoms associated with the amount of wine used in this study such as unusual nausea, headache, vomiting, weakness, or mental confusion. Subjects will be given the contact information for the lead investigator in case of questions or problems. Excessive alcohol consumption associated with alcoholism is well understood to be detrimental both psychologically and physically. Individuals identified to have alcohol problems will not be included in this study (identified through CAGE Questionnaire).

Furthermore, before being allowed to leave the study site, a breathalyzer test will be performed on all subjects to confirm that blood alcohol concentrations are 0.0%.

IV. Benefits

The subject will receive results of all tests and $30.00 compensation upon full completion of the study. The NC Quit Line (1-800-Quit-Now or 1-800-784-8669) is available to individuals that would like to quit smoking. I understand that no promise or guarantee of benefits have been made to encourage participation. Larger societal benefits include potential progress toward methods to minimize detrimental effects from oxidative stress associated with certain disease processes.

V. Extent of Anonymity and Confidentiality

The identity of subjects will not be disclosed in any published documents or shared with anyone but the experimenters.

VI. Compensation

Thirty dollars compensation will be given upon full completion of the study. If as a result of a research project, the investigator determines that the subject should seek counseling or medical treatment, a list of local services will be provided. In the event of physical injury resulting from the research procedures, immediate first-aid is provided free of charge. No funds have been set aside for medical treatment of any injury or illness resulting from this project and the subject assumes full responsibility for any costs.

VII. Freedom to Withdraw

The subject is free to withdraw from this study at any time without penalty.

VIII. Approval of Research

This research project has been approved, as required, by the Institutional Review Board of Appalachian State University.

_____ _____

IRB Approval Date Approval Expiration Date

IX. Subject's Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities:

1). The subject will attend an orientation session at 168B University Hall prior to the start of the study and agrees to be randomized into Wine or Control groups initially, complete the respective protocol, washout for 1-wk, and then crossover into opposing treatment and complete the protocol.

2). The subject agrees to drink 10 oz red wine (wine group) and sit as a control (without wine) and smoke one cigarette a total of four times during the entire study. Subjects agree to remain in the lab for 3-h afterwards for testing and safety reasons and undergo testing of blood alcohol concentration via portable breathalyzer. While a subject in this project, subjects agree to avoid the use of alcohol and over the counter medications and supplements not recommended by a physician 24-h prior to testing and caffeine and nicotine the morning of the test. Should any medications be prescribed by a physician during the course of the study, subject will notify an investigator immediately. Subject agrees and understands that depending upon the drug, it may be necessary for the subject to be released from the study

without compensation. Body composition (% body fat) will be determined using BIA at Baseline and Pre-acute session. Blood vessel measurements will be obtained at each blood draw.

3. On the day of acute testing and prior to the test, subjects will not eat anything past midnight the previous day before coming to the lab and avoid all caffeine and cigarettes the morning of the test.

4. Blood samples will be collected at Pre, 1-h post, and 3-h post on two separate occasions.

X. Subject's Permission ***(May be modified in the case of minors or members of other vulnerable populations.)***

I have read and understand the Informed Consent and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent:

_____Date_____

Subject signature

_____ Date _____

Witness (Optional except for certain classes of subjects)

Should I have any questions about this research or its conduct, I may contact:

Lisa McAnulty office phone 828-262-2630

mcanultyl@appstate.edu

Primary Investigator

Julie Taubman 828-262-7981
taubmanjl@appstate.edu

Administrator, IRB Telephone e-mail

Graduate Studies and Research

Appalachian State University

Boone, NC 26608

Subjects must be given a complete copy (or duplicate original) of the signed Informed Consent.

Institutional Review Board

Approval Date: ___4/27/2010_____

Expires On: _4/26/2011____

Study #: _10-0178__

Initials: _____L.M._____

APPENDIX C

Health Questionnaire

Subject ID: _____ Interviewer's Name:_____

Subject's Name:_____ Date (mm/dd/yy):_____

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

Personal	Family	Medical History
□	□	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)

HEALTH AND MEDICAL HISTORY QUESTIONNAIRE

10. Please indicate the approximate number of alcoholic beverages **per week**. (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) _____

HEALTH AND MEDICAL HISTORY QUESTIONNAIRE

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)					

