The Effects of Sumac on Saturated Fat-induced Inflammation in Human Vascular Smooth Muscle Cells and Isolated Mesenteric Arteries from Rats

by

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ABSTRACT

Cardiovascular disease (CVD) is characterized by impaired vasodilation and the development of atherosclerosis. A diet high in saturated fat, such as palmitate, contributes to this by promoting inflammation and oxidative stress in human vascular smooth muscle cells (VSMC). The inflammation cascade that occurs increases pro-inflammatory cytokines, like tumor necrosis factor alpha (TNF-alpha) and increases proinflammatory enzymes like cyclooxygenase 2 (COX-2) contributing to inflammation, oxidative stress, blood pressure shifts, and atherosclerosis. Palmitate has been found to upregulate TNF-alpha, and COX-2.

In various studies, sumac, a Mediterranean spice and known antioxidant, has been shown to have antioxidant properties through its ability to inhibit reactive oxygen species (ROS) such as superoxide. Sumac has also been found to reduce TNF-alpha. Results from a study of hypertensive human subjects fed a sumac supplement showed a decrease in blood pressure.

In the current study, COX-2 levels were determined to evaluate the level of inflammation in response to palmitate when primary aortic human vascular smooth muscle cells (HAoVSM) were treated with sumac. The treatments included: vehicle (bovine serum albumin), 100 µM palmitate, and 10, 20, 40, 60, and 80 µg/mL sumac. Sumac did not alter COX-2 protein levels between vehicle and sumac groups. Additional studies were designed to examine whether 80 µg/mL sumac could reverse impaired vasodilation caused by 10 weeks of high fat intake, consisting of 60% of total calories from fat, in Sprague-Dawley rats. Mesenteric arteries were isolated and exposed to sumac. High fat diet (HFD) arteries had impaired vasodilation compared to arteries from
chow-fed fats. HFD arteries exposed to sumac had similar endothelium-dependent vasodilation responses as those not exposed to sumac, however, there were trends for improved vasodilation. I suggest that sumac likely exhibits antioxidant capabilities that prevent superoxide from decreasing the bioavailability of nitric oxide in the vasculature, thus promoting endothelium-dependent vasodilation and preventing the creation of more harmful reactive oxygen species. Isolated arteries from chow fed rats developed irreversible vasodilation when exposed to sumac and were therefore not responsive to pre-constriction with phenylephrine (PE) likely related to nitrates and gallic acid naturally present in sumac whereby inhibiting PE.
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**DEFINITIONS**

**Antioxidant**: substances that breakdown oxidants or convert them to less reactive products

**Atherosclerosis**: the build-up of plaque on blood vessel walls that narrows the lumen and leads to hypertension

**BW**: body weight

**Diastolic blood pressure**: the bottom number in the blood pressure ratio that explains the amount of pressure in the arteries between heart beats

**Endothelial**: the inner layer of cells that lines blood vessels

**ENOS**: endothelial nitric oxide synthase

**Hypertension**: blood pressure measurement of 140/90 mmHg or higher

**NO**: nitric oxide

**Oxidant**: molecules with unpaired electrons that are very reactive and can steal electrons from other molecules forming more reactive molecules that can cause damage to the body

**Oxidative stress**: increased molecules with unpaired electrons (free radicals) that scavenge other molecules and damage healthy cells

**Reactive Oxygen Species (ROS)**: chemically reactive species containing oxygen that are capable of damaging DNA, RNA, proteins, and may cause cell death

**Shear stress**: pressure on the blood vessels due to increased blood flow
Systolic blood pressure: the top number in the blood pressure ratio that identifies how much pressure is put on the blood vessel during contraction of the heart as it pushes blood through the vessels.

T2DM: type 2 diabetes mellitus

Vascular smooth muscle cells (VSMC): a smooth muscle layer distal to the endothelial layer that makes up the majority of the blood vessel wall and is responsible for regulating vascular tone.

