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by

was prepared under the direction of the Master's Thesis Advisory Committee. It is accepted by the committee members in partial fulfillment of the requirements for the degree Master of Science in the Byrdine F. Lewis College of Nursing and Health Professions, Georgia State University. The Master's Thesis Advisory Committee, as representatives of the faculty, certify that this thesis has met all standards of excellence and scholarship as determined by the faculty.

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ABSTRACT

Several chronic conditions, including cardiovascular disease, diabetes, and cancer have been linked to oxidative stress and inflammation in the body. Angiotensin (Ang) II, a hormone that increases blood pressure, has been shown to induce inflammation and oxidative stress in adipose tissue. Berries have well documented anti-inflammatory and antioxidant properties. Our goal was to determine if adding blackberries or raspberries to the diet mitigates Ang II-induced inflammation and oxidative stress in the epididymal and retroperitoneal white adipose tissue. At 8 weeks of age, male Sprague-Dawley rats were assigned to one of five groups: 1) control, 2) Ang II, 3) Ang II + 10% blackberry diet, 4) Ang II + 10% raspberry diet, or 5) Ang II + 5% blackberry and 5% raspberry combination diet. The rats were fed their respective diets for four weeks at which point rats had osmotic minipumps implanted that delivered either saline or Ang II (270 ng/kg BW/min). The rats consumed their assigned diets for an additional three weeks. Adipose tissue was collected, and expression of genes involved in inflammatory and antioxidant pathways was measured by real-time PCR (*Il1b*, *Ccl2*, *Nqo1*, *Gpx1*, *Nrf2*, and *Hmox1*). Ang II did not significantly affect expression of genes involved in the inflammatory or antioxidant pathways. Likewise, while berry consumption tended to reduce expression of some inflammatory genes, the berries had no significant effect on expression of the genes measured. Though some studies have found berries to mitigate inflammation and oxidative stress in rat models, our results were nonsignificant. The discrepancy may be due to the concentration of Ang II, the exposure time of the berry diet, the type of berries used, or the concentration of freeze-dried berry powder used in the study. Therefore, our data suggest further research investigating the impact of berry consumption on inflammation and oxidative stress in adipose tissue.

EFFECTS OF BLACKBERRY AND RASPBERRY CONSUMPTION ON MARKERS OF
OXIDATIVE STRESS AND INFLAMMATION IN ADIPOSE TISSUE OF ANGIOTENSIN
II-TREATED RATS

by

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Under the Direction of Dr. Desiree Wanders

A Thesis submitted in Partial Fulfillment of the Requirements for the Degree of
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Department of Nutrition

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ABBREVIATIONS

| | |
|-------------------------------|-------------------------------------|
| Ang I | Angiotensin I |
| Ang II | Angiotensin II |
| ANOVA | Analysis variance |
| AT1R | Angiotensin II type 1 receptor |
| AT2R | Angiotensin II type 2 receptor |
| CO ₂ | Carbon dioxide |
| CVD | Cardiovascular disease |
| CAT | Catalase |
| cDNA | Complementary deoxyribonucleic acid |
| CHOP | C/EBP homologous protein |
| ER | Endoplasmic reticulum |
| EWAT | Epididymal white adipose tissue |
| GSR | glutathione reductase |
| GPX1 | glutathione peroxidase 1 |
| Hmox1 | heme oxygenase 1 |
| HDL | high-density lipoprotein |
| H ₂ O ₂ | hydrogen peroxide |
| HO | hydroxyl radical |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IL-10 | Interleukin 10 |

| | |
|-----------------------------|---|
| IL-1 β | Interleukin-1 β |
| IL-6 | Interleukin 6 |
| KIHD | Kuopio Ischaemic Heart Disease Risk Factor |
| MCP-1 | monocyte chemoattractant protein-1 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| Nqo1 | NAD(P)H dehydrogenase [quinone] 1 |
| NOX | NADPH oxidase |
| NOX1 | NADPH oxidase 1 |
| NOX4 | NADPH oxidase 4 |
| NO | Nitric oxide |
| NOS3 | Nitric oxide synthase 3 |
| Nfe2l2 | Nuclear factor erythroid 2-related factor 2 |
| PCR | Polymerase chain reaction |
| ROS | Reactive oxygen species |
| RAAS | Renin-angiotensin-aldosterone system |
| RPWAT | Retroperitoneal white adipose tissue |
| RNA | Ribonucleic acid |
| SIRT3 | sirtuin-3 |
| O ₂ ⁻ | Superoxide anion |
| SOD | Superoxide dismutases |
| SOD1 | Superoxide dismutase 1 |

SOD2

Superoxide dismutase 2

TNF- α

Tumor necrosis factor

VCAM-1

Vascular cell adhesion molecule 1

WAT

White adipose tissue

I. INTRODUCTION

Systemic oxidative stress and inflammation are linked to a number of chronic diseases, including cardiovascular disease, type 2 diabetes, and cancer. Angiotensin (Ang) II, a hormone most known for its hypertensive effects, has been shown to induce inflammation and oxidative stress in adipose tissue. Adipose tissue is the organ that stores energy when caloric excess occurs, and this energy can be used when the body is in periods of caloric restriction.¹ Adipose tissue plays an important role not only in housing energy, but in interacting with the body's inflammatory system, sympathetic nervous system, and renin-angiotensin-aldosterone system (RAAS).² The main role of the RAAS is the regulation of blood pressure and electrolyte balance.³ The main components of the RAAS are renin, Ang II, and aldosterone.⁴ The RAAS is stimulated by the activation of angiotensinogen.² Angiotensinogen is a hormone known classically to be produced by the liver, but it is also expressed in adipose tissue.² Angiotensinogen, which is typically inactive, can be cleaved by renin to form Ang I. Angiotensin I, also an inactive form of the hormone, is cleaved by angiotensin converting enzyme to form Ang II.² Angiotensin II is a vasoconstrictor, meaning it increases blood pressure and can lead to hypertension.^{2,5} Angiotensin II can also increase blood pressure by stimulating aldosterone release, which increases sodium reabsorption, and stimulates the release of arginine vasopressin, which increases water reabsorption.⁴ The action of Ang II on the brain also stimulates thirst and increased water intake leading to increased blood pressure.⁴ The two G-protein coupled receptors, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R), mediate the effects of Ang II in RAAS.^{6,7} One of the receptors for Ang II, AT1R, is expressed by adipocytes and its activation can lead to problems in cell differentiation, formation, and function.² The RAAS has a significant influence on inflammatory reactions and oxidant injury considering the ability of Ang II to activate NADPH

