The Effects of Raspberry Consumption on Angiotensin II-induced Oxidative Stress and Fibrosis in the Kidney of Sprague-Dawley Rats, A Secondary Analysis

Lena Lear

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THE EFFECTS OF RASPBERRY CONSUMPTION ON ANGIOTENSIN II-INDUCED OXIDATIVE STRESS AND FIBROSIS IN THE KIDNEY OF SPRAGUE-DAWLEY RATS, A SECONDARY ANALYSIS

by

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Under the Direction of Dr. Rafaela G. Feresin

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

Master of Science

The Byrdine F. Lewis College of Nursing and Health Professions

Department of Nutrition

Georgia State University

2022
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ABBREVIATIONS

3-NT 3-Nitrotyrosine
ACE Angiotensin Converting Enzyme
Ang Angiotensin
ANOVA Analysis of Variance
ARBs Angiotensin Receptor Blockers
AT$_1$R Angiotensin II Receptor Type 1
AT$_2$R Angiotensin II Receptor Type 2
BDE Boerhavia diffusa Extract
BSA Bovine Serum Albumin
BUN Blood Urea Nitrogen
Cat Catalase
CI Confidence Interval
CKD Chronic Kidney Disease
DAMPs Damage Associated Molecular Patterns
DCT Distal Convoluted Tubule
eGFR Estimated Glomerular Filtration Rate
GPx-1 Glutathione Peroxidase 1
GSH Glutathione
GSSG Glutathione Disulfide
HO-1 Heme Oxygenase 1
IL Interleukin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mercapturate Pathway</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated Protein Kinase</td>
</tr>
<tr>
<td>Mas</td>
<td>Mitochondrial Assembly Protein</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<tr>
<td>NF-κB</td>
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<td>NOX</td>
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<td>NRF2</td>
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<tr>
<td>PAMPS</td>
<td>Pathogen Associated Molecular Patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCT</td>
<td>Proximal Convoluted Tubule</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-Angiotensin Aldosterone System</td>
</tr>
<tr>
<td>RB</td>
<td>Raspberry</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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ABSTRACT

Kidney disease currently affects one in seven American adults, with over 130,000 newly diagnosed adults in 2019 alone. Although there are several risk factors attributed to the development of chronic kidney disease, high blood pressure is considered the second most common risk factor behind diabetes, causing 29% of cases. The elevated levels of angiotensin II associated with high blood pressure can lead to increased levels of oxidative stress, causing damage to the kidneys. The progression of kidney disease can be slowed by following doctors’ orders of prescribed medications to manage the comorbidities of CKD and consuming foods that assist in prolonging renal function through lessening the filtration work required of the kidney.

Many foods associated with prolonged kidney function are plant based with high polyphenol content, and studies show an association between polyphenol intake and reduced risk of CKD. Polyphenols have bioactive properties that target oxidative stress, an underlying driver of CKD development and progression. Raspberries, a concentrated source of polyphenols including ellagitannins and anthocyanins, are often on approved lists for individuals with CKD.

The objective of this study was to examine whether dietary supplementation with raspberries attenuates angiotensin (Ang) II-induced oxidative stress in the kidneys of rats. Eight-week-old male Sprague-Dawley rats were fed an AIN-93M diet (control and Ang II groups) or AIN-93M diet supplemented with 10% w/w freeze-dried raspberry (RB + Ang II) for seven weeks. At week 4, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control) or Ang II (270 ng/kg body weight/min) for an additional three weeks. Protein expression of antioxidant enzymes in the kidneys was assessed by western blot. Expression of 3-nitrotyrosine was assessed through immunohistochemistry in the kidney. Fibrosis was assessed in kidney tissue through Masson Trichrome Staining. Raspberry supplementation increased the expression of Mas
I receptor, a receptor heavily involved in anti-inflammatory pathways. Raspberry supplementation also increased the expression of transcription factor NRF2, as well as protein expression of antioxidant enzymes HO-1 and NQO1. Expression of oxidative biomarker 3-nitrotyrosine was increased in the Ang II group and mitigated with the raspberry supplementation, while fibrosis showed no change with raspberry supplementation. Our findings indicate that supplementation with raspberry has the potential to significantly increase the expression of antioxidant enzymes in an animal model of Ang II-induced oxidative stress.
CHAPTER 1

INTRODUCTION

There has been a consistent increase in the cases of chronic kidney disease (CKD) in United States since the 1980’s. According to the National Kidney Foundation, 37 million American adults are affected by CKD\textsuperscript{10} with almost a 15\% prevalence of CKD in the United States.\textsuperscript{11} With the current obesity rates continuing to rise and the population continuing to age, it is expected that the prevalence of CKD in the United States will increase.\textsuperscript{12} CKD also imparts a significant financial burden with $250 billion spent on CKD care in patients 65 and older in the year 2011 alone.\textsuperscript{13} The pathogenesis of CKD is characterized by kidney injury, which may occur in response to activation of the renin-angiotensin aldosterone system (RAAS). The RAAS typically acts as a homeostatic response in hypotensive states; however, individuals with an upregulated RAAS have increased systemic circulation of angiotensin (Ang) II. Ang II is a potent vasoconstrictor that increases oxidative stress, inflammation, and fibrosis in the kidney which are hallmark features of CKD.\textsuperscript{14,15}

Several studies have shown a significant decrease in the incidence of chronic inflammatory diseases among individuals who consistently consume a diet high in fruits and vegetables.\textsuperscript{16-18} This attenuation is partially attributed to the polyphenols present in fruits and vegetables that act as antioxidants by reducing reactive oxygen species (ROS).\textsuperscript{19} Berries, specifically raspberries, are widely consumed and high in polyphenols. Although there are anti-inflammatory medications available, these medications do not come without side effects. A benefit of consuming raspberries as a preventative measure is providing a natural approach with the avoidance of additional risks presented by medication use. Raspberries also have a moderate amount of potassium compared to other fruits,\textsuperscript{20} making them an excellent source of nutrients for individuals with CKD without the additional stress on the kidneys that is required to filter excess potassium.\textsuperscript{21}
The objective of this study is to investigate the extent to which raspberry consumption affects oxidative stress and fibrosis induced by Ang II in the kidneys of male Sprague-Dawley rats. We hypothesize that animals consuming a raspberry-supplemented diet will have reduced Ang II-induced oxidative stress and fibrosis in their kidneys.

**Aim 1:** Assess the effects of raspberries on oxidative stress in the kidneys of Sprague-Dawley rats treated with Ang II.

**Aim 2:** Assess the effects of raspberries on fibrosis in the kidneys of Sprague-Dawley rats treated with Ang II.
CHAPTER 2

LITERATURE REVIEW

2.1 Kidney Anatomy and Function

The kidneys are a pair of apple sized, brownish purple colored organs located in the lower back with one on either side of the spine. They are made up of an outer cortex, several medulla, major and minor calyces, and the renal pelvis. The medullae consist of over 1 million functional units called nephrons that are involved in filtering water and waste products including urea and uric acid from the blood, as well as excretion and absorption of minerals from food and drink.