Human aortic vascular smooth muscle cells (HAoVSMC): smooth muscle cells located in the aorta.
CHAPTER 1

INTRODUCTION

A chronic disease such as cardiovascular disease (CVD) is a pro-inflammatory disease that is a major cause of death. CVD is caused by inflammation that occurs in the vascular system. CVD is the leading cause of death globally at 31% of all deaths in 2013, accounting for 17.3 million deaths yearly. CVD is in part characterized by atherosclerosis. This is the process by which plaque forms in the blood vessels leading to altered vascular morphology and function. This includes impaired vasodilation and vasoconstriction as well as narrowing of the vessel. This increases the potential for a blood clot to travel and cause a blockage. Atherosclerosis is characterized by inflammation that occurs in both the endothelial cells as well as the VSMC within the vasculature. There are many causes of vascular inflammation and atherosclerosis. Conditions such as dyslipidemia, hypertension, and hyperglycemia contribute to the initiation and progression of atherosclerosis.

CVD is also characterized by oxidative stress, an overproduction of free radicals, and this remains a critical issue. Various mechanisms cause oxidative stress in the body. Increased consumption of dietary fats such as palmitate, a saturated fatty acid, can lead to inflammation as well as increased prevalence of oxidative stress due to cytokine release from adipose tissue. This is described as lipid peroxidation, which then causes the activation of NF-κB and thus the generation of reactive oxygen species (ROS). These accumulate in the vasculature, cause inflammation and contribute to atherosclerosis. Oxidative stress also reduces the bioavailability of the vasodilator nitric oxide (NO) thereby promoting constriction of blood vessels and hypertension. Chronic inhibition of
NO prevents vasodilation and can result in endothelial damage and CVD. Adequate antioxidant activity in the body is therefore pertinent to reducing oxidative stress, and thus, the risk of conditions like CVD.

*Rhus coriaria* L. is a plant, also known as sumac. It is found in the Middle East and used as a spice in its powdered form. The drupes are typically dried, ground and sprinkled on salad and meats for a lemony, tart flavor. Plants like sumac have been used medicinally by indigenous people and have putative abilities to lower blood glucose and cholesterol as told by folklore. In various cell-free models, sumac has been shown to be an antioxidant. It is capable of inhibiting ROS like superoxide and xanthine oxidase, which attack the body by causing oxidative stress. Sumac has the potential to protect the body from lipid peroxidation as well. Specifically, sumac has been found to contain oleic fatty acids, organic acids, nitrates, and various antioxidants such as: tannins and phenols, which have been shown to reverse lipid peroxidation as well as scavenge superoxide. There has been one study that looked at the effect of a different, and less widely available, species of sumac called *Rhus hirta*. Researchers found that the oxidant, superoxide, was decreased, however this study did not involve cells. Researchers concluded that sumac had a greater antioxidant effect than vitamin C or green tea. The dose of sumac was not provided and this study was conducted in a cell-free solution. Another study mixed superoxide with xanthine oxidase and exposed the mixture to *Rhus coriaria* powder (282 µg/mL) and it was found that sumac scavenged superoxide and inhibited xanthine oxidase, which is a mechanism for producing superoxide. This is an important observation as superoxide is a major ROS known to scavenge NO and thus reduce its availability for vasodilation. In one study it was concluded that the tannins
were responsible for lowering vascular smooth muscle cell migration, which suggests that sumac may prevent atherosclerosis, thus decreasing inflammation and oxidation to help prevent CVD.\textsuperscript{3} Further, a couple studies have shown a decrease in TNF-alpha when cells were exposed to sumac with one study involving human breast cancer cells.\textsuperscript{61,100} Recent research consisting of randomized clinical trials where the intervention group is fed sumac, show improvement in markers of CVD risk such as serum lipid in patients with either elevated lipids or type 2 diabetes mellitus.\textsuperscript{4-6} Many of the studies that have been conducted on subjects with T2DM show that blood glucose is decreased in the sumac-fed groups. Finally, it has been shown that sumac has the potential to reduce blood pressure as seen in hypertensive patients. The mechanism by which we suspect sumac is working is through its antioxidant capacity which would restore NO bioavailability and hence normalize flow-mediated vasodilation and blood pressure.\textsuperscript{59}