oxidases and pro-inflammatory cytokines leading to inflammation.⁸ This activation of NADPH oxidases has been shown to be present in white adipose tissue (WAT), leading to inflammation and oxidative stress in this tissue specifically.⁷ White adipose tissue (WAT) is the organ that stores energy as triglycerides for use when energy is in high demand.^{9,10} Chronic diseases, including diabetes mellitus, hypertension, dyslipidemia, obesity, and metabolic syndrome, have all been shown to be associated with chronic inflammation and oxidative stress in tissues.⁸ Therefore, local adipose tissue RAAS may play a bigger role, as it relates to the effects of Ang II on oxidative stress and inflammation, than previously considered. Treatment for lowering inflammation and oxidative stress is becoming seemingly essential to relieve this global burden of disease and dietary modifications are frequently being assessed to target elevated Ang II.¹¹

Increasing fruit intake, specifically berries, which contain high amounts of phytochemicals, such as polyphenols, has been shown to contribute to cardiovascular health.¹² Flavonoids found in fruits and vegetables have been linked to lower cardiovascular disease (CVD) related mortalities.¹³ The polyphenol content in berries contributes to their antioxidant properties and anti-inflammatory effects. Though some benefits can be found using life style change and food, while under stress it is hypothesized that the body can promote pro-inflammatory cytokine release and lead to a state of inflammation.¹⁴ It is not clear whether hypertension is a cause or effect of increased levels of these pro-inflammatory cytokines.^{14,15} The potential for berries to reduce Ang II-induced inflammation and oxidative stress in adipose tissue has implications for the chronic conditions associated with elevated Ang II, including hypertension and cardiovascular disease.

The primary study was conducted focusing on the ability of berry consumption to reduce blood pressure and reduce Ang-II induced cardiac damage. Knowing that Ang II may affect other organs, multiple tissues were collected for secondary analysis. The objective of our secondary

analysis is to determine if blackberries or raspberries reduce oxidative stress and inflammation in adipose tissue of Ang II-treated rats. Accordingly, the following specific aims will be pursued.

Specific Aim #1. Determine the effects of Ang II and berry consumption on inflammation in epididymal and retroperitoneal white adipose tissue.

Hypothesis #1. Angiotensin II will induce inflammation in epididymal and retroperitoneal white adipose tissue, and berry consumption will mitigate this effect.

Specific Aim #2. Determine the effects of Ang II and berry consumption on oxidative stress in epididymal and retroperitoneal white adipose tissue.

Hypothesis #2. Angiotensin II will induce oxidative stress in epididymal and retroperitoneal white adipose tissue, and berry consumption will mitigate this effect.

II. REVIEW OF THE LITERATURE

A. Adipose Tissue

Adipose tissue is the organ that stores energy for use when the body is in caloric deficit.¹ Acting as an insulator, adipose tissue also assists the body with temperature regulation.¹⁶ Adipocytes are the primary cell type that makes up adipose tissue, and as more energy storage accumulates in the body, adipocytes increase in size and number.^{1,17} Adipocyte hypertrophy is when the adipocytes increase in size, which is common in those with obesity and type 2 diabetes, and both are associated with an increased risk of hypertension and CVD.^{17,18}

During states of metabolic stress, such as with obesity, the adipose tissue can contribute to the inflammatory state of the body by producing and secreting inflammatory cytokines.¹ Adipocytes themselves are capable of producing both inflammatory and anti-inflammatory cytokines.^{1,10} Further, adipose tissue contains immune cells which, depending on the metabolic state of the body, can release inflammatory or anti-inflammatory cytokines.¹⁰ The inflammatory state of WAT has been linked to a number of chronic conditions, including insulin resistance, type 2 diabetes, hypertension, CVD, and cancer.¹⁹

Visceral adipose tissue is the fat tissue that surrounds inner organs, while subcutaneous adipose tissue is the fat tissue beneath the skin and distributed throughout the body.^{20,21} Two visceral WAT depots are of importance to our study. The epididymal adipose tissue located in the gonadal region and the retroperitoneal adipose tissue located behind the peritoneum are similar in rodent and human models.^{9,20} Human research has shown an increase in visceral adipose tissue to play a role in diseases related to obesity, insulin resistance, and hypertension.^{21,22} Unlike visceral adipose tissue, subcutaneous adipose tissue expansion is associated with improved insulin sensitivity and decreased risk of metabolic dysfunction.²⁰

1. Link Between Adipose Tissue Inflammation and Oxidative Stress and Hypertension

The hypertrophy of the adipose tissue causes stress and increased production of inflammatory cytokines.¹ More specifically, the endoplasmic reticulum (ER) and mitochondria of the adipocytes are affected when exposed to stress.¹ The hypertrophy of adipocytes causes dysfunction of the mitochondria and can promote reactive oxygen species (ROS) production.¹ ER stress is also attributed to ROS production.¹ NADPH oxidases are a type of enzymatic complex that acts as a host defense, specifically when infection or stress is present, and they play a role in cellular signaling and gene expression regulation.²³ NADPH oxidase can serve as an inflammatory mediator and promote the release of ROS.²³ ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO) are known markers of oxidative stress.^{1,24,25}

Other host defense systems include antioxidants that can be separated into three groups: superoxide dismutases, peroxidases, and thiol-redox proteins.²⁶ Superoxide dismutases (SOD) including SOD1 and SOD2 play an antioxidant role by causing dismutation of O_2^- therefore preparing them for metabolism.²⁶ Peroxidases such as glutathione peroxidase (GPx1) and catalase (CAT) function to detoxify ROS, specifically peroxides.²⁶ Thiol-redox proteins are a group of antioxidant genes that play a huge role in metabolizing ROS using a thiol-redox mechanism including glutathione reductase (GSR).²⁶ Other genes such as nuclear factor erythroid 2-related factor 2 (Nfe2l2), heme oxygenase 1 (Hmox1), and nitric oxide synthase 3 (NOS3) assist in regulation of antioxidant defense and preventing inflammation.