HEALTH AND MEDICAL HISTORY QUESTIONNAIRE

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	Somewhat	Extremely
Physically active	physically active	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

Name of personal physician:_____

Phone #:_____

Address:_____

APPENDIX D

Demographic Data

Age	22	23	21	21	21	21	31	31
Height	71”	71”	73”	65”	70”	67”	69”	67”
Weight	144	226	134	148	284	149	165	150
Smoking length	4 years	7 years	6 years	8 years	4 years	3 years	15 years	15 years
Smoking days/month	30	7	10	30	30	15	30	30
Cigarettes per day	8	10	10	2.5	3.5	1.5	20	13.5

APPENDIX E

Ferric Reducing Ability of Plasma assay instructions

FERRIC REDUCING ABILITY OF PLASMA (FRAP)

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)

a. weigh 3.1 grams sodium acetate trihydrate

b. add 16 ml acetic acid (1N) (Molarity = Normality/Valence)

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 (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

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)

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 250 ml acetate buffer

b. add 25 ml TPTZ solution

c. add 25 ml ITX solution

This amount of solution is sufficient for 86 tests.

7 STDs x 2 = 14

1 BLK x 2 = 2

35 samples x 2 = 70

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. **(Note: Consider using Trolox as STD). you will have to calculate how to make the 1000uM trolox.**

	1000uM Troloxate solution	DI
1000 uM	5 mL	0 mL
800 uM	4 mL	1 mL
600 uM	3 mL	2 mL
400 uM	2 mL	3 mL
200 uM	1 mL	4 mL
100 uM	0.5 mL	4.5 mL
0 uM	0.0 mL	5.0 mL

6. Warm FRAP solution to 37 deg C

7. For BLK, samples, and ascorbate STDs, add 100 ul of DI, plasma sample, or STD, respectively to 3.0 mL FRAP reagent and vortex.

8. Incubate tubes at 37 deg C for 4 min

9. Measure absorbance (593 nm) immediately after 4 min.

10. Report plasma reducing potential as ascorbate acid equivalents obtained from reference curve of ascorbate concentration (593nm). ie... 1 umol ascorbate = one reducing equivalent.

APPENDIX F

Oxygen Radical Absorbance Capacity assay instructions

Standard Operating Procedure of Oxygen Radical Absorbance Capacity ($ORAC_{FL}$)

Written by Xianli Wu, ACNC

Last Modified: 10/31/2005

1. INSTRUMENTS AND SUPPLIES

1.1. Necessary Instruments and Supplies:

- Microplate Reader with or without injectors and desktop PC with relevant software
- Centrifuge and/or microcentrifuge
- Water bath, 0-100 °C
- Refrigerator, 4°C
- Freezer, -20°C
- Vortex apparatus
- Pan balance
- pH meter
- Stir plate, magnetic
- Analytical balance
- Polypropylene or Polystyrene Assay Plate, 96 Well Clear Flat Bottom (Costar, Falcon etc.)
- Pipettemen, 100-1000 µL, 50-200 µL, 10-100 µL (Eppendorf)
- Pipette Tips, 20-200 µL, 101-1000 µL (Fisher)
- Volumetric flasks, 1000, 100, 50, 25, 10, 2 mL
- Graduated cylinders, 1000 mL, 250 mL and 100 mL.
- Conical tubes 50 mL, 15 mL (Fisher)
- Eppendorf tubes 1.8 mL
- Sealing film (if plate does not include a cover)
- Plastic transfer pipettes (Fisher)

1.2. Optional Instruments and Supplies:

- Automated Microplate Pipetting System with desktop PC and software

- Repeating Multi-Channel Pipette, 20-200 µL with compatible tips and reagent reservoir
- Timer
- Orbital Shaker

2. REAGENTS AND SOLVENTS

- Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich Chemicals, Item 238813- 5G, CAS# 53188-07-1, F.W. 250.29
- Fluorescein disodium salt, Aldrich Chemicals, Item 166308-100G, CAS# 518-47-8, F.W. 376.28
- AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride, Wako Chemicals, Item 992-11062 RN# 2997-92-4. F. W. 271.193.
- Potassium Phosphate Dibasic, Fisher Chemical, CAS# 7758-11-4, F.W. 174.18.
- Potassium Phosphate Monobasic, J.T. Baker, item 4008-01, CAS# 7778-77-0, F.W. 136.09
- diH2O