The two studies conducted for this paper examined whether sumac could improve inflammation in human coronary artery VSMC and vasodilation in isolated arteries from animals fed a high fat diet for 10 weeks. The protein intensity of the inflammatory marker COX-2 in human coronary artery VSMC was measured. Vasodilation in ex vivo arteries isolated from rats fed a high fat diet was measured as well. For this study, small mesenteric arteries were isolated from the rats. These arteries are very susceptible to stress and show great vascular resistance. It is proposed that the ability of sumac to decrease superoxide would lend to improvements in blood pressure by increasing NO bioavailability for vasodilatory activities. To examine this, we evaluated whether sumac could reverse palmitate-induced inflammation in human coronary artery VSMCs since palmitate is a saturated fatty acid with research supporting its ability to cause
inflammation. Finally, we aimed to quantify vasodilation in rat arteries treated with sumac and isolated from rats fed a high fat diet. The intent of this study was to evaluate the vasodilatory response to the endothelium-dependent vasodilator acetylcholine (ACh) in an environment of inflammation and oxidative stress. Therefore, this study has the potential to lay the ground work for future research that may greatly benefit people with risk factors for CVD by offering a cost-effective strategy, a simple dietary intervention, to reverse the most deleterious complications associated with poor dietary choices. If a natural antioxidant, such as sumac, can either prevent or reverse oxidation and inflammation, as well as improve vasodilation, it has the potential to be a non-pharmacological intervention as compared to the use of synthetic antioxidants like BHA and BHT.

H1: Sumac will reverse palmitate-induced inflammation in human vascular smooth muscle cells.

H2: Sumac will improve vasodilation in ex vivo arteries isolated from rats fed a high fat diet.

Null1: Sumac will not reverse palmitate-induced inflammation in HVSMC.

Null2: Sumac will not improve vasodilation in ex vivo arteries isolated from rats fed a high fat diet.
CHAPTER 2

REVIEW OF LITERATURE

Cardiovascular Disease

Cardiovascular disease (CVD) remains the leading cause of death globally at 31% of all deaths in 2013. CVD accounts for 17.3 million deaths yearly. In America, over 800,000 people died of CVD in 2014, equating to about 1 out of 3 deaths. Cardiovascular disease remains a threat to Americans considering the prevalence of fat in the diet. A diet high in fat, particularly saturated fat, plays a major role in the development of CVD. High saturated fat intake increases plasma low density lipoprotein (LDL) and this raises the risk for CVD. In 18 out of 27 countries evaluated (the USA included), at least 50% of the population’s saturated fat intake exceeded the World Health Organization’s recommendation of <10% of calories per day.

Pathobiology of CVD

CVD has a complex pathophysiology with deleterious consequences. There are various diseases that characterize CVD such as atherosclerosis. Atherosclerosis is associated with a high incidence of morbidity and mortality. It is a multifaceted disease that alters vascular morphology and function. It is a gradual process which involves the activity of various cells from the formation of a fatty streak, to a plaque, and finally to a ruptured plaque leading to thrombosis. The evolution and consequences of atherogenesis occurs due to the involvement of the endothelial and smooth muscle cells together with T-lymphocytes, mast cells, dendritic cells, and monocytes that migrate into
the vessels as well as the products that they secrete including: cytokines, enzymes, and chemokines.\(^7\)

Overall, vascular health deteriorates due to a trigger of an inflammatory cascade within VSMC. Vascular inflammation can be triggered, for example by the presence of saturated fat in the arteries where receptors on the VSMC walls, like toll-like receptor 4 (TLR4), are activated resulting in further activation of downstream signaling cascades where the cytokine tumor necrosis factor alpha (TNF-alpha) activates nuclear transcription factor B (NF-κB), which increases cyclooxygenase 2 (COX-2) expression. TNF-alpha promotes the following: VSMC migration and initiation, adhesion molecule expression in endothelial cells, activation of inflammatory cells, and increases in plasma triglycerides by increasing fatty acids (Figure 1).\(^1\) This is significant in the progression of atherosclerosis and heart disease.\(^1\) COX-2 is a proinflammatory enzyme responsible for producing eicosanoids such as the prostaglandin, PGE2, which cause inflammation, pain, platelet aggregation in blood vessels, and blood pressure shifts. PGE2 is one of the more inflammatory prostaglandins.\(^2\)