After consumption of food and drink, the body absorbs the needed minerals, leaving the excess minerals in the blood for removal. Filtration of the blood is performed in the glomerulus of the nephron where the cardiovascular system meets the renal system at the renal artery. The exchange of minerals occurs in the nephron, initiating in the glomerulus with the movement of filtrate across the porous glomerular capillaries through podocytes. These “foot cells” have filtration slits that allow for the flow of filtrate to enter the glomerular capsule. The filtrate continues to the proximal convoluted tubule (PCT) where water and solutes are reabsorbed, and unnecessary materials are removed. The filtrate then flows through the loop of Henle where it initially continues the work of the PCT; however, as it continues, the purpose shifts to only absorbing and excreting water to maintain appropriate hydration status. Lastly, the filtrate flows through the distal convoluted tubule (DCT) where its main role is acid-base balance and sodium balance.

Filtered blood leaves through the renal vein, and the remaining material in the tubules forms the final urine. Urine then flows through the collecting ducts, to the calyces, on to the renal pelvis where it is then transferred through the ureter to the urinary bladder (Figure 1). The final urine is stored in the urinary bladder until it is expelled during urination.
Figure 1. Structure of the Nephron

The kidneys are involved in many functions outside of waste removal, including hormone release through the adrenal cortex to regulate blood pressure in the renin-angiotensin-aldosterone system (RAAS), conversion of inactive vitamin D to active vitamin D, as well as participation in red blood cell production. The granular cells of the nephron are responsible for the release of renin, while the adrenal cortex, located superior to the kidney, releases aldosterone. These hormones are largely influential in the regulation of blood pressure through initiation of the RAAS cascade through renin and the direct effects of aldosterone.

2.2 Renin Angiotensin Aldosterone System

RAAS (Figure 2) is a homeostatic system involved in maintaining blood pressure through systemic vasoconstriction, increased plasma volume and sodium reabsorption in the kidneys. When functioning properly, this system can be instigated by several factors including a decrease in arterial blood pressure recognized by renal granular cells, macula densa cell signaling due to
low NaCl concentrations, and/or stimulation of granular cells by renal sympathetic nerves. In response to any or all of these stimuli, the granular cells of the kidney release renin, a protein that converts the plasma protein angiotensinogen, an α2-globulin protein with a variable length about 118 amino acids, from the liver, to Ang I, a decapeptide, through hydrolysis of the leucine-valine bond. Angiotensin converting enzyme (ACE), an endothelial-bound carboxypeptidase from the lungs, converts Ang I, an inactive peptide, to Ang II, an active octapeptide that acts as a hormone. Ang II increases blood pressure by acting on several tissues including smooth muscle to constrict blood vessels, the adrenal cortex to release aldosterone which stimulates sodium retention, and the thirst center of the brain to increase fluid intake and fluid retention in response to vasopressin. Through constricted blood vessels, sodium and water retention, and amplified water intake, blood pressure rises.

![Figure 2. Renin-Angiotensin Aldosterone System](image)

However, in instances of chronic hypertension, there is a concern with an unnecessarily upregulated RAAS through impaired endothelial function and vascular insulin metabolic signaling. RAAS activation not only leads to increased blood pressure, but it also stimulates fibrosis and inflammation through specific receptors such as angiotensin II type 1 (AT₁R).
Consequences of upregulated RAAS leading to chronic hypertension can include increased risk of cardiovascular events such as heart attack or heart failure, stroke and risk of developing chronic kidney disease or the progression of the disease.\textsuperscript{28}

A homologue of ACE, expressed most commonly in vascular endothelial cells of the kidney and heart, has been acknowledged and named ACE2. ACE2, also a carboxypeptidase, generates Ang1-9 from Ang I and Ang1-7 from Ang II.\textsuperscript{29} The actions of the ACE2/Ang-(1-7)/Mas axis are potential therapeutic targets that act in the management of RAAS through anti-inflammatory, antifibrotic mechanisms.\textsuperscript{30} This is discussed further in section 2.4.6.

2.3 Angiotensin (Ang) II

Angiotensin II, an octapeptide acting as a hormone, is one of the main driving forces behind the RAAS.\textsuperscript{31,32} Ang II is produced not only for generalized anti-hypotensive effects in the body in response to several stimuli but also locally by individual tissues, as well as intracellularly to have a more targeted assault.\textsuperscript{27,33} In instances of hypotension, the effects of Ang II can be lifesaving; however, in situations of chronic tissue exposure, its effects can be detrimental to many of the body’s systems.\textsuperscript{2}

Many of the tissues in the body express Ang II receptors to allow for the cascade of the RAAS to be effective in several bodily organ systems. More specifically, Ang II is pivotal in the process of excretion and reabsorption in the kidneys through the binding of Ang II to two different G-protein coupled receptors: AT\textsubscript{1}R and AT\textsubscript{2}R.\textsuperscript{34,35} AT\textsubscript{1}R and AT\textsubscript{2}R are expressed in many tissues including the heart, kidney, brain, and vascular smooth muscle.\textsuperscript{27,35} The kidney is an important target for antihypertensive efforts because they contain all steps of RAAS through angiotensinogen synthesis in the proximal lumen, renin production in the juxtaglomerular apparatus, ACE production in the tubules and AT\textsubscript{1}R and AT\textsubscript{2}R expression throughout.\textsuperscript{36} AT\textsubscript{1}R are heavily active
in the RAAS cascade to propel the intended outcomes of vasoconstriction in vascular smooth muscles and sodium retention in renal tubular cells to increase blood pressure. However, the binding of Ang II to AT$_1$R can also lead to proinflammatory, profibrotic outcomes. In contrast, AT$_2$R performs anti-inflammatory, antifibrotic actions when Ang II bound. AT$_2$R possess renoprotective properties through inhibition of renin synthesis and release. The expression of AT$_2$R is at its highest in the unborn fetus with a significant decline in expression in the neonatal period and into maturation, majority of which remains in the uterus, ovary, adrenal cortex, and specific areas of the brain. As a consequence of this predominance of AT$_1$R expression over AT$_2$R, chronic upregulation of Ang II can result in oxidative stress, inflammation and fibrosis leading to deleterious outcomes in the kidneys through the binding of Ang II. This activation is responsible for the cascade of several signaling pathways including mitogen-activated protein kinase (MAPK) and nicotinamide adenine dinucleotide phosphate (NADPH), whose upregulation can lead to the oxidative stress and inflammation.

Tissue-specific Ang II expression can heavily influence the health and function of the related tissue through cell proliferation, cytokine production and management of inflammatory pathways. More specifically, in the kidney, concentrations of Ang II are significantly higher than plasma concentrations, making the effects of Ang II far more substantial. For example, intrarenal RAAS leading to chronic upregulation of Ang II and subsequent elevated aldosterone concentrations has been shown to contribute to kidney tissue injury through its mineralocorticoid receptor activation, leading to the potential development and progression of CKD.