3. REAGENT PREPARATION

3.1. Phosphate Buffer

- ***Buffer Stock Solution:***

  Solution A: 75 mMolar Monopotassium phosphate

  - Accurately weigh 10.21 grams of Monopotassium Phoshate into a weigh dish
  - Transfer the monopotassium phosphate into a 1000 mL volumetric flask
  - Add approximately 700 mL DI water and a magnetic stir bar. Stir on a magnetic stirplate until the phosphate is dissolved
  - Remove the stir bar and make up to volume with DI water

  Solution B: 75 mMolar Dipotassium phosphate

  - Accurately weigh 13.06 grams of Dipotassium Phoshate into a weigh dish
  - Transfer the Dipotassium phosphate into a 1000 mL volumetric flask
  - Add approximately 700 mL DI water and a magnetic stir bar. Stir on a magnetic stirplate until the phosphate is dissolved
  - Remove the stir bar and make up to volume with DI water

- ***Buffer Working Solution:***
  - Place a magnetic stir bar into a 1000 mL beaker. Pour 800 mLs of solution B into the 1000 mL beaker. Insert the pH electrode and begin reading the pH of the solution.

- Transfer 200 mLs of solution A into a graduated cylinder. Transfer the A solution into the 1000 mL beaker containing B and monitor the change in pH.
- Slowly add additional solution A as needed to reach a final solution pH of 7.4
- Store the 7.4 Buffer in a one liter amber glass bottle.

3.2. Trolox Standards

- ***Trolox Stock Solution:***

  Dissolve 25 mg (0.025 g) of Trolox in 100 mL phosphate buffer working solution to make 1 mM trolox, then dilute to 500 µM. Aliquot 1.5 mL in 1.8 mL Eppendorf tubes and store at –70°C until use.

- ***Trolox Working Solution:***
- Thaw an aliquot of Trolox stock solution. Take 1 mL of 500 µM Trolox in a 15 mL tube; add 9 mL of working buffer and vortex to make 50 µM Trolox solution. Make serial dilutions of the 50 µM Trolox solution with working buffer solution to produce 25, 12.5, 6.25 µM Trolox standards. Keep remaining Trolox solution at 4°C.

3.3. Fluorescein Solution

- ***Fluorescein Stock solution:***
  - **Stock solution #1:** dissolve 0.0225 g in 50 mL of working phosphate buffer and mix well.
  - **Stock solution #2:** dissolve 50 µL of stock solution #1 in 10 mL of working buffer and vortex. Aliquot stock solution #2 into 1.8 mL Eppendorf tubes and store at –20°C until use.

- ***Fluorescein Working Solution:***

  Pipette 800 µL of stock solution #2 into 50 mL phosphate buffer (for two runs) in a 50 mL conical tube. Before use, incubate in the water bath at 37°C until thoroughly heated. This solution can be kept in the water bath for many hours**.**

3.4. AAPH Solution

The phosphate buffer is preincubated at 25 °C. Dissolve 0.108 g of AAPH into 5 mL of incubated phosphate buffer immediately before the start of the assay. This produces an AAPH solution containing 79.6 µmol/mL. A 20 µL aliquot provides 1.6 µmol AAPH per well. It is important to keep the AAPH in the refrigerator at 4^{o}C before adding the warm buffer. The AAPH reaction is temperature-dependent. This procedure was proven with the BMG plate reader to significantly reduce apparent temperature effects.

NOTE: If –20°C and –70°C storage facilities are not available, fresh reagent solutions have to be made freshly on a daily basis.

4. PREPARE AND LOAD 96-WELL MICROPLATE

- Set up a paper layout to determine the order of your samples. In order to avoid possible positional errors, a "forward-then-reverse" order is recommended. It is highly recommended to leave the edge wells empty or blank (phosphate buffer working solution) to reduce the impact of "edge effect" on samples and standards particularly if your instrument exhibits some temperature effects on the outside wells. The following is an example of sample layout in a 96-well microplate:

X	B	B	B	B	B	B	B	B	B	B	B
B	T1	T2	T3	T4	B	S1	S2	S3	S4	S5	B
B	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	B
B	B	B	B	T1	T2	T3	T4	B	B	B	B
B	S16	S17	S18	S19	S20	S20	S19	S18	S17	S16	B
B	S15	S14	S13	S12	S11	S10	S9	S8	S7	S6	B
B	S5	S4	S3	S2	S1	B	T4	T3	T2	T1	B
B	B	B	B	B	B	B	B	B	B	B	B

X: 200 µL Fluorescein working solution for gain adjustment of plate reader.