**Figure 1**: Mechanism of Inflammation in human vascular smooth muscle cells. Saturated fats trigger the toll like receptor 4 on the membrane. This activates the nuclear transcription factor kappa B which activates tumor necrosis factor alpha. This upregulates cyclooxygenase 2 which creates pro-inflammatory prostaglandins and reactive oxygen species such as superoxide.
Atherosclerosis is manifested by the buildup of lesions in the arterial walls that are related to shear stress. Blood vessel walls are comprised of three layers including: tunica intima, media, and adventitia. The tunica intima consists of a monolayer of endothelial cells as well as the basal lamina that they produce. The media layer is separated by an internal elastic lamina. The tunica media consists of multiple layers of smooth muscle cells surrounded by basal and elastic lamina. The tunica adventitia layer is separated by an external elastic lamina. The majority of this layer consists of fibroblasts, microvessels, mast cells, and lymphatic vessels and nerves.78

The process of plaque formation in the blood vessel walls can be initiated in various ways. Dyslipidemia, pro-inflammatory cytokines, hypertension, and hyperglycemia (products of glycoxidation specifically) are a few major contributing factors to atherosclerosis.78 The initiation of atherosclerosis involves the endothelial cells due to their exposure to blood flow. Increased levels of low density lipoproteins (LDL), C-reactive protein, and TNF-alpha in the plasma lead to the movement of LDL into the tunica intima layer of blood vessels. LDLs are lipid transporters in the blood that contain triglycerides and cholesterol. LDLs can undergo oxidative modification and become modified lipoproteins (MLp) which deposit in the tunica intima. This deposition encourages endothelial dysfunction.9,78,79 An immense inflammatory response follows that includes the entering of monocytes into the tunica intima where they become macrophages, take up MLp, and form foam cells which are characteristic of fatty streaks. Smooth muscle cells migrate from the tunica media to the intima, proliferate and form a fibrous cap to stabilize the plaque (fibro-lipid plaque). The migrating smooth muscle cells
are attracted to the artery walls by platelet-derived growth factor, which is released by activated platelets. As the atherosclerotic lesion forms, the adventitia, outer layer, of the artery is infiltrated with inflammatory cells such as macrophages and t-lymphocytes. During the progression of atherosclerosis, the plaque calcifies, and cell death occurs. This may then rupture leading to thrombosis.\textsuperscript{78}

Arachidonic acid stems from omega-6 (linoleic acid), a proinflammatory essential fatty acid. COX-2 then uses substrates from arachidonic acid to create PGE2, and thus leads to inflammation. Vascular inflammation can also be triggered when arachidonic acid is metabolized, and thus oxidized, creating free radicals\textsuperscript{69}. In addition to the COX-2 pathway, the 5-lipoxygenase pathway increases expression of TNF-alpha and produces leukotrienes which cause inflammation by increasing microvascular permeability.\textsuperscript{11}

Inflammation is also upregulated in endothelial cells via lipopolysaccharides (LPS). LPS comprise the outer membrane of gram negative bacteria. These can potentially move into the vessels and activate an immune response. TLR4 on pericytes (the contractile outer layer of endothelial cells) is triggered and thus upregulates proinflammatory cytokines, chemokines, and induces NF-kB.\textsuperscript{89}

**Palmitate**

One specific fat that is a major contributor to inflammation is palmitate.\textsuperscript{11,12,84} It is a saturated long-chain fatty acid consisting of 16 carbons. It is found in palm oil, palm kernel oil, milk, butter, cheese, and red meat. Fatty acids contain a hydrocarbon chain with a terminal carboxyl group. Saturated fatty acids are solid at room temperature, contain no double bonds between molecules and are, therefore, saturated with hydrogen
molecules. Ingestion of excess fat leads to the storing of fat in adipose tissue in the form of triglycerides, the main form of fat found in food and stored in the body. Triglycerides consist of a glycerol molecule plus three fatty acids. When triglycerides are broken down, the free fatty acids undergo beta-oxidation to produce acetyl-coA. The glycerol is broken down to glucose and then acetyl-coA. The acetyl-CoA then goes on to the citric acid cycle and electron transport chain to produce ATP for energy. Beta oxidation (or fatty acid oxidation) occurs primarily in the mitochondria. This is a multi-step process where fatty acids are broken down to acetyl CoA, which enters the TCA cycle, where it is oxidized and produces CO2.