2.4 Oxidative Stress

The production of reactive oxygen species (ROS) and free radicals is a normal part of oxygen metabolism; however, if the homeostatic antioxidant response is unable to accommodate
for the amount of ROS and free radicals being produced, an imbalance can occur.\textsuperscript{43} Oxidative stress is the result of continuous excessive amounts of ROS in the body, which can be due to an inefficiency in the biological antioxidant response to stabilize free radicals or increased activity of pro-oxidant enzymes through environmental stressors, genetics, or lifestyle choices. The inefficiency in the biological response is usually due to the effects of exogenous influences increasing the presence of systemic ROS.\textsuperscript{44} Ang II is a potent stimulator of the NADPH pathway, a major contributor to the production of ROS leading to oxidative stress.\textsuperscript{45} Although ROS is expected as a product of metabolic processes, disproportionate amounts can lead to the oxidation and damage of cells throughout the body, including kidney cells.\textsuperscript{46} Glutathione and cysteine are thiols that perform the role of antioxidant by acting as reducers of free radicals.\textsuperscript{47} Pro-oxidant enzymes can be credited to exogenous and endogenous factors including diet, drugs, environmental pollution, cellular metabolism and pathogens. These factors, when experienced in high amounts, can encourage the increased presence of ROS, and in turn cause an increase in expression of oxidative stress which is associated with the progression of many diseases.\textsuperscript{7,48} More specifically, as CKD progresses, the antioxidant activity in the body is dramatically decreased, lessening the body’s natural ability to mitigate oxidative stress through the reduction of free radicals and oxidation of ROS.\textsuperscript{49} The cardiac output associated with oxygen carrying to the kidneys is between 20-25\%, dramatically increasing the risk for oxidative stress and tissue damage.\textsuperscript{50}

2.4.1 Glutathione (GSH)

Glutathione is a tripeptide found in the cytosol of the cell containing L-cysteine, L-glutamic acid and glycine, and it acts as an antioxidant expressed by all organs for ROS scavenging.\textsuperscript{51} GSH is synthesized in the cell through the $\gamma$-glutamyl cycle comprised of six reactions.\textsuperscript{52} Initially, cysteine combines with glutamate through an amide bond, catalyzed by glutamate cysteine ligase
enzyme. Glutathione synthetase then adds a glycine to the original dipeptide to form the final product of GSH.\textsuperscript{53} GSH can either be free or bound, typically in its reduced state when bound.\textsuperscript{52} When oxidized, GSH is converted to glutathione disulfide (GSSG), a reversible oxidation. Excessive amounts of ROS will lower the GSH/GSSG ratio through decreased GSH levels as a result of the ROS scavenging, thus, decreased GSH can be indicative of oxidative stress.\textsuperscript{54} The majority of GSH research investigates its ability to detoxify several free radicals and peroxides; however, it is also involved in the detoxification of xenobiotics including food additives, flavorings or pollutants.\textsuperscript{52,55} The result of this detoxification is mercapturic acids that are excreted in the feces and urine.\textsuperscript{52} The mercapturate pathway (MAP) is heavily expressed in the cortical renal proximal tubular cells and is associated with chronic kidney disease progression.\textsuperscript{56} Kwon et al. demonstrated that the cytoprotective effects of GSH are heavily reliant on the activation of the nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 (NRF2/HO-1) signaling, a pathway involved in antioxidant gene transcription.\textsuperscript{51}

2.4.2 Glutathione Peroxidase-1 (GPx-1)

Glutathione peroxidase is a 23 kDa antioxidant enzyme with four subunits that each contain a seleno-cysteine residue.\textsuperscript{57} GPx has five isoforms and is found in all organs predominantly in the cytosol and mitochondria in cells.\textsuperscript{58,59} The main function of GPx is the conversion of the free radical hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to water and oxygen to protect against damage and potential progression to oxidative stress.\textsuperscript{48} Vera et al. demonstrated that enhancing the GPx pathways can protect the endothelium against damage from uremia, a characteristic commonly seen in individuals with CKD.\textsuperscript{60} GPx-1 is the main isoform found in the kidney with 96\% of GPx activity within the kidney coming from GPx-1.\textsuperscript{58}

2.4.3 Superoxide Dismutase (SOD1 and SOD2)
Superoxide dismutase acts as a nitric oxide scavenger for a protective effect against the damages of oxidative stress. In the redox pathways, SOD catalyzes the conversion of the highly damaging radical superoxide (O$_2^-$) to H$_2$O$_2$, hence the name, superoxide dismutase.$^{54,61}$ SOD1 is a 17 kDa protein found in the cytosol that contributes to 80% of SOD activity, while SOD2, a 22 kDa protein, is found mostly in the mitochondria and is moderately expressed.$^{48,62}$ Decreased levels of SOD indicate an imbalance in ROS to antioxidant ratio and signals the presence of oxidative stress.$^{63}$ There are specific single-nucleotide polymorphisms among SOD2 that can indicate an increased risk for kidney function decline.$^{48}$

2.4.4 Catalase (Cat)

Catalase, with a molecular weight of 240 kDa in mammals, is a scavenger of ROS typically found in peroxisomes and highly expressed in the kidneys. Similar to the function of GPx-1, catalase is responsible for converting H$_2$O$_2$, the product of SOD, to water and oxygen, further lessening the oxidative stress potential.$^{62,64}$ In comparison to GPx-1, catalase acts more as a high capacity scavenger due to its efficiency in H$_2$O$_2$ detoxification.$^{65}$ Sunami et al found that catalase deficiency in mice with unilateral ureteral obstruction exhibited higher injury and fibrosis in the tubules and interstitium of the kidneys.$^{66}$

2.4.5 3-nitrotyrosine (3-NT)

3-nitrotyrosine is an oxidative stress biomarker typically produced through nitration of a tyrosine residue and nitrogen dioxide; however, other nitrating agents are capable of the reaction including peroxynitrite, nitric oxide, and certain peroxidases. The production of 3-nitrotyrosine is post-translation, meaning the production is not determined through gene transcription. The presence of inflammatory conditions increases the nitration of tyrosine due to the high levels of nitrates and nitrites.$^{67}$
2.4.6 Mitochondrial Assembly Protein (Mas) Receptor 1

Mas receptor 1 is a g-protein coupled receptor with a molecular weight of 37 kDa, found on the surface of several cell types including kidney cells, cardiomyocytes and adipocytes.\textsuperscript{30,68-70} When activated by the binding of Ang-(1-7), a product of ACE2 degradation of Ang II, mas receptor 1 acts in opposition of the AngII/ AT\textsubscript{1}R inflammatory pathway associated with chronic RAAS upregulation. The anti-inflammatory, anti-fibrotic effects of the ACE2/Ang-(1-7)/Mas axis are largely involved in regulating leukocyte recruitment and inflammatory cytokine production.\textsuperscript{30} Studies have shown an inverse correlation between Ang-(1-7) and renal fibrosis, renal oxidative stress, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity, a protein complex associated with ROS production.\textsuperscript{71,72} The cascade initiated by the binding of an Ang-(1-7) to mas 1 receptor incites the transcription of antioxidant enzymes heme oxygenase 1 (HO-1) and NADPH: quinone acceptor oxidoreductase 1 (NQO1) through the cytoprotective NRF2/Keap1 pathway.\textsuperscript{73,74}