Translation of mRNA occurs in the ER followed by protein folding, but under inflammation and stressful conditions caused by hypertrophy, dysfunction of the ER results in improper protein folding which can alter normal cellular and regulatory functions.^{1,17} Stress of the ER in the adipocyte can also cause C/EBP homologous protein (CHOP) and lactate concentrations

to increase.¹⁷ Multiple sources show that in mouse models, accumulation of CHOP under stress causes adiponectin levels to decrease.^{17,27} Overall, the damaging effects of oxidative stress and ROS accumulation has been linked to the development of disease including hypertension.²⁵ Identifying these processes and markers of stress is important as we will examine how berries may impact these mechanisms.

Highlighting the importance of adiponectin's protective properties can help us link the reduction in adiponectin to complications such as hypertension. Adiponectin is a crucial protein when it comes to reducing adipose tissue inflammation because of its anti-inflammatory properties and ability to enhance production of nitric oxide (NO).¹ Although NO is an oxidant, it can be beneficial for endothelial function. Research shows that NO plays a critical role in proper endothelial function and anti-inflammatory responses.^{1,28} Endothelial cell adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, and impaired NO production are markers of inflammation and oxidative stress.¹ When adiponectin levels are deficient, the reduction of NO occurs and these endothelial cell adhesion molecules are present.¹ Tumor necrosis factor (TNF)- α , a pro-inflammatory molecule, is produced by adipocytes and immune cells in adipose tissue.¹ TNF- α is a pro-inflammatory cytokine, and similar to the other processes occurring from adipose hypertrophy, TNF- α causes endothelial dysfunction and downregulation of NO.^{1,28} However, adiponectin is shown to reverse some of those inflammatory effects.¹ This leads us to believe adiponectin plays an important role in maintaining proper endothelial function. TNF- α , along with other pro-inflammatory cytokines including interleukin (IL)-1 β produced by macrophages and, to a lesser extent, adipocytes, is known to stimulate endothelial cell adhesion molecule expression and ROS production leading to the negative inflammatory effects on the endothelial cell.^{18,29} IL-1 β in adipose tissue has recently

been associated with insulin resistance due to its function in repression of insulin signal transduction.²⁹ Insulin signaling is also found to be reduced by IL-6 and TNF- α .²⁹

These inflammatory cytokines can contribute to the systemic inflammation found in people with hypertension. Multiple studies have shown that increased cytokines, including IL-6, IL-1 β , and TNF- α , are associated with a significant increase in arterial pressure and hypertension.^{30,31} Although it is sometimes unclear whether the increase in inflammatory cytokines cause hypertension, or whether the hypertensive state leads to the increase in inflammatory cytokines. Recognizing the pro-inflammatory cytokines expressed in adipose tissue can help us confirm the correlation between the hypertensive state and inflammation. Working to understand the significance of the anti-inflammatory properties of blackberries and raspberries will assist in counteracting the dysfunction in endothelial cells caused by increased expression of inflammatory cytokines in adipose tissue.

2. Angiotensin II and Inflammation

The RAAS is the basis for regulation of systemic arterial pressure, using Ang II to act on the sympathetic nervous system and smooth muscle throughout the body.³² Angiotensin II is a hormone shown to cause increased TNF- α and O₂⁻ production and vasoconstriction, all of which increase blood pressure, causing hypertension and endothelial dysfunction.^{1,2} Pro-inflammatory ROS are also stimulated by the release of Ang II.³³ Angiotensin II stimulates inflammation by enhancing cytokine production, increasing oxidative stress and expression of monocyte chemoattractant protein (MCP)-1.^{16,34} MCP-1 is a chemokine that stimulates inflammatory responses and is well known to be linked to CVD causing adhesion, tissue factor, and inflammatory molecule production.³⁴ Recent studies have also shown that mice injected with Ang II (1 μ g/mouse) have decreased high-density lipoprotein (HDL) levels, which may contribute to the dyslipidemia

commonly seen in patients with other comorbidities such as obesity, hypertension, and cardiovascular disease.^{16,35} A study focused on the perivascular adipose tissue in Ang II-induced hypertensive mice examined the role of increased inflammation and SIRT3 (sirtuin-3) deficiency.³⁶ The study found that Ang II-induced inflammation in the adipose tissue was accelerated by the SIRT3 deficiency and additional inflammasomes and cytokines, including IL-1 β , were activated further promoting inflammation.³⁶ This is important as it may show a relationship between SIRT3 and its ability to regulate inflammatory cytokines that cause oxidative stress and inflammation. Similarly, while assessing chronic inflammation, Menikdiwela et al. found IL-6 to be a major inflammatory cytokine expressed in the adipose tissue of mice overexpressing Ang II.³⁷ Overall, research shows that Ang II has a role in influencing inflammation in adipose tissue which, in turn, may lead to hypertension and eventually cardiovascular disease.

3. Angiotensin II and Oxidative Stress

Known markers of oxidative stress include the ROS previously discussed including O₂⁻, H₂O₂, and \cdot OH. When these ROS and antioxidant defenses, such as superoxide dismutases, peroxidases, and thiol-redox proteins, are out of balance this leads to development of oxidative stress.^{26,38} Angiotensin II is one factor that generates ROS causing oxidative stress and further dysfunction throughout the body.³⁹ Camargo et al. studied Ang II-induced hypertensive rats and showed that increased ROS caused oxidative damage.⁴⁰ Oxidative damage can lead to endothelial dysfunction, renal injury, and cardiovascular remodeling all confirming the effects of Ang II-induced hypertension.^{39,41} The study showed that Ang II increased ROS through Nox expression, specifically increased Nox4 in the ER showing the correlation between hypertension and vascular ER stress.⁴⁰ NADPH oxidases, including Nox1 and Nox4, are oxidases both involved in ROS

production and the prooxidant pathway.²³ Griendling and colleagues found that Ang II activates NADPH and NADH oxidases leading to superoxide formation in vascular smooth muscle cells.⁴² This process is not only seen in vascular smooth muscle cells, but also in many other tissues including white adipose. Looking at both human and animal models, Ang II which is the major precursor to the RAAS, has been shown to be expressed in the WAT.^{7,43} Ang II has the ability to activate NADPH oxidases, inducing ROS production, which can activate the NF- κ B pathway and lead to inflammation and oxidative stress in WAT.⁷ These studies help show the influence of Ang II on oxidative stress and highlights the potential role in the pathophysiology of hypertension. Some dietary interventions are being explored as a complementary approach to be used as an adjunct to standard pharmacologic treatment for Ang II-induced hypertension and its effect on inflammation and oxidative stress.