B: Blank, phosphate buffer working solution.

S: Sample

T: Trolox standards, from T1 to T4, from 6.25 µM to 50 µM.

- Pipette 20 µL of sample, blank and Trolox standard solutions into appropriate wells. Also pipette 200 µL of fluorescein working solution into the selected well for the gain adjustment.

<u>FOR PLATE READERS WITH 2 INJECTORS (eg. BMG Fluostar Galaxy/Optima plate reader):</u>

- Place 25 mL Fluorescein working solution in the plate reader so that it corresponds to Pump #1.

- Place the correct amount of AAPH solution in position so that it corresponds to Pump #2.

5. SETTING THE PLATE READER

5.1. BMG Fluostar galaxy/optima plate reader

Plate Reader Startup

- From the Start Menu, select: Programs> BMG technologies> Fluostar galaxy/optima> Fluostar control. Click "incubator" icon and set the tempe  at 37 °C.

-  Click on the "open door" icon after the color of the "incubator" icon turns from red to green. Cover the plate with a lid or sealing film, insert the microplate, and click the "close door" icon. Leave the plate in the chamber for ten minutes for pre-heating.

-  Click on the "Test Protocol" icon (The icon is located next to the setup icon on the Fluostar Optima brand instrument. Use the "Test" icon for the Fluostar Galaxy brand instrument) to create or open an ORAC Fluostar Test Protocol. Click on "new" or open an already existing test protocol.

Layout

Set up the layout exactly like the paper layout. Trolox standards will be labeled as "sample." Fluorescein will be a "standard."

Basic Parameters

Positioning delay: 0.3s

No. of kinetic windows: 1

No. of cycles: 35

Measurement Starting Time: 0.0 s

No. of flashes per cycle: 15

Cycle time: Depending on the layout.

Filters and integration: Fluorescence Intensity

No. of multichromatics: 1

Gain: will change depending on each Gain Adjustment

Pause before cycle: 0

Excitation filter: 485 nm

Emission filter: 520 nm

Calculation range: start from 1, stop at 35

Click on "check timing" icon (lower left corner). Note the cycle time.

Concentration/Volume/Shaking

Volume 1: 200 µL, Pump speed: 420 µL/s

Volume 2: 20 µL, Pump speed: 420 µL/s

Shaking mode: orbital

Shaking width: 4 mm

Additional Shaking: before each cycle

Shaking time: 8 s

Injection and timing for instruments with automatic pipettors as part of the plate reader:

Volume group 1 injection cycle: 2

Volume group 2 injection cycle: 4

Volume group 1 injection start time: 0.0 s

Volume group 2 injection start time: 0.0 s.

- Place the injection needles and the tubes into their appropriate solution. The solution should pump back into its tube. Pump 1 is for Fluorescein and pump 2 is for AAPH. Next, click on the 'Prime' icon to prime the two pumps and remove any air bubbles. Prime each needle at least 3 times. 
- Open the microplate chamber and remove the lid or film. Close the chamber again.

-  Click the 'measure' icon or select 'measure' under the Measure menu. Select the correct test protocol. Enter plate and sample identifications and check the gain adjustment for the fluorescein well. The normal range of gain value is 35 ± 5 for Galaxy and 1700 ± 100 for Optima, at 90% required value.
- Open the microplate chamber, cover the plate and leave it in the instrument for ten minutes. Then, open the chamber once more, remove the cover, close the chamber, and start the test run.

- **NOTE**
  - Make sure the incubator is on and the temperature is set to 37°C.
  - Make sure the cables are turned so as to read from the bottom of the plate.
  - Back flush old reagents from pumps after each run.
  - Two injection needles and measurement head need to be washed thoroughly at least every two days if the machine is fully used.