2.4.7 Nuclear Factor Erythroid 2-Related Factor 2 (NRF2)

NRF2 is a transcription factor at 110 kDa involved in the transcription of genes coding for antioxidant and detoxifying enzymes. In the presence of ROS, the NRF2 pathway is stimulated to express genes that promote glutathione synthesis. The presence of NRF2 can be used as an indicator of the presence of oxidative stress.\textsuperscript{54} In normal conditions, NRF2 is captured by Keap1 and degraded by a ubiquitin ligase complex; however, in conditions of high oxidative stress, Keap1 is inactivated and unable to bind NRF2. This allows NRF to translocate to the nucleus and activate the expression of genes for several antioxidant pathways.\textsuperscript{50} Additionally, NRF2/Keap1 pathway interferes with the NF-κB pathway, preventing the phosphorylation of NF-κB, and therefore preventing its translocation to the nucleus to induce the gene expression of several inflammatory
cytokines. Nezu and Suzuki reported that renal tubules of mice with ischemia-reperfusion injury were protected against damage and interstitial fibrosis with the activation of NRF2 transcription factor. The transcription of NRF2 is integral for the gene expression of antioxidants including glutathione and NADPH, as well as the reduction of pro-oxidants through activation of enzymes including HO-1 and NQO1.

2.4.8 Heme Oxygenase-1 (HO-1)

HO-1 is a 28 kDa NRF2-dependent enzyme found in the cytosol that acts as a cell protectant in conditions of high oxidative stress through the inhibition of ROS formation. It is the rate-limiting enzyme for the degradation of heme into carbon monoxide (CO) and free iron. Additionally, HO-1 catalyzes the degradation of biliverdin to bilirubin. CO and bilirubin are anti-inflammatory compounds that are involved in the protective characteristics of HO-1 by stunting the progression of the NF-κB pathway.

2.4.9 NADPH: Quinone Acceptor Oxidoreductase 1 (NQO1)

NQO1, found in the cell cytosol, is a 31 kDa target gene for NRF2 to further the antioxidant pathway through its enzymatic capabilities. The downregulation of NQO1 is seen in many models of kidney disease in response to reduced activity of NRF2, and in turn, activation of the NRF2/NQO1 pathway was nephroprotective. In one study in streptozotocin-induced diabetic nephropathy mice, NQO1 knockout showed higher glomerular injury, increased blood urea nitrogen levels and podocyte deformation compared to wild type.

2.4.10 Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidases (NOX)

NOX enzymes are membrane-bound protein complexes that increase the presence of ROS through the transfer of electrons across the cell membrane. The electrons are transferred from NADPH to molecular oxygen and form either H$_2$O$_2$ or hydroxyl radicals (OH$^-$). This ROS
production can be triggered by the presence of Ang II and aldosterone. NOX1, NOX2, and NOX4 are just three of the seven NOX isoforms found at 65, 60, and 67 kDa, respectively. Although they all share several similar properties, they differ in activation of certain enzymes and the ROS that is generated. NOX1, NOX2 and NOX4 all interact with p22\textsuperscript{phox} transmembrane protein. However, NOX1 and NOX2 need cytosolic organizer subunits and G-protein Rac to be active, while NOX4 does not. NOX4 is the most highly expressed in the kidney. NOX1 and NOX2 are mostly responsible for O\textsuperscript{2-} production, and NOX4 is mostly responsible for H\textsubscript{2}O\textsubscript{2} production.\textsuperscript{78}

### 2.5 Inflammation

Inflammation is an immunological response to trauma or foreign bodies in the system. The inflammatory response goes into effect to allow for immune cells to target the damaged tissue and encourage repair and healing\textsuperscript{40} through the initial release of M1 macrophages and later of M2 macrophages in the advanced stages of inflammation to induce fibrotic healing.\textsuperscript{79} M1 macrophages are associated with pro-inflammatory cytokine production, while M2 macrophages are associated with anti-inflammatory cytokine production.\textsuperscript{80} Inflammation is studied in two categories: acute and chronic. When acute inflammation is not resolved and returned to a homeostatic normalcy, it can further advance into a chronic inflammatory response.\textsuperscript{81} Angiotensin II can lead to pro-inflammatory effects that perpetuate the onset of this chronic inflammatory response through its binding to AT\textsubscript{1}R.\textsuperscript{32,37} This response then can progress to development of a multitude of diseases including CKD through the degradation of cells and tissues. These stressors cause a cascade of inflammatory pathways including the nuclear factor (NF)-\kappaB pathway that trigger the synthesis of pro-inflammatory cytokines.\textsuperscript{81}

### 2.6 Chronic Kidney Disease


Chronic kidney disease is multi-systemic disorder characterized by damage present in the nephrons of the kidney resulting in reduced kidney function. There are many causes including unregulated hypertension, poorly managed diabetes, or acute kidney injury, all leading to inflammation. Although the population of obese and overweight is growing, cases of chronic kidney diseases are stabilizing due to awareness and proper treatment.

Chronic kidney disease is diagnosed and staged through urine and blood tests. An initial blood test is performed to measure circulating creatinine, a waste product of muscle turnover. Additionally, urine tests are performed to measure albumin:creatinine ratio and to check for blood and protein. After a confirmation of CKD, estimated glomerular filtration rate (eGFR) is established based on how effective the kidneys are at filtering waste in one minute (Table 1). The eGFR is used to determine the stage of the disease and the necessary approach for treatment.

<table>
<thead>
<tr>
<th>Table 1 Stages of CKD&lt;sup&gt;82&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR(ml/min/1.73m&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>&gt;60 (with other signs of kidney damage)</td>
</tr>
<tr>
<td>60-89 (with other signs of kidney damage)</td>
</tr>
<tr>
<td>45-59</td>
</tr>
<tr>
<td>30-44</td>
</tr>
<tr>
<td>15-29</td>
</tr>
<tr>
<td>&lt;15 (majority function loss)</td>
</tr>
</tbody>
</table>

Unmanaged hypertension is one of the leading risk factors for chronic kidney disease, and it is estimated that 80-85% of individuals with chronic kidney disease also have hypertension. Pharmacological treatment of hypertension is typically administered through ACE inhibitors or Ang II receptor blockers (ARBs) with the overall intention behind the treatment being control of an upregulated RAAS by blocking the effects of Ang II. With the control of Ang II comes the
reduced risk of the development or progression of chronic kidney disease through hypertension management.