B. Blackberry and Raspberry Intervention Studies

Berries have been showcased for their beneficial properties contributing to better overall cardiovascular health.¹² Berries are known to have a high polyphenol content. Polyphenols, commonly found in plants, are antioxidants that protect the body from oxidative stress and inflammation caused by ROS.⁴⁴ Separated into two categories, polyphenols can be classified by flavonoids or nonflavonoids.⁴⁵ From there they differ in classification depending on their function and chemical structure and can be broken into many subclasses based on phenol units.^{44,45} Flavonoids play a role in antioxidant activity and their structural backbone consists of the C6-C3-C6 configuration with linkage of ring A and ring B by a heterocyclic ring.^{44,45} Flavonoids include flavonols, flavan-3-ols, flavanones, flavones, isoflavones, and anthocyanins.^{44,45} Nonflavonoids include phenolic acids, stilbenes, and lignans that present with a chemical structure of an aromatic ring that can contain several hydroxyl groups.⁴⁵ Polyphenols that contain an amide group are

known as polyphenol amides and demonstrate antioxidant and anti-inflammatory functions.⁴⁵ The high content of polyphenols and micronutrients in berries may be responsible for mediating the mechanisms for improved CVD outcomes including upregulation of NO synthase, decreased oxidative stress, and the inhibition of inflammatory gene expression.¹²

In multiple studies, fruit and vegetable intake, specifically high in flavonoids, have been shown to decrease the risk of cardiovascular disease.^{13,46,47} The Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study found that over a span of 12 years, the group of men consuming a higher intake of berries showed lower risk of cardiovascular, noncardiovascular, and all-cause mortality than the group consuming fewer berries.⁴⁶ The intake of fruits, berries, and vegetables was controlled for in the study. Results of the study showed that the men that died of CVD within 5 years consumed 41% less fruits, berries, and vegetables when compared to the men that survived.⁴⁶ Another study, focusing on higher flavonoid intake, was associated with a reduced risk of death from coronary heart disease and CVD.⁴⁷ The cohort assessed during this study presented with hypertension, among other comorbidities, as a risk factor for coronary heart disease mortality at baseline.⁴⁷ Results from this study concluded that intake of total flavonoids, anthocyanidins, flavanones, flavones, and proanthocyanidins had an inverse association with total CVD mortality.⁴⁷

One study examining the effects of berry intake on oxidative stress found that blueberry consumption contributed to a significant decrease in lipid hydroperoxides, a measure of oxidative stress.⁴⁸ These favorable effects, shown to contribute to improved markers of oxidative stress, may help as the exploration continues to uncover an effective solution in managing oxidative damage in the body. Jensen et. al discovered use of a supplemental juice blend of fruit and berries increased serum antioxidants and decreased serum lipid peroxidation in healthy people.⁴⁹ Lipid peroxidation,

seen in development of disease, is a process in the body where ROS destroy fatty acids leading to tissue damage.⁵⁰ A similar study focused specifically on blackberry, raspberry, and black raspberry polyphenols found a reduction in Ang II-induced ROS levels and cellular deterioration.²⁴ Therefore, determining whether berries protect against Ang II-induced oxidative stress and inflammation in adipose tissue may provide some insight into their potential as a therapeutic for hypertension.

In relation to our study, we will focus on the beneficial properties found in a whole food approach using whole blackberries and raspberries. We chose to focus on blackberries and raspberries particularly because of their antioxidant capacity and high polyphenol content. Many current research studies are focused on other berries, including blueberries and strawberries; therefore, our research will fill a gap in the literature when it comes to other types of berries. Blackberries and raspberries both belong to the Rosaceae family and are known for high levels of bioactive compounds.⁵¹ Bioactive compounds in berries include phenolic acids, flavonoids, and ascorbic acid that all contribute favorable antioxidant properties.⁵¹ Flavonoids, including anthocyanins, and phenolic acids are what contribute to the health benefits and antioxidant properties found in berries.⁵¹ Ascorbic acid contributes to the neutralization of free radicals.⁵¹ Though raspberries and blackberries are similar in ascorbic acid content, raspberries contain about half of the phenolic content of blackberries.⁵¹ Raspberries are also frequently used in diets to manage the beginning stages of hypertension.⁵² Blackberries contain metabolites that could protect cells against oxidative damage plus, the high content of polyphenols provides anti-inflammatory properties.⁵¹ Considering this, the increased capacity of antioxidants in blackberries and raspberries may play a role in the reduction of Ang II-induced hypertension by neutralizing free radicals and preventing oxidative damage.

Table 1. Polyphenol content of Blackberry and Red Raspberry, raw ⁵³ *

| Polyphenols | Blackberry (mg/100g FW) | Red Raspberry (mg/100g FW) |
|---------------------------|------------------------------------|---------------------------------------|
| Flavonoids | | |
| Anthocyanins | | |
| Cyanidin 3-O-glucoside | 138.72 | 37.61 |
| Flavanols | | |
| (-)-Epicatechin | 11.48 | 5.05 |
| Flavonols | | |
| Quercetin 3-O-galactoside | 4.10 | 11.0 |
| Phenolic acids | | |
| Hydroxybenzoic acid | | |
| Ellagic acid | 43.67 | 76.10 |
| Hydroxycinnamic acid | | |
| 3-Caffeoylquinic acid | 4.53 | 0.57 |

* List of major polyphenols present, not comprehensive list

C. Conclusion

The burden of hypertension has been steadily increasing over the last decade in the U.S. Adipose tissue inflammation and oxidative stress have been linked to hypertension and CVD. Berries have been shown to ameliorate inflammation and oxidative stress in various *in vitro* and *in vivo* models. This study will determine whether blackberries and raspberries can alter effects of Ang II on oxidative stress and inflammation in visceral white adipose tissue and will test the following hypotheses: (1) Ang II will induce inflammation in epididymal and retroperitoneal white adipose tissue, and berry consumption will mitigate this effect; and (2) Ang II will induce oxidative stress in epididymal and retroperitoneal white adipose tissue, and berry consumption will mitigate this effect.