Free fatty acids (FFAs) are utilized by the body and through metabolism they yield great amounts of ATP. However, increased plasma FFAs negatively affect the vasculature in by causing inflammation. Free fatty acids are released from the adipocytes where they are stored, leading to inflammation, the creation of ROS, and ultimately atherosclerosis. Researchers found that elevated plasma FFAs are associated with increased inflammatory markers as well as endothelial dysfunction, vascular inflammation, and thrombosis in healthy subjects. Fatty acids are more recently found to directly activate receptors on the plasma membrane of monocytes. Research has shown that saturated fatty acids can act as ligands and bind to TLR4 eliciting receptor activation in vascular smooth muscle cells. COX-2 is not only activated by this innate immune response when pathogens (non-sterile inflammation) are present, but is also activated by free-fatty acids (sterile inflammation). Specifically, palmitate has been found to upregulate COX-2. One study identified that palmitate exerts a dose-dependent response on COX-2 in human VSMC. Others demonstrate that palmitate
increases TNF-alpha as well.\textsuperscript{85} Furthermore, palmitate induces inflammation in various other ways. Palmitate may also upregulate inducible nitric oxide synthase (iNOS), an inflammatory enzyme that when activated produces excessive NO in vascular cells.\textsuperscript{15} Palmitate may also alter the morphology of VSMCs.\textsuperscript{11} There is evidence that palmitate increases ROS such as superoxide within endothelial cells via the mitochondrial electron transport chain.\textsuperscript{88} Finally, nicotinamide adenine dinucleotide phosphate (NADPH) aids in the creation of ROS and is created by FFAs in the citric acid cycle.\textsuperscript{19} Specifically, NADPH oxidase is expressed in the vascular wall and is essential for LDL oxidation and thus the production of ROS.

Increased consumption of dietary fats such as palmitate, increases free radical activity and oxidation which leads to inflammation and damage to the body. Therefore, oxidative stress is a prominent factor in CVD mainly due to this inflammatory process.\textsuperscript{16}

**Vascular Smooth Muscle and Endothelial cells**

Vascular smooth muscle cells provide structural integrity for blood vessel walls. They contract and relax to regulate luminal diameter for the purpose of maintaining blood pressure. VSMCs are comprised of an outer cell wall, a plasma membrane, and a protein embedded lipid bilayer that forms a barrier to separate cell contents from the extracellular environment. Peripheral proteins bind to the inner or outer surface of the bilayer. VSMCs evolve through proliferation and migration for the function of long-term structural remodeling by synthesizing extracellular matrix components.\textsuperscript{20}

VSMCs are found in one of two phenotypes, contractile and synthetic. Smooth muscle cell phenotypic changes are modulated by mechanical stimulation, platelet-
derived growth factor, angiotensin II, and interleukins. During inflammation and instances of blood vessel damage, VSMCs switch from the more common contractile form to the synthetic form. In atherosclerosis, the synthetic form of VSMCs exacerbate the disease by narrowing the blood vessel lumen as well as providing substrates for the retention of lipoproteins. Lipoproteins transport triglycerides which contain saturated fatty acids (SFA).

SFAs induce vascular inflammation through their effects on VSMCs. In one study, when exposed to 250 µM of palmitate, rat aortic smooth muscle cell proliferation, migration, and phenotypic change significantly increased. Researchers found this phenotypic change by observing a decrease in the contractile markers: SM alpha-actin and SM22alpha resulting in a more synthetic form. This phenotypic change is significant for atherogenesis. Further, increased levels of SFA are predictive of cardiovascular events.