2.7 Dietary Approaches for Chronic Kidney Disease

Chronic kidney disease has no cure, and instead is managed through the treatment of its comorbidities by medications along with a specific diet that minimizes stress on the kidneys. Although a small portion of the population with chronic kidney disease has genetic basis, the majority of individuals with CKD have nutritional causes paired with poorly controlled comorbidities. The core of a specialty kidney diet is to limit the intake of potassium, sodium, phosphorus, and protein while monitoring the patient’s blood urea nitrogen (BUN) and creatinine levels. BUN is the measurement of urea nitrogen in the blood, a waste product of protein breakdown in the liver. Creatinine is a waste product of muscle turnover that circulates in the blood. The kidneys are responsible for removal of these waste products through urine production, and poor kidney function can lead to increased BUN to Creatinine ratios. The need to restrict dietary factors lies in the amount of work the kidney must perform to properly filter them along with their usual waste removal functions. Initially, this dietary approach limited the amount of fruits, vegetables, nuts and grains available for consumption due to potassium and phosphorus content. Additionally, lean meats were often included in the diet as long as phosphorus and protein were monitored. However, more recent studies have shown the amount of potassium and phosphorus absorbed by the body is far less in plant-based sources than animal-based sources due to their bioavailability. In addition to the benefits of a plant-based diet in managing mineral intake, plant-based diets have been shown to help manage diabetes and hypertension, two of the main comorbidities associated with chronic kidney disease. A large part of the benefits of a plant-based diet is its anti-inflammatory properties that have been shown to be nephroprotective.
2.8 Benefits of Polyphenols

Polyphenols are a class of phytochemicals found only in plants, and include four main classes: flavonoids, phenolic acids, stilbenes and lignans.\textsuperscript{6,92} Flavonoids can be further broken down into more than 4,000 varieties and are characterized by the amount of hydroxyl groups they contain. However, these varieties fall into six sub-categories including flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanins.\textsuperscript{93} Phenolic acids can also be further broken down into benzoic acids including ellagic acid and hydrocinnamic acids including caffeic acid.\textsuperscript{92} Polyphenols have at least one aromatic ring and hydroxyl group and contain ROS scavenging abilities.\textsuperscript{6} The antioxidant capabilities of polyphenols were developed through evolutionary adaptations of the plants to protect against threats such as fungi, bacteria and insects.\textsuperscript{6}

Polyphenols exhibit many health-related benefits including blood pressure regulation, gut microbiome diversification, improved platelet and endothelial function, and inflammation mitigation.\textsuperscript{92,94} Although their antioxidant capabilities were considered polyphenols’ most beneficial characteristic, it has been more recently shown that the concentrations needed to reach tissues for this benefit are not achievable \textit{in vivo}. Instead, it is more apparent that polyphenols can regulate inflammation through the modulation of inflammatory pathways by limiting the synthesis of certain cytokines.\textsuperscript{92} It is believed that through this capability, polyphenols are potentially able to aid in the treatment of inflammation-related diseases.\textsuperscript{6}

2.9 Raspberry Polyphenols and Inflammation, Oxidative Stress and Chronic Disease

Red raspberries (\textit{Rubus idaeus}) are a small red fruit that have been gathered for consumption for centuries.\textsuperscript{20} They are a great source of fiber and vitamin C with a moderate amount of kilocalories and potassium, making them a recommended fruit for individuals with compromised kidney function (Table 2).\textsuperscript{95}
Table 2 Raspberry Nutrient Profile

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilocalories</td>
<td>52</td>
</tr>
<tr>
<td>Protein</td>
<td>1.2g</td>
</tr>
<tr>
<td>Fat</td>
<td>.65g</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>11.9g</td>
</tr>
<tr>
<td>Fiber</td>
<td>6.5g</td>
</tr>
<tr>
<td>Calcium</td>
<td>25mg</td>
</tr>
<tr>
<td>Potassium</td>
<td>151mg</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>26.2 mg</td>
</tr>
<tr>
<td>Folate</td>
<td>21μg</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>33 IU</td>
</tr>
</tbody>
</table>

Red raspberries are also one of the richest sources of polyphenols in the berry family, particularly of the flavonoid sub-class including the anthocyanin, gallic acid, and the favonol sub-class including the tannin, ellagic acid, that are present in the skin of the raspberry (Table 3). Polyphenols are acknowledged as efficient scavengers of ROS as well as significant contributors to antioxidant pathways leading to attenuation of oxidative stress. Through this mitigation of oxidative stress, inflammation is in turn lessened systemically. Additionally, polyphenols that are found in red raspberries are capable of attenuating the synthesis of certain cytokines, therefore also aiding in the reduction of systemic inflammation.
Table 3 Raspberry Polyphenol Profile\textsuperscript{9}

<table>
<thead>
<tr>
<th></th>
<th>mg/100g FW</th>
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</thead>
<tbody>
<tr>
<td><strong>Phenolic Acids</strong></td>
<td></td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>2.12</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>2.30E-04</td>
</tr>
<tr>
<td><strong>Chlorogenic acids</strong></td>
<td></td>
</tr>
<tr>
<td>5-O-caffeoylquinic acid</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>Flavonoids</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Flavonols</strong></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
</tr>
<tr>
<td>Cyanidin 3-O-glucoside</td>
<td>14.89</td>
</tr>
<tr>
<td>Cyanidin 3-O-sophoroside</td>
<td>37.61</td>
</tr>
<tr>
<td>Delphidin 3-O-glucoside</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Flavan-3-ols</strong></td>
<td></td>
</tr>
<tr>
<td>(-)-Epicatechin</td>
<td>5.05</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>0.58</td>
</tr>
</tbody>
</table>

There have been several studies demonstrating the potential benefits of red raspberry polyphenols on oxidative stress and inflammation mitigation. For example, Noratto et al.\textsuperscript{98} demonstrated that obese diabetic (db/db) mice fed a 5.3% raspberry supplemented diet for 8 weeks had a significant reduction in plasma IL-6, hypothesized to be a result of increased GPx. Wu et al.\textsuperscript{99} investigated the effects of red raspberry extract on oxidative stress \textit{in vitro}, and their results demonstrated that pretreatment with 250 µg/mL red raspberry extract in activated hepatic stellate cells mitigated oxidative stress through modulation of the NRF2/HO-1 pathway. Additionally, Feresin et al.\textsuperscript{100} showed senescence induced via Ang II in vascular smooth muscle cells was attenuated by pretreatment with 200 µg/mL raspberry polyphenol extracts. The raspberry polyphenol extract also increased the expression of SOD1, SOD2 and GPx1, indicating its ability to potentially increase scavenging of free radicals and reactive oxygen species.
CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents

Radioimmunoprecipitation assay (RIPA), phosphatase inhibitor cocktail 1 and 2, protease inhibitor cocktail were purchased from Sigma Aldrich, St. Louis, MO. Rabbit polyclonal against: GAPDH (3683S), SOD2 (13141), HO-1 (82206) from Cell Signaling, Danvers; MA; NOX1 (ab131088), NOX 2 (ab180642), NOX4 (ab133303), ACE1 (ab216476), ACE2 (ab15348), 3-nitrotyrosine (ab7048) were from Abcam (Boston, MA); GPx-1 (AF3798) from R&D Systems (Minneapolis, MN); AT1R (NBP1-77078), AT2R (NBP1-77368) Mas Receptor (NBP1-78444), NRF2 (NBP1-32822) were from Novus Biologicals (Centennial, CO). Mouse polyclonal against: β-actin (3700) from Cell Signaling, Danvers, MA; NQO1 (NB200-209) from Novus Biologicals, Centennial, CO; rabbit secondary antibody (65-6120; Invitrogen, Carlsbad, CA) or mouse secondary antibody (7076S; Cell Signaling, Danvers, MA); Weigert’s Iron Hematoxylin Solution (HT1079), Biebrich Scarlet-acid Fuchsin (HT151); Phosphotungstic/Phosphomolybdic Acid Solution (HT152- & HT153); Aniline Blue Solution (HT154) from Sigma-Aldrich, St. Louis, MO. DAB antibody substrate (11724 & 11725; Cell Signaling, Danvers, MA).