III. MATERIALS AND METHODS

A. Animals

All experiments were reviewed and approved by the Georgia State University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (age 8 weeks) were obtained from Envigo (Indianapolis, IN). Rats were individually housed on a 12-hour light cycle. Animals had *ad libitum* access to food and water. The rats were maintained on a semi-purified casein-based diet (AIN-93M) in which soybean oil was replaced with corn oil since soybean oil contains phytoestrogens that can potentially exert additional protective effects. After one-week acclimation, rats were assigned to one of five treatment groups (n=9-10/group): 1) Control, 2) Ang II, 3) Ang II + 10% blackberry diet, 4) Ang II + 10% raspberry diet or 5) Ang II + 5% blackberry and 5% raspberry combination diet. The rats were fed these diets *ad libitum* for four weeks, at which point they underwent surgery for implantation of osmotic minipumps. The minipumps delivered vehicle (0.9% saline) to the control group while the other four groups received Ang II (270 ng/kg body weight/min) for an additional three weeks. Upon completion of the seven-week intervention, rats were sacrificed by CO₂ inhalation. Epididymal WAT (EWAT) and retroperitoneal WAT (RPWAT) were collected, and flash frozen in liquid nitrogen, and were subsequently stored at -80°C for later analyses.

B. RNA Isolation, Reverse Transcription, and Real-time PCR

Total RNA was isolated from EWAT and RPWAT using an RNeasy Mini Lipid Tissue Kit (QIAGEN Inc.). RNA quantity and quality were measured with the Nanodrop spectrophotometer (Thermo Scientific). One microgram of total RNA was reverse transcribed to produce cDNA (Promega Corporation). Gene expression was measured by real-time PCR (LightCycler 480; Roche Life Science) by measurement of SYBR Green (Bio-Rad Laboratories). mRNA expression

of genes of interest were normalized to cyclophilin expression. The following six genes were measured: *Il1b*, *Ccl2*, *Nqo1*, *Gpx1*, *Nfe2l2*, and *Hmox1*. The genes that were measured, along with their primer sequences, are listed in Table 2.

Table 2. List of genes measured in EWAT and RPWAT, along with their primer sequences, and pathway involvement.

| Gene Symbol | Gene/Protein name | Forward Primer (5' --> 3') | Reverse Primer (5' --> 3') | Pathway |
|---------------|--|----------------------------|----------------------------|-------------------------|
| <i>Il1b</i> | Interleukin 1 beta | GACTTCACCATGGAACCCGT | GGAGACTGCCCATCTCTCGAC | Pro-inflammatory marker |
| <i>Ccl2</i> | MCP-1 (monocyte chemoattractant protein 1) | CCAGAAACCAGCCAACCTCTCA | CCAGAAGCGTGACAGAGACC | Pro-inflammatory marker |
| <i>Nqo1</i> | NAD(P)H dehydrogenase [quinone] 1 | ATTGTATTGGCCACGCAGA | TCATATCCCAGGCCACCTGA | Antioxidant pathway |
| <i>Gpx1</i> | Glutathione Peroxidase 1 | CAGTCCACCGTGTATGCCTT | GTAAAGAGCGGGTGAGCCTT | Antioxidant pathway |
| <i>Nfe2l2</i> | Nuclear factor erythroid 2-related factor 2 (Nrf2) | TTGTAGATGACCATGAGTCGC | ACTTCCAGGGGCACTGTCTA | Antioxidant pathway |
| <i>Hmox1</i> | Heme Oxygenase 1 | TTAAGCTGGTGATGGCCTCC | GTGGGGCATAGACTGGGTTC | Antioxidant pathway |

C. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 8.0.2. Data were analyzed using a one-way ANOVA followed by Tukey's multiple comparison's test and is represented as the mean \pm SEM. Shapiro-Wilk test was used to verify normality. For data that are not normally distributed, the Kruskal-Wallis test was used and is represented using a 95% confidence interval. Significance was set *a priori* at $p < 0.05$.

IV. RESULTS

To examine the effects Ang II had on the blackberry, raspberry, and combination diets, we measured expression of genes in the inflammatory and antioxidant response pathways through real-time PCR.

A. Oxidative Stress

1. EWAT

The genes involved in the antioxidant pathway that were measured in EWAT included *Gpx1*, *Hmox1*, *Nfe2l2*, and *Nqo1*. While Ang II had no effect on the expression of the antioxidant transcription factor *Nfe2l2*, raspberry consumption tended to increase its expression (Fig. 1A) ($P = 0.19$). Compared to the control group, *Nfe2l2* expression was slightly, but not significantly, higher in the raspberry group resulting in a fold change of 2.37. ($P = 0.19$; Fig. 1A) (CON = 1.0 ± 0.18 vs RB = 2.37 ± 0.65). The expression of *Nqo1*, *Hmox1*, and *Gpx1* (genes involved in the antioxidant pathway) in EWAT were not affected by Ang II administration or berry consumption (Fig. 1).

2. RPWAT

The markers of oxidative stress involved in the antioxidant pathway observed in RPWAT included *Gpx1*, *Hmox1*, *Nfe2l2*, and *Nqo1*. In RPWAT, Ang II had no effect on expression of the antioxidant pathway gene *Hmox1* when compared to the control with a fold change of 1.22. (Fig. 2A). The expression of *Hmox1* also was unaffected by berry consumption (Fig. 2A). Similarly, neither Ang II nor berry consumption had an effect on expression of the antioxidant transcription factor, *Nfe2l2* (Fig. 2B). Antioxidant pathway genes, *Nqo1* and *Gpx1*, displayed no trend in

expression with data being not normally distributed. There were no significant differences in *Nqo1* (Fig. 2C) or *Gpx1* expression (Fig. 2D) between the groups.

B. Inflammation

1. EWAT

The pro-inflammatory markers measured in the EWAT included *Il1b* and *Ccl2*. Ang II did not affect *Ccl2* expression when compared to the control (fold change of 1.07 compared to control; Fig. 3A). Likewise, berry consumption did not affect *Ccl2* expression in EWAT (Fig. 3A). Similarly, Ang II did not significantly affect *Il1b* expression when compared to the control (fold change of 1.06 compared to control; Fig. 3B). *Il1b* expression was highest in the blackberry group when compared to the control ($P = 0.73$), though there were no significant differences between groups (Fig. 3B).