The endothelium is a simple monolayer of cells lining the lumen of blood vessels. This layer acts as a barrier and is metabolically active. Endothelial cells are important for the transportation of metabolic substrates between the blood and interstitial space. One major role that the endothelium plays is in controlling vasodilation and vasoconstriction. When the endothelium becomes dysfunctional, a plethora of complications occur including excessive oxidative stress, increased blood pressure, inflammation, cell proliferation, atherogenesis, thrombosis, and altered lipids. Endothelial dysfunction is an early stage of atherosclerosis in that it upregulates NF-κB and other inflammatory pathways. Similar to vascular dysfunction, endothelial dysfunction can occur due to various circumstances. With a high fat diet, cytokines are released from adipose tissue
and can become oxidized creating ROS. An accumulation of ROS in the vasculature contributes to increased vasoconstriction by reducing the bioavailability of NO when it is scavenged by superoxide.\textsuperscript{2} Chronic inhibition of NO leads to endothelial damage and eventually CVD.\textsuperscript{95} During times of elevated blood glucose, proteins and lipids can be non-enzymatically glycated and produce advanced glycation end products (AGEs) which cluster on the vessel walls (Wautier). The presence of ROS leads to leukocyte attraction and adhesion to the vessel walls as well. The vessels become stiff and contribute to endothelial dysfunction as seen by NO resistance as well as a reduction in cGMP-dependent protein kinase-1, which plays a role in vasodilation.\textsuperscript{96}

**Nitric Oxide Synthase (NOS)**

Nitric oxide synthase (NOS) is an enzyme that produces NO, which is significant for modulating vascular tone. There are three isoforms of NOS including inducible, endothelial and neuronal. Inducible NOS (iNOS), is expressed when induced by cytokines and remains constantly active, releasing an abundance of NO. Thus, when the body is in a state of inflammation, iNOS produces a fair amount of NO which, when over-produced, causes the body harm through excessive vasodilation and cell damage.\textsuperscript{24}

NOS is also found as neuronal NOS (nNOS) and expressed in neurons in the brain. In the central nervous system, nNOS plays a role in central control of blood pressure. In the peripheral nervous system it acts as a neurotransmitter mediating the relaxation of gut peristalsis and vasodilation.\textsuperscript{24}

Endothelial NOS (eNOS) expressed in endothelial cells. NO derived from eNOS dilates blood vessels by entering the VSMC to activate soluble guanylyl cyclase (SGC)
which converts GTP to cyclic GMP and cAMP. Finally, elevated cAMP results in blood vessels dilation. In the vascular lumen eNOS provides protection against platelet aggregation and adhesion on the vascular cell walls. Further, it prevents VSMC proliferation and creation of matrix molecules by inhibiting the release of platelet-derived growth factor. eNOS protects against early stages of atherosclerosis by reducing the expression of the chemoattractant protein MCP-1 gene involved in leukocyte adhesion to vascular and endothelial cells as well as leukocyte migration into the vascular wall. eNOS protects against later stages of atherogenesis as it prevents VSMC proliferation and migration. When there is an increase in oxidative stress there is a reduction in eNOS, thus decreasing endothelium-dependent vasodilation. The increases the susceptibility of the vessels to plaque formation.

Generation of NO involves removal of electrons from NADPH via flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This occurs in the carboxy-terminal reductase domain where electrons are transferred to the heme as a part of the amino-terminal oxygenase domain. This is where L-arginine binding occurs. The electrons from NADPH are used to oxidize L-arginine to L-citrulline in the endothelial cells via eNOS to create NO.

During times of inflammation, when monocytes and macrophages are present, eNOS goes through a process called NOS uncoupling. This process is characterized by the action of NADPH increasing superoxide (O2-) which reacts with NO forming peroxynitrite (ONOO-). ONOO- aids in the oxidation of tetrahydrobiopterin (BH4), an eNOS cofactor, thus reducing its availability for eNOS. eNOS is then uncoupled and NO production decreases. Further, in endothelial cells, iNOS competes with eNOS for BH4.
Finally, asymmetric dimethylarginine (ADMA) is heightened after eating a high fat meal and ADMA competes for NOS, which leads to vascular dysfunction due to the decreased bioavailability of NO.\textsuperscript{23}