3.2 Study Design

This study is a secondary analysis of kidney tissue from a diet intervention study on male Sprague-Dawley rats treated with Ang II. Eight-week-old male Sprague-Dawley rats were fed an AIN-93M diet (control and Ang II groups) or AIN-93M diet supplemented with 10% w/w freeze-dried raspberry (RB + Ang II) for seven weeks. Diet macronutrient and micronutrient characteristics were matched between treatment groups. At week 4, rats were implanted with subcutaneous osmotic minipumps (Alzet, model 2004) that delivered 0.9% saline (control) or Ang
II (270 ng/kg body weight/min) (Bachem) for an additional three weeks. Ang II dosages were determined for hypertensive outcomes and effects on cardiovascular function.

3.3 Western Blot Tissue Analysis

Harvested kidney tissue was frozen and stored at -80°C. To prepare the tissue for western blot, 0.03g of tissue was added to 300 µL of complete RIPA buffer mixed with 1:100 dilution of phosphatase inhibitor cocktail 1 and 2 and protease inhibitor cocktail and incubated on ice for 10 minutes to allow for tissue breakdown. Tissue was then homogenized until fully dissolved in complete RIPA. Lysate was centrifuged at 16,000 x g for 20 minutes at 4°C. The pellet was discarded, and the supernatant removed and stored on ice for analysis.

Tissue supernatant was quantified by way of DC protein assay kit. To prepare for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis application, 70 µg of sample was mixed with 4x Laemml buffer + 10% 2-mercaptoethanol (BioRad Laboratories, Hercules, CA). Samples were briefly vortexed and centrifuged prior to a dry bath for 10 min at 70°C in a dry heating block. Samples were loaded in a polyacrylamide gel for electrophoresis. At the completion of electrophoresis, gels were transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in either TBS-T (50 mmol/L Tris, 150mol/L Nacl, 0.2% Tween-20, pH 7.4) + 5% non-fat dry milk or TBS-T + 5% bovine serum albumin (BSA) and washed in TBS-T.

Membranes were incubated overnight in 4°C with primary antibodies against: NOX1, NOX2, NOX4, GPx-1, NRF2, SOD2, CAT, HO-1, NQO1, ACE1, ACE2, AT1R, AT2R and Mas receptor 1 (dilution determined according to manufacturer’s instruction). Membranes were washed for (3) 5-minute intervals in TBS-T then probed with secondary antibody in TBS-T + 5% BSA for 1 h. After an additional (3) 5-minute washes, chemiluminescent imaging of target proteins were
performed with Immobilon Forte Western HRP Substrate (EMD Millipore, Billerica, MA) using ChemiDoc Imaging Systems (Bio-Rad Laboratories, Hercules, CA). Image bands were quantified in Image Lab 6.0.1 (BioRad Laboratories, Hercules, CA).

3.4 Immunohistochemistry

Kidney tissues that were formalin-fixed and paraffin-embedded were used for histology. Kidney tissue was sliced and mounted on slides, deparaffinized and rehydrated. Slides were boiled in sodium citrate buffer solution for antigen retrieval at 100°C for 10 min. Slides were cooled at room temperature then washed in PBS for (3) 3-min intervals. Slides were incubated in 3% H2O2 for 20 min under dark conditions, followed by (3) PBS 3-minute washes. Slides were blocked with appropriate blocking buffer and incubated overnight at 4°C in 3-nitrotyrosine (dilutions according to manufacturer’s instructions). The following day, slides were washed in PBS for (3) 5-min intervals and incubated in secondary antibody for (1) h at room temperature under dark conditions. This was followed by (3) 5-min washes in PBS. DAB antibody substrate was added under a microscope to observe color change. After change was observed, slides were rinsed with DDI water and counterstained for 30 sec in hematoxylin. Slides were washed with tap water followed by DDI water, dehydrated, and the coverslip was mounted.

3.5 Masson Trichrome

Kidney tissue was sliced and mounted on slides, deparaffinized and rehydrated. Slides were stained using working Weigert’s Iron Hematoxylin Solution, rinsed with DDI water, followed by Biebrich Scarlet-acid Fuchsin and rinsed again with DDI water. Slides were stained with working Phosphotungstic/Phosphomolybdic Acid Solution followed by Aniline Blue Solution. The slides were rinsed with DDI water, dehydrated, and the coverslip was mounted.

3.6 Statistical Analysis
Data distribution was analyzed using the Shapiro-Wilk test. Normal distributed data was analyzed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test for multiple comparisons and presented as mean ± standard of deviation (SD). Data not normally distributed was analyzed using the Kruskal-Wallis test and presented as mean ± 95% confidence interval (CI). Significance was determined at $P \leq 0.05$. Data was analyzed using GraphPad Prism version 8.4.0 (La Jolla, CA) Effect size was determined through Cohen’s d ($d$) in data with $P$-values at 0.05 or higher but still exhibiting change.
CHAPTER 4

RESULTS

4.1 Raspberry supplementation had no effect on NOX isoform expression

Renal expression of NOX1 (Fig. 3A & B) and NOX4 (Fig. 3A & D) was not significantly different among groups. NOX2 expression was significantly increased by Ang II compared to control group (1.29 ± 0.24 vs 1.00 ± 0.15-fold, n=9, P = 0.03) but raspberry treatment was not able to attenuate this increase (1.31 ± 0.34 vs 1.29 ± 0.24-fold, n=9, P = 0.99) (Fig. 3A & C).

Figure 3. Raspberry supplementation had no effect on NOX isoform expression. Eight-week-old Sprague-Dawley male rats were fed an AIN-93M diet (control and Ang II groups) alone or supplemented with 10% w/w freeze-dried RB (RB + Ang II) for seven weeks. At week 4, rats were
implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control) or Ang II (270 ng/kg body weight/min) for an additional three weeks. Kidney protein expression of NOX1 (A & B), NOX2 (A & C), and NOX4 (A & D) were assessed by western blot. Values are mean ± 95% confidence interval (CI). *P ≤ 0.05.