2. RPWAT

The pro-inflammatory markers observed in RPWAT included *Il1b* and *Ccl2*. Ang II did not affect the expression of *Ccl2* when compared to the control (fold change of 1.25 compared to control; Fig. 4A). The combination diet group showed the lowest expression of *Ccl2*, but there were no significant differences in *Ccl2* expression between any groups (Fig. 4A). Neither Ang II nor berry consumption altered *Il1b* expression (Fig. 4B). Like in the EWAT, *Il1b* expression was higher in the blackberry group compared to the raspberry group (fold change of 1.32 in blackberry compared to raspberry group), but there were no significant differences in *Il1b* expression between groups (Fig. 4B).

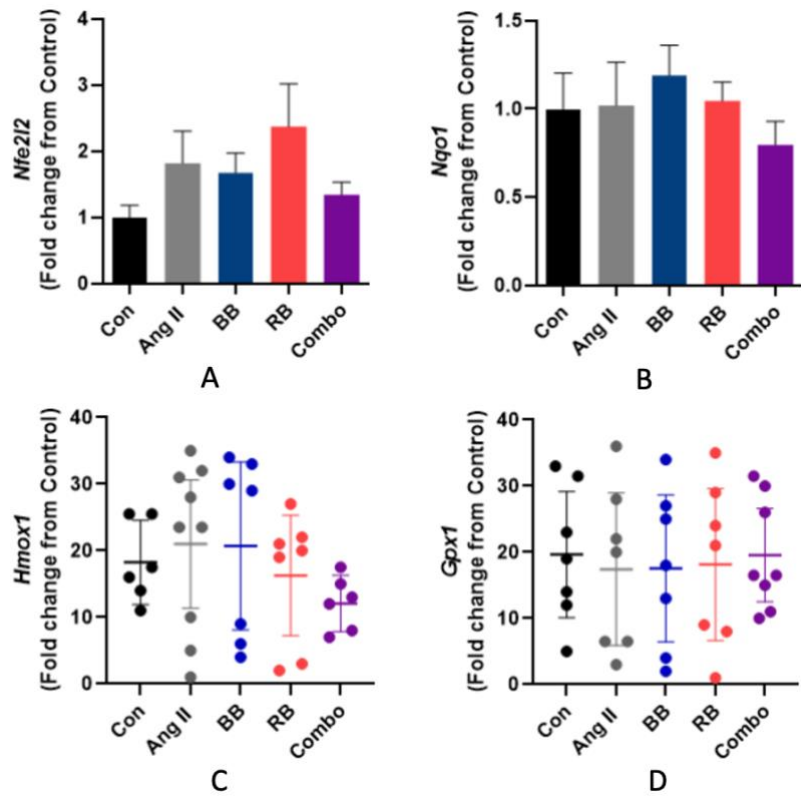


Figure 1: Expression of Oxidative Stress Markers in EWAT. mRNA expression of antioxidant pathway genes, *Hmox1* (A), *Nfe2l2* (B), *Nqo1* (C), and *Gpx1* (D), in EWAT of Ang II-treated rats (n = 9 - 10/group) on a variation of diets: 10% freeze-dried blackberry powder (BB), 10% freeze-dried raspberry powder (RB), or combination diet of 5% freeze-dried blackberry powder and 5% freeze-dried raspberry powder (Combo). Values are (A-B) means \pm SEM or (C-D) means \pm 95% CI.

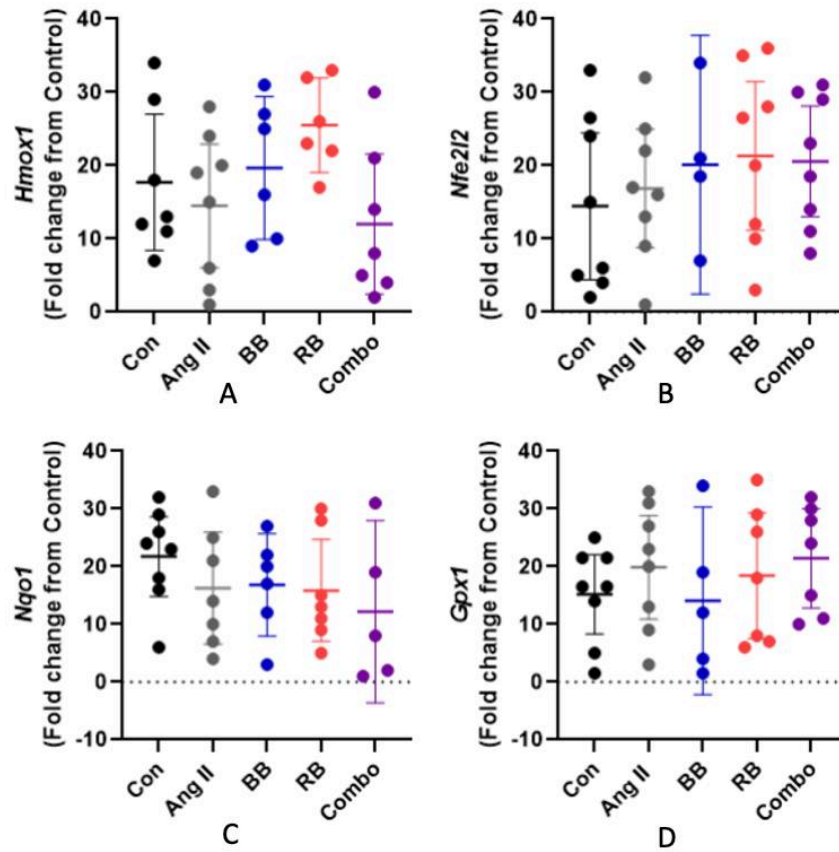


Figure 2: Expression of Oxidative Stress Markers in RPWAT. mRNA expression of antioxidant pathway genes, *Hmox1* (A), *Nfe2l2* (B), *Nqo1* (C), and *Gpx1* (D), in RPWAT of Ang II-treated rats (n = 9 - 10/group) on a variation of diets: 10% freeze-dried blackberry powder (BB), 10% freeze-dried raspberry powder (RB), or combination diet of 5% freeze-dried blackberry powder and 5% freeze-dried raspberry powder (Combo). Values are means \pm 95% CI.