**Oxidation**

Reactive oxygen species are free radicals that cause oxidative stress. Superoxide and malondialdehyde are ROS and thus markers of oxidative stress. Xanthine oxidase, NADPH oxidases (Nox1 and Nox2), uncoupled eNOS, and the mitochondrial respiration chain can all produce superoxide in the vascular wall.\textsuperscript{19} Xanthine oxidase from the mitochondria allows hypoxanthine and xanthine to come together to create superoxide.\textsuperscript{30} Superoxide can be converted to hydrogen peroxide which can then spontaneously convert to a hydroxyl radical. Hydroxide (OH\textsuperscript{-}), in particular, is a very strong oxidant that has the potential to degrade cells in the body. As previously mentioned, superoxide can react with NO to form ONOO\textsuperscript{-}. These are just a few of the many ROS relevant to the vasculature.\textsuperscript{24} Xanthine oxidase adheres to endothelial cells\textsuperscript{97} and its activity increases in the presence of atherosclerosis.\textsuperscript{98} Further, inhibition of xanthine oxidase increases NO-mediated vasodilation in hypercholesterolemic animal subjects.\textsuperscript{97}

Lipids can be oxidized. Lipid peroxidation is mediated by various mechanisms some of which include: NADPH oxidases, uncoupled eNOS, cyclooxygenases, and cytochrome P450. Potential end products include hydroxy-fatty acids and oxysterols. Another by product is malondialdehyde, a highly reactive oxidant.\textsuperscript{24}

Hydroxyl radical production initiates lipid peroxidation.\textsuperscript{26} Low density lipoprotein particles pose a threat to overall health as heightened incidence of LDL oxidation is
correlated with increased risk of CVD. Clinical trials have shown that when LDL are decreased, the incidence of cardiovascular events is decreased as well.

Various cardiovascular risk factors contribute to vascular oxidative stress and reduced NO production. Hyperlipidemia, hypertension, and hyperglycemia are a few of the major risk factors. These pathologies lead to the activation of NADPH oxidase, thus leading to eNOS uncoupling and therefore decreased NO. Hyperglycemia also leads to NADPH oxidase activation by first stimulating mitochondrial ROS production. Malondialdehyde, specifically, is more abundant in type 2 diabetes mellitus (T2DM). Not only does hyperglycemia lead to eNOS uncoupling via NADPH oxidase but also through a mechanism that decreases L-arginine. Hypertension and hyperlipidemia lead to the same reaction as well. Hyperlipidemia not only leads to the reduction of NO via eNOS uncoupling but also by directly reducing eNOS activity which decreases NO production.

**Antioxidants**

The human body can host many free radicals that cause oxidative stress and can lead to inflammation and cardiovascular disease. The process of oxidation first requires a substrate. Subsequently, it needs a free radical, an initiator, intermediate products, and final products. Altogether, oxidation is divided into initiation, propagation, and termination reactions. Antioxidants are imperative to the survival of all human cells. Antioxidants are beneficial in that they halt the propagation step. They may act as a reducing agent where they donate hydrogen, by interrupting the peroxyl radical chain, or by inhibiting the formation of singlet oxygen. Antioxidants scavenge free radicals to
donate a hydrogen atom for the purpose of stabilizing and deactivating them. The
initiation step is propelled by singlet oxygen or free radicals for example. It is the
creation of free radicals. The propagation step is characterized by the formation of peroxy
radicals. The number of free radicals does not increase, however, the free radicals form
into more harmful ROS. Termination is when free radicals are eradicated such as when a
peroxy radical interacts with an antioxidant. Natural antioxidants are typically derivatives
of flavones, isoflavones, flavonols, and catechins, for example.99

A great number of free radicals in the body can result in the weakening of
antioxidant enzymes like superoxide dismutase and glutathione peroxidase. After the
formation of superoxide, superoxide dismutase (SOD) converts it to H2O2, which is then
converted to O2 and H2O via a catalase.31 Glutathione peroxidase is an antioxidant
produced by the body that is made up of amino acids. It is a major source of antioxidant
activity in most cells and has the ability to convert ONOO-, hydrogen peroxide (H2O2),
and linoleic acid hydroperoxide (LOOH) into less harmful substances. The body needs
these antioxidants to function correctly and to prevent cell damage. Ultimately, oxidative
stress is caused by an increased number of free radicals, a depression of antioxidant
enzymes, and/or a lack of dietary antioxidant consumption.24