4.2 Raspberry supplementation increased NRF2 and antioxidant enzyme expression

The expression of the transcription factor NRF2 was not changed with Ang II treatment (1.20 ± 0.60 vs 1.00 ± 0.22-fold, n=9, P = 0.83) (Fig. 4A & B). Nonetheless, raspberry supplementation significantly increased NRF2 expression compared to control (1.87 ± 1.11 vs 1.00 ± 0.22-fold, n=9, P = 0.05). Similarly, Ang II did not affect the expression of HO-1 and NQO1; however, raspberry supplementation increased the expression of both (1.59 ± 0.36 vs 1.00 ± 0.22-fold, n=9, P = 0.09 (Fig. 4A & C) and 1.52 ± 0.50 vs 1.00 ± 0.16-fold, n=9, P = 0.004 (Fig. 4A & E), respectively) compared to control. Although the increase in HO-1 was not statistically significant, there was a significant effect size compared to control (d= 1.11). There were no significant differences in the expression of GPx1 (Fig. 4A & D), SOD2 (Fig. 4A & F) and CAT (Fig. 4A & G).
Figure 4. Raspberry supplementation increased the expression of transcription factor NRF2 and antioxidant enzymes HO-1 and NQO1. Eight-week-old Sprague-Dawley male rats were fed an AIN-93M diet (control and Ang II groups) alone or supplemented with 10% w/w freeze-dried RB (RB + Ang II) for seven weeks. At week 4, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control) or Ang II (270 ng/kg body weight/min) for an additional three weeks. Kidney protein expression of antioxidant enzymes, HO-1 (A & C), GPx1 (A & D), NQO1 (A & E), SOD2 (A & F), and Catalase (A & G), and their major transcription factor, NRF2 (A & B), were assessed by western blot. Values are mean ± 95% confidence interval (CI). *P ≤ 0.05.

4.3 Effects of raspberry supplementation on ACE isoforms and Angiotensin Receptors

ACE1 was significantly increased with Ang II (1.34 ± 0.36 vs 1.00 ± 0.22-fold, n=9, P = 0.04; $d=1.14$) and raspberry supplementation (1.53 ± 0.25-fold, n=9, $P=0.001$) (Fig. 5A & B) in comparison to control. There was no significant difference in the expression of ACE2 (Fig. 5A & C) or AT$_1$R (Fig. 5A & E) in response to Ang II or supplementation with raspberry. Ang II treatment did not affect AT$_2$R expression (1.28 ± 0.67 vs 1.00 ± 0.44-fold, n=4, $P=0.8$) (Fig. 5A & F); however, raspberry supplementation significantly increased AT$_2$R expression compared to control (2.13 ± 0.69-fold, n=4, $P=0.05$; $d=1.95$) (Fig. 5A & F). Mas Receptor 1 expression was not changed after treatment with Ang II. Nonetheless, raspberry supplementation significantly increased renal expression of Mas receptor 1 compared to control (1.33 ± 0.31 vs 1.00 ± 0.07-fold, n=9, $P=0.006$) and Ang II (1.10 ± 0.15-fold, n=9, $P=0.05$; $d=0.94$) (Fig. 5A & D).
Figure 5. Raspberry supplementation increased expression of Mas Receptor 1, AT1R, AT2R, ACE1, had no effect on ACE2. Eight-week-old Sprague-Dawley male rats were fed an AIN-93M
diet (control and Ang II groups) alone or supplemented with 10% w/w freeze-dried RB (RB + Ang II) for seven weeks. At week 4, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control) or Ang II (270 ng/kg body weight/min) for an additional three weeks. Kidney protein expression of ACE1 (A & B), ACE2 (A & C), and Ang II receptors Mas receptor 1 (A & D), AT1R (A & E), and AT2R (A & F), were assessed by western blot. Values are mean ± 95% confidence interval (CI). *P ≤ 0.05.

4.4 Raspberry supplementation reduced presence of oxidative stress but did not mitigate fibrosis development

The expression of oxidative stress biomarker 3-nitrotyrosine was increased with Ang II and mitigated with raspberry supplementation. Trichrome staining of the tissue did not show increased fibrosis development in the kidney in response to Ang II treatment.

Figure 6. Raspberry supplementation reduced the presence of 3-nitrotyrosine, a biomarker of oxidative stress. Eight-week-old Sprague-Dawley male rats were fed an AIN-93M diet (control and Ang II groups) alone or supplemented with 10% w/w freeze-dried RB (RB + Ang II) for seven weeks. At week 4, rats were implanted with subcutaneous osmotic minipumps that delivered 0.9% saline (control) or Ang II (270 ng/kg body weight/min) for an additional three weeks. Kidney protein expression of oxidative stress biomarker, 3-nitrotyrosine (A), was assessed by immunohistochemistry. Fibrosis was assessed through Masson Trichrome Staining (B).
CHAPTER 5

DISCUSSION

Previous studies have shown the anti-inflammatory effects of berry-derived polyphenols in both human and animals; therefore, the goal of this study was to investigate the effects of consumption of raspberries, which are rich in polyphenols, on oxidative stress and inflammation induced by Ang II in the kidneys of male Sprague Dawley rats. We showed, in response to raspberry polyphenol supplementation and Ang II administration, an increase in the expression of MAS receptor 1, an increase in the activation of the KEAP1/NRF2 pathway and an increase in the expression of HO-1 and NQO1 antioxidant enzymes. Ang II significantly increased NOX2, a pro-oxidant enzyme, and nitrotyrosine, an oxidative stress biomarker. Although NOX2 was not down in the raspberry supplemented group, the group was not significantly different from control, which would indicate a level of mitigation. We speculate that raspberry exerts its main effects through the NRF2 cascade, the principal transcription factor of glutathione, leading to antioxidant enzyme production and increased ROS neutralization. This could potentially be through increased expression of AT2R and Mas 1 receptor; therefore, increasing phosphorylation of NRF2, freeing it from KEAP1. This allows NRF2 to translocate to the nucleus of the cell and promote the transcription of cytoprotective and detoxifying enzymes such as enzymes HO-1 and NQO1 (Figure 8). Additionally, with the increased expression of Mas 1 receptor, you can speculate that Mas receptor agonist Ang (1-7) could potentially antagonize AT1R, leading to reduced inflammatory and fibrotic outcomes. It is likely that the raspberry polyphenols exert direct effects on the proteins mentioned, however, under basal conditions, it is possible the protective proteins would already be elevated.
Figure 7. Proposed mechanism of Action  Raspberry supplementation may work through increase function of the Ang1-7/Mas receptor axis through increase of Mas 1 expression. This would allow for an increase in the binding of Ang 1-7 to promote NRF2 translocation and gene transcription of antioxidant enzyme production. Additionally, potentially through increased expression of Ang II receptor, AT2R, as a result of raspberry supplementation, further antioxidant effects can be seen.