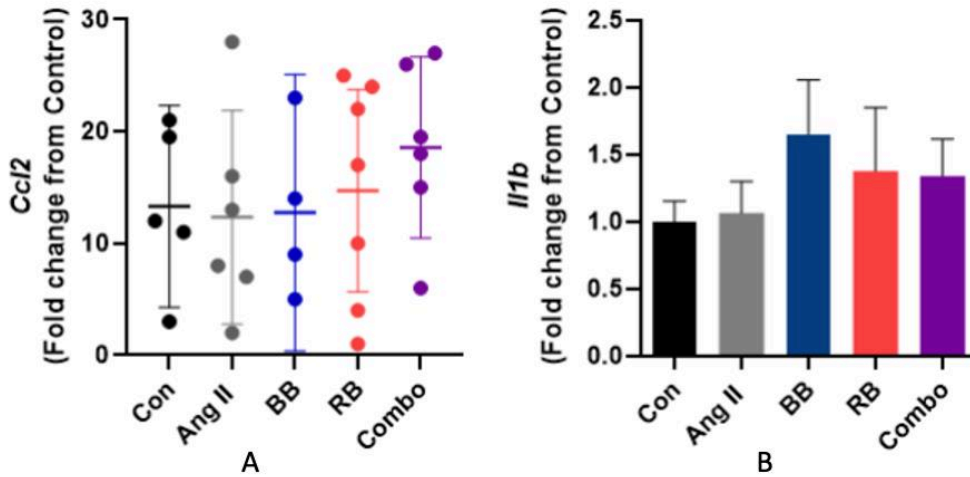


Figure 3: Expression of Inflammation Markers in EWAT. mRNA expression of inflammatory markers, *Ccl2* (A) and *Il1b* (B), in EWAT of Ang II-treated rats (n = 9 - 10/group) on a variation of diets: 10% freeze-dried blackberry powder (BB), 10% freeze-dried raspberry powder (RB), or combination diet of 5% freeze-dried blackberry powder and 5% freeze-dried raspberry powder (Combo). Values are (A) means \pm SEM (B) or means \pm 95% CI.

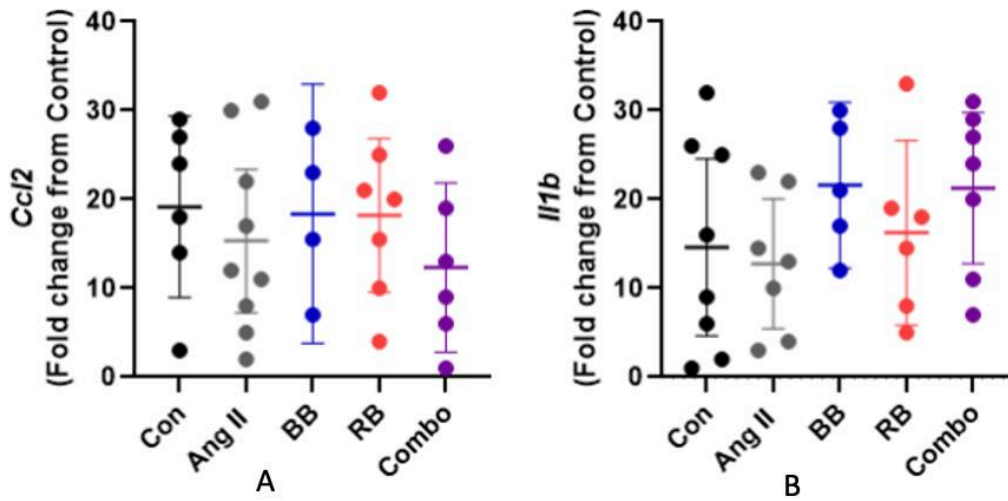


Figure 4: Expression of Inflammation Markers in RPWAT. mRNA expression of the inflammatory markers, *Ccl2* (A) and *Il1b* (B), in RPWAT of Ang II-treated rats (n = 9 - 10/group) on a variation of diets: 10% freeze-dried blackberry powder (BB), 10% freeze-dried raspberry powder (RB), or combination diet of 5% freeze-dried blackberry powder and 5% freeze-dried raspberry powder (Combo). Values are means \pm 95% CI.

V. DISCUSSION

The results indicate that Ang II does not consistently increase markers of inflammation, nor does it affect genes associated with the antioxidant response in adipose tissue. Further, berry consumption had no significant effects on markers of inflammation and antioxidant genes in visceral adipose tissue. These findings do not support our hypotheses. The lack of a consistent impact of Ang II on inflammatory markers could be due to the low dose and relative short duration of Ang II used in this study. Similarly, the amount of berries consumed could play an important role in their anti-inflammatory and antioxidant properties. Further, given that there was no inflammatory state induced by Ang II in the adipose tissue, it would be difficult for the berries to reduce inflammatory markers.

The Ang II concentration used in our study was 270 ng/kg body weight/min. A study examining increased arterial pressure induced by Ang II only saw increases in the pro-inflammatory marker, IL-6, at dosages of 400-1000 ng/kg/min in mice after 28 days.⁵⁴ The concentration of Ang II used in our study, 270 ng/kg BW/min, may have been too low to induce inflammation and oxidative stress in the adipose tissue of rats. Similarly, our results do not align with the claims Menikdiwela et al. made relating to the impact of Ang II on the expression of pro-inflammatory genes in adipocytes.³⁷ In EWAT and RPWAT, our study found oxidative stress and inflammatory markers were not significantly affected by Ang II when compared to the control. Menikdiwela et al. found that Ang II increased inflammation and ER stress in adipocytes.³⁷ The Ang II concentration used for treatment varied from 10, 50, and 100 nM and the study concluded concentrations above 10 nM did not induce ER stress.³⁷ The adipocytes were exposed to Ang II for 24 to 48 hours.³⁷ The differences in the effects Ang II had on inflammation could be due to the study being done *in vitro* and focusing on adipocytes rather than adipose tissue. The cells that

make up adipose tissue include not only adipocytes, but also fibroblasts, endothelial cells, macrophages, stromal cells, immune cells, and pre-adipocytes.⁵⁵ The diversity of cell types in adipose tissue could be a major reason why increased inflammation was seen in the adipocytes alone when compared to adipose tissue. In this *in vitro* experiment, the adipocytes are directly exposed to the Ang II. Without the complexity of the organ systems present in an *in vivo* model, it is impossible to directly compare the two. Therefore, the *in vitro* model produces poor generalizability and transferability in comparison to our *in vivo* model.