Injection and timing for instruments without automatic pipettors as part of the plate reader:

- Add 200 µL Fluoroscein working solution to each well using an 8 channel micropipettor.
- Place cover on microplate and incubate plate and contents at 37 ^{o}C for at least 20 minutes.
- Verify that plate reader is warmed up to 37 ^{o}C and ready to read samples.
- Add 20 µL AAPH working solution using an 8 channel micropipettor.

NOTE: Timing of the addition of the AAPH is critical and the time to add all columns should be kept to a convenient minimum. An automated pipetting system was timed and it took about 8 seconds per column for a total of 1 min 40 seconds to pipette the entire plate. This was sufficient time such that a difference in the initial reading was observed due to the reaction occurring in the 1st column compared to the 12th column. A sample layout of "forward then reverse" is critical in this case to account for timing differences.

6. DATA ANALYSIS AND CALCULATIONS

6. 1. Calculation of Area Under the Curve (AUC)

The relative area under curve (AUC) was calculated as

$$AUC = (0.5 + f_5 / f_4 + f_6 / f_4 + f_7 / f_4 + \ldots + f_i / f_4) \times CT$$

Where f_i = fluorescence reading at cycle *i* (i.e. f_4 = initial fluorescence reading at cycle 4), and CT = cycle time in minutes.

The net AUC was obtained by subtracting the AUC of the blank from that of a sample.

NOTE: The AUC calculated in this SOP is a relative AUC, which means the initial point was treated as 1, so the other data points would range from 0 to 1.0. In terms of the calculation, the AUC was calculated as the sum of many small trapezoids. The formula is as follows:

$$AUC = [(f_1/f_1 + f_2/f_1) \times t]/2 + [(f_2/f_1 + f_3/f_1) \times t]/2 + [(f_3/f_1 + f_4/f_1) \times t]/2 + \ldots + [(f_{i-1}/f_1 + f_i/f_1) \times t]/2 = [0.5 + f_2/f_1 + f_3/f_1 + \ldots + f_{i-1}/f_1 + (f_i/f_1)/2] \times t$$

6. 2. Calculation of $ORAC_{FL}$ value

The final $ORAC_{FL}$ values were calculated by using a quadratic regression equation ($y = a + b\chi + cx^2$) between Trolox concentration (μM) and the net area under the FL decay curve (net AUC). The quadratic regression was used in the range of 6.25-50 μM Trolox. Data is expressed as micromoles of Trolox Equivalents (TE) per liter (for liquid sample) or per gram (for solid sample) of sample (μmol TE/L or μmol TE/g).

The data was analyzed by Microsoft Excel (Microsoft, Roselle, IL).

A quality control standard of gallic acid or uric acid can be included in each run. It is important to monitor the net area of the standards and the regression coefficients.

USE THIS EQUATION TO SOLVE FOR FINAL CONCENTRATIONS: $x = -b \pm \sqrt{b^2-4ac+4cy}$

7. Key References

1. Cao, G., Alessio, H. M., Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic. Biol. Med.* **1993**, 14, 303-311.
2. Cao, G.; Prior, R. L. Measurement of oxygen radical absorbance capacity in biological samples. *Methods Enzymol.* **1998**, *299*, 50-62.
3. Ou, B., Hampsch-Woodill, M., Prior, R. L. Development and validation of an improved Oxygen Radical Absorbance Capacity Assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, 49, 4619-4626.
4. Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273-3279.

APPENDIX G

FRAP Data

Subject	Wpre	Wpost45	Wpost245	Cpre	Cpost45	Cpost245
2	948.875	947.625	870.125	847.625	868.875	868.875
3		1108.875	1070.125	1006.375	1027.625	1032.625
6		1037	984.5	1083.875	1110.75	1107
7	1220.75	1213.25	1189.5	1042	1003.875	1061.375
11	1234.5	1268.875		1154.5	1220.125	1193.875
12	1348.875	1507	1449.5	1368.875	1317	1344.5
14	1367.625	1627.625	1497.625	1408.25	1457.625	1180.75
15	944.5	1431.375	966.375	1172	1110.75	1087.625

FRAP = Ferric Reducing Ability of Plasma, Wpre = wine pre consumption, Wpost45 = wine 45 minutes post consumption, Wpost245 = wine 2 hours and 45 minutes post consumption, Cpre = control pre consumption, Cpost45 = control 45 minutes post consumption, Cpost245 = control post 2 hours and 45 minutes.