In our study, we saw an increase in the expression of NRF2 in response to raspberry supplementation compared to control. A study by Manna et al.\textsuperscript{10} also found that the ellagitannins, a polyphenol found abundantly in raspberries, played a role in enhanced NRF2 phosphorylation compared to a streptozotocin alone treatment group, most likely due to the reduced expression of Keap1. The study used a diabetic nephropathy model induced by streptozotocin to investigate the effects of ellagitannins derived from pomegranate peels on progression of the disease and its associated inflammation. The enhanced phosphorylation of NRF2 increased dose dependently, further supporting the involvement of the extract in translocation of NRF2.
In response to raspberry supplementation in our study, we saw a significant increase in the expression ACE1 and antioxidant enzyme HO-1 compared to control. The opposite was seen in a study by Kang et al.\textsuperscript{103} investigating the effects of gallic acid, a phenolic acid found in raspberries.\textsuperscript{104} showed the inhibition of ACE1. In human umbilical vein endothelial cells, gallic acid from green algae, was administered in dosages ranging from 16.5-132 \( \mu \)g/ml. Three dimensional simulations of gallic acid activity were preformed to determine its processes, and it was exhibited that gallic acid blocks the actions of ACE1 by binding to its active site pocket. The difference in our results could be explained by the supplementation of gallic acid versus the supplementation of the whole raspberry, most likely resulting in a higher dose of gallic acid supplementation in this study compared to ours. A similar study by Sivasinprasasn et al.\textsuperscript{105} using human umbilical vascular endothelial cells showed more comparable results to our study. Cells pretreated with cyanidin-3-glucoside (5-20\( \mu \)M), a polyphenol present in raspberries, for 2 h prior to addition of Ang (10\textsuperscript{-6} M) for 24 h promoted NRF2 signaling as well as increased expression of HO-1.

Supplementation of raspberries in our study had no effect on the expression of antioxidant enzymes CAT, SOD and GPx1. However, in contrast, a study by Prathapan et al.\textsuperscript{106} showed polyphenols derived from \textit{Boerhavia diffusa} extract (BDE) significantly increased the production of antioxidant enzymes CAT, SOD and GPx, compared to the Losartan treatment group, an AT\textsubscript{1}R blocker commonly prescribed to treat hypertension. The study investigated the effects of polyphenols derived from BDE on Ang II-induced cardiac hypertrophy in Wistar rats compared to treatment with Losartan. Ang II was administered subcutaneously daily for seven days at a dosage of 1.5 mg/kg/day. These outcomes seen are partially attributed to increased NRF2 translocation. Our study showed increased expression of total NRF2, however, nuclear and cytosolic NRF2 was
not measured. The Ang II dose of this study was significantly higher than our dose by over 3 times administered one time per day. In our study, Ang II was administered at a consistent dose of 270 ng/kg body weight/min throughout the day, and it delivered just under .40 mg/kg/day compared to this study’s 1.5 mg/kg/day. Additionally, Prathapan et al\textsuperscript{106} administered the dose of BDE at 100 mg/kg orally through gastric intubation, making the consumption of polyphenols consistent between groups. Our study allowed the animals to consume the polyphenols \textit{ad libitum}, allowing for a more generalizable outcome. However, on average, based on daily food weights, rats from our study were consuming about 68.7mg/kg of polyphenols per day. This is equivalent to about 2.5 cups of raspberries per day for a 60-kg human or 11mg/kg of bodyweight.

Our results showed raspberry supplementation to increase the activity of the Ang1-7/Mas receptor axis, and in addition to that, we saw an inhibitory effect of raspberry supplementation in the expression of 3-nitrotyrosine. A study investigating the effects of the polyphenol resveratrol in aging mice kidneys by Jang et al\textsuperscript{107} showed similar effects through increased expression of Ang (1-7)/Mas receptor axis as well as reduced 3-nitrotyrosine. The study supplemented standard mice chow with 40 mg/kg resveratrol to be eaten \textit{ad libitum}. Consumption was followed through daily food weighs. The aged mice were observed for a six-month period and sacrificed at two years old. One significant difference between studies was the antifibrotic effects seen as a result of the resveratrol treatment by Jang et al. The difference in fibrosis production and mitigation could be attributed to the extended length of this study compared to ours.

Transcription factor NRF2 and antioxidant enzyme HO-1 were increased in the AngII + RB group compared to control in our study. Similarly, a study investigating the effects of polyphenols on diabetic nephropathy model in rats showed the potential of phenolic compound chlorogenic acid to increase the translocation of NRF2, therefore, increasing the expression of HO-
1. Bao et al\textsuperscript{108} induced diabetic nephropathy through streptozotocin administration and a high-fat diet in male Sprague-Dawley rats, and chlorogenic acid was dosed at 10 mg/kg/day for 8 weeks. Similar to our study, the polyphenols were presented as a pre-treatment and provided prior to disease development. Although the polyphenolic dose was less than our dose of an average of 24 mg/kg/day, this study reflected similar outcomes in HO-1 expression. Additionally, we saw an increase in the expression of NRF2, however we did not look at nuclear and cytosolic NRF2 to determine translocation.

Endothelial dysfunction is a condition often seen in individuals with compromised kidney function, especially in the aging population. The threat of endothelial dysfunction to kidney health largely relies on the influx of ROS and an inflammatory response that leads to vascular damage within the kidneys.\textsuperscript{109} Our study showed, in the AngII + RB group, an increase in the expression of Ang II receptor AT\textsubscript{2}R and a reduced expression of 3-nitrotyrosine, two markers often affected by endothelial dysfunction in renal models.\textsuperscript{110,111} A study by Dal-Ros et al.\textsuperscript{112} in middle-aged rats found that polyphenols from red wine significantly decreased the expression of 3-nitrotyrosine, NOX1, and AT\textsubscript{1}R and AT\textsubscript{2}R compared to control. Aged rats were fed solvents containing red wine extract at a dosage of 100 mg/kg/day in their drinking water for 4 weeks. The other treatment group received an antioxidant and NOX inhibitor at the same dosage of 100 mg/kg/day. The model of oxidative stress and inflammation although different, still showed the anti-inflammatory effects of polyphenols in models of oxidative stress and inflammation. In contrast to our study, we did not see a significant difference in AT\textsubscript{1}R or NOX1 in response to raspberry supplementation. This dissimilarity in results could be explained by the variance in model as well as the source of polyphenols.
As with all studies, the current study also has limitations. For example, the length of time animals were pre-exposed to the raspberry supplementation may not have been long enough to mitigate the effects of the angiotensin II treatment in the kidneys. Additionally, the angiotensin II dosage was established based on previous studies for hypertension and was not specific to oxidative stress in kidneys. Also, lack of a raspberry control group limits our conclusions on the direct effects of raspberry compared to the control. For these reasons, the results of this study are limited in their generalization.

In conclusion, raspberries, a commonly consumed and widely available fruit, have the ability to increase antioxidant pathways in the kidneys. We hypothesize that these pathways are functional through the activation of Mas receptor 1 leading to the translocation of NRF2 to the nucleus of the cell, stimulating the gene expression of antioxidant enzymes HO-1 and NQO1. It is hypothesized that these effects are seen due to the bioactive compounds present in raspberries. These results suggest a possible role for raspberries as an alternative and complementary therapy to prevent oxidative stress in the kidneys. Human studies are needed to confirm these effects.
REFERENCES