Many of the antioxidant markers in EWAT were increased in the berry groups when compared to the control and Ang II groups. Though no significance was found, a slight trend upward in antioxidant markers within the berry groups could be related to the anti-inflammatory effects of berries. Specially in the EWAT, the expression of *Nfe2l2*, *Nqo1*, and *Hmox1* saw slight trends upward in berry groups, though not significant. When the primary study observed the cardiac tissue in these rats, focusing on the ability of raspberries to ameliorate cardiac oxidative stress in Ang II-infused rats, NRF2 protein expression was found to be increased in the heart tissue of rats that were consuming a raspberry diet.⁵⁶ The primary study also saw an increased NQO1 protein expression in rats consuming a raspberry diet.⁵⁶ The increasing trend of NRF2 and NQO1 protein expression in the raspberry groups may be explained by the major role of NRF2 as an antioxidant transcription factor.⁵⁷ Our secondary analysis, did not find significance in the expression of these antioxidant markers, this could be due to our studies observing different tissues. Antioxidant markers can vary in each tissue, the presence of oxidative stress and antioxidant capacity of each tissue is tissue-specific.⁵⁸ A study done by Starr, found adipose tissue to be a major source of IL-6.⁵⁹ Though, our study did not find *Il6* to be expressed highly in the epididymal adipose tissue of the rats. Our study used Ang II to induce inflammation, while the

study done by Starr used bacterial endotoxin lipopolysaccharide to induce inflammation.⁵⁹ The difference in treatment to induce systemic inflammation may be why the tissue-specific markers differed.

In RPWAT, the expression of *Hmox1* was not significant in the blackberry and raspberry groups when compared to the Ang II group. Heme oxygenase-1 is known for its association with anti-inflammatory products, biliverdin and carbon dioxide, and its role in protection against oxidative stress.⁶⁰ Though, the mechanism by which *Hmox1* mediates the anti-inflammatory effects is still unknown.⁶⁰ Resveratrol, a polyphenol, found in berries, displays anti-inflammatory properties related to its ability to induce *Hmox1* expression by *Nfe2l2* activation.^{61,62} This could suggest why *Nfe2l2* were increased in some of the berry groups, though *Hmox1* expression was not significant. The variable response to the Ang II treatment and diet across the animals, does not allow for a direct association between the berry groups and anti-inflammatory properties related to the expression of *Hmox1* and *Nfe2l2*. To determine if a clear association exists, the antioxidant pathways should continue to be examined, with a focus on the expression of *Hmox1*, *Nfe2l2*, and *Nqo1*.

Similar to our study, multiple studies have found no effect on inflammatory markers using berry supplementation. One study looked at inflammatory markers in adipose tissue of Wistar rats, supplementing a high fat, high sucrose diet with 6% w/w of a freeze-dried strawberry-blueberry powder (5:1).⁶³ The study found no differences in serum levels of TNF- α and IL-6 between the control and high fat sucrose group, though MCP-1 concentrations were reduced in the group supplemented with the strawberry-blueberry powder.⁶³ Similarly, our study found no TNF- α or IL-6 to be detected in the adipose tissue and no difference in inflammatory markers in our control versus the berry groups.

Our study used a higher concentration of freeze-dried berry powder and evaluated different types of berries. To test the ability strawberries have on attenuating inflammation, Parelman et al. focused on a mouse model feeding mice a low-fat or high-fat diet supplemented with 2.6% freeze dried strawberry powder.⁶⁴ The strawberry supplemented diet had no effect on expression of TNF- α , IL-6, IL-10, and IL-1B proteins in splenocytes.⁶⁴ This supports our results in that the berry supplementation did not attenuate the effects of inflammation, though different tissues were analyzed in these studies and a lower percentage of freeze-dried berries were used.

In contrast, another study observed inflammatory cytokines in high-fat-diet-fed Wistar rats supplemented with 10% blueberry powder for 8 weeks.⁶⁵ This study found a reduction in IL-1 β and TNF- α in the adipose tissue.⁶⁵ These outcomes could be due to the difference in polyphenol content of blueberries, blackberries, and raspberries. Our study ensured that the phenolic compounds in the diet were only coming from the freeze-dried berries; therefore, the soybean oil was replaced with corn oil. The study that found a reduction in IL-1 β and TNF- α in adipose tissue did not specify the type of fat used in the diets given to the rats.⁶⁵ If this study was using soybean oil, which contains phenolic compounds, this may have caused their results to be significant. By eliminating the possibility of the phenolic compounds present in soybean oil affecting our results, this strengthens the results of our study. Similarly, our study used the same percentage of freeze-dried berries. Although our study only exposed the rats to the berry diets for 7 weeks and a different berry was used, this could impact the concentration of phenolic compounds in the diet and therefore affect significance of the data.

A. Limitations

The primary goal of the study was to observe the effects that Ang II and berry consumption had on reducing blood pressure and reducing Ang II-induced cardiac damage. Our secondary

analysis was only focused on observing inflammation and oxidative stress in the adipose tissue. The study was not originally designed to observe the effects of berries and Ang II on adipose tissue; therefore, this is a major limitation in this secondary analyses study. Another limitation is that Ang II did not induce inflammation in the adipose tissue. This could have been due to the concentration of Ang II used in our study was (270 ng/kg body weight/min) and the duration of the intervention (three weeks). While higher concentrations of Ang II may have elicited greater inflammatory response, the dose may not be physiologically relevant. Another consideration is the duration of berry consumption. The rats consumed their assigned berry diet for seven weeks, four weeks before Ang II administration, and three weeks after. This may have been too short of exposure to the berry diet to see changes in inflammation and oxidative stress markers. The concentration of freeze-dried blackberry and raspberry powder may have not been ideal to see beneficial effects. The rats were fed either a 10% freeze-dried raspberry powder, 10% freeze-dried blackberry powder, or the combination diet which included 5% raspberry and 5% blackberry. Other concentrations of berries, both higher and lower than 10%, may have yielded more significant results in our study. Lastly, using an animal model may create poor generalizability when it comes to the application to humans. The differences in diet, genetic make-up, and unpredictable human response limit the translatability of our study to humans. However, conducting the study in a preclinical model allowed us to examine effects of Ang II and berry consumption on a number of tissues, which would have proved difficult or impossible in human subjects.

VI. FUTURE DIRECTIONS

Recommendations for future research includes analyzing the effects of berries given at a lower or higher concentration than previously examined. Future investigation into the effects of Ang II and berries on inflammation and oxidative stress in different tissues (heart, kidney, liver, and brain) and their mechanisms of action is essential.

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