APPENDIX H

ORAC Data

Subject	Wpre	Wpost45	Wpost245	Cpre	Cpost45	Cpost245
2	39.85483	37.86606	40.00897	40.43463	37.38255	39.56575
3	N/A	27.87225	34.43399	32.72482	45.71433	14.99088
6	N/A	48.13496	49.41785	36.35119	47.24731	20.87101
7	32.28554	41.87714	47.39213	26.54822	21.82394	52.85438
11	34.43399	37.09556	N/A	20.87101	37.09556	32.28554
12	31.17731	43.36692	28.77338	32.72482	44.40942	45.20204
14	36.35119	41.8714	37.86606	46.1891	46.26145	51.63489
15	48.26434	28.77338	46.06616	31.17731	28.31945	25.31793

ORAC = Oxygen Radical Absorbance Capacity, Wpre = wine pre consumption, Wpost45 = wine 45 minutes post consumption, Wpost245 = wine 2 hours and 45 minutes post consumption, Cpre = control pre consumption, Cpost45 = control 45 minutes post consumption, Cpost245 = control post 2 hours and 45 minutes.

APPENDIX I

Vascular Data

See table on next page.

Subject #	sbpa	sbpb	sbpc	sbpd	sbpe	sbpf	dbpa	dbpb	dbpc	dbpd	dbpe
1	124						84				
2	110	132	126	110	116	110	62	89	86	70	75
3	121	120	120	118	122	123	71	75	72	69	70
4	135	125	127				73	80	63		
6	132	123	132	124	126	126	79	82	83	78	78
7	130	120	132	129	126	124	85	81	78	79	80
11	126	130	122	121	121	123	78	80	84	82	73
12	121	124	119	126	123	122	75	71	72	63	62
14	124	122	125	123	120	123	66	70	73	72	70
15	120	122	120	128	120	128	79	79	65	78	70

dbpf	aixa	aixb	aixc	aixd	aixe	aixf	cpwva	cpwvb	cpwvc	cpwvd
	15									
68	13	19	-8	14	4	6	7.7	7.5	9.2	6.6
75	3	-4	-3	0	-6	3	5.8	6.5	7.1	5.4
	13	14	11				8.7	12.1	10.9	
79	22	26	22	27	21	29	8.9	7.9	8.1	7.6
78	21	9	5	18	19	17	11.3	11.1	9.5	9.7
74	11	7	12	14	13	15				
72	11	-4	4	0	4	17	6.7	8.4	7.5	8.2
69	17	5	-2	23	19	21	7.1	6.5	7.4	9.3
76	18	-3	11	12	4	3				

cpwve	cpwvf	ppwva	ppwvb	ppwvc	ppwvd
		9.5			
7.7	6.9	7.8	8.8	7.6	6.5
6.2	6.7	6.8	6.2	8.5	5.6
		9.8	10.7	9.3	
6.8	8.4	7.8	9.5	8	9.1
9.6	9.9	10.6	9.9	10.3	9.5
8.8	8.8	8.3	7.5	8.7	8
8.3	7.9	7.2	6.5	8	9.4
			6.5		9.4
			6.5		

sbp = systolic blood pressure, dbp = diastolic blood pressure, aix = augmentation index, cpwv = central pulse wave velocity, ppwv = peripheral pulse wave velocity; a, b, c = the 3 measures taken during wine; d, e, f = the 3 measures taken during control

VITA

Rohan Elizabeth Krehbiel was born in Cincinnati, Ohio on August 17, 1987. Her parents are Anne and Dick Krehbiel, and she has one younger brother, Paul. She attended St. Ursula Villa for grade school and Ursuline Academy for high school. In the fall of 2005, she started as a freshman at the University of Kentucky in Lexington, Kentucky where she was a member of Sigma Kappa sorority and an avid supporter of all the Kentucky Wildcat sports teams. She graduated in May 2009 with a Bachelor of Science in Dietetics and a minor in business. She moved to Boone, North Carolina in the fall of 2009 to start the combined Master's Degree and Dietetic Internship program at Appalachian State University. The M.S. was awarded in May 2011. In June 2011, she is planning to take the Registered Dietitian exam and hopes to pursue a career as a Registered Dietitian. During her career, she hopes to help her patients understand the importance of food and how to foster a healthy relationship with food.