**Sumac Origin**

Sumac is a spice that is very prevalent and grows wild in the Mediterranean and
Middle East. It is also grown and consumed in the United States. Sumac is mainly
prepared by drying the fruits and grinding them down to a powder. However, sumac can
also be consumed as an extract or by consuming the leaves.32 Sumac is part of the *Rhus*
genus, which is within the cashew family, also known as, Anacardiaceae. The word sumac comes from “sumaga,” meaning red in Syriac. There are about 250 species of sumac in the *Rhus* genus. This genus is not to be confused with poison sumac, which should not be ingested.\(^3\)\(^4\) Within the *Rhus* genus, there are some species that are considered ornamental and should also not be consumed. *Rhus coriaria* L., is a nonornamental species and is commonly consumed. It is characterized by a shrub-like appearance and bears clusters of red, fuzzy berries.\(^3\)\(^2\) Much of the current research on sumac involves *Rhus coriaria* L., however there are some studies conducted using *Rhus hirta*, a much less available species but also a species that can be consumed.

Across the globe, a multitude of spices, made from plants including *Rhus coriaria* L., are used in cooking to add flavor and color. Further, there has been a long-time belief that spices such as *Rhus coriaria* L. can contribute to health benefits by alleviating symptoms associated with diabetes, diarrhea, dysentery, inflammation, cancer, stroke, and oral-diseases. *Rhus coriaria* L. has also been used as an antimicrobial, antifungal, antiviral, and antioxidant agent.\(^3\)\(^5\)

**Constituents of Sumac**

The health benefits of sumac may be related to its makeup of oleic fatty acids, organic acids (gallic and phenolic acid), nitrates, and antioxidants as well as its ability to alter carbohydrate digestion.\(^3\),\(^1\)\(^6\),\(^3\)\(^2\)-\(^3\)\(^4\),\(^3\)\(^6\)-\(^3\)\(^8\) Using high performance liquid chromatography (HPLC), researchers have found that sumac contains 211 phytochemical compounds including: tannins, anthocyanins, isoflavonoids, flavonoids, phenols and organic acids.\(^3\)\(^5\)
Antioxidant qualities are present in the fruit, leaves, and whole plant in both water and methanol extracts.65

**Antioxidant role of sumac**

The antioxidant qualities of sumac can play a major role in human health. An *in vitro* study of isolated primary human lymphocyte cells suggested that sumac has enormous antioxidant qualities that appear to protect against oxidative DNA damage. In this study an ethanolic sumac extract was used. Human subjects consumed 3 grams of sumac over 3 days. Following the isolation of the cultivated lymphocytes, they were exposed to various sumac extract concentrations including 10, 20, and 40 µg/mL for 1 hour. Hydrogen peroxide induced DNA migration was significantly decreased by 30%. The cells showed a dose-dependent response where comet formation was decreased as sumac was increased.39 A comet assay, also known as a single cell gel electrophoresis, is used to measure and evaluate DNA damage. This is seen through fluorescent staining where the damaged DNA separates from the intact DNA and forms a comet tail.77 Researchers find that an ethanol extract of sumac has greater antioxidant activity than butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and alpha tocopherol when looking at 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays37,38, a free radical commonly used to measure how compounds behave as antioxidants. Total antioxidant status (TAS) was increased in a study conducted on adult male rabbits that were fed sumac powder for 3 months. With a sample of blood, researchers measured TAS with an assay kit that measures the ability to fight an oxidant that is introduced.34 Another study found that sumac leaf extract had an antioxidant effect on chondrocyte cells, cells found in cartilage, which maintains the matrix.42 Tannins were found in sumac as well and have
been shown to have an anti-carcinogenic effect.\textsuperscript{34,43} Hydrolysable tannin derivatives were the most abundant antioxidants found in sumac fruits.\textsuperscript{44} It was also found that tannins inhibited VSMC migration in rats. This was concluded after a 10-day trial where researchers extracted tannins from sumac powder and exposed the sumac to rat cells \textit{in vitro}.\textsuperscript{16} Inhibition of VSMC migration is significant for the pathogenesis of atherosclerosis.\textsuperscript{16} The tannins in sumac were found to be gallotannins. These have anti-inflammatory abilities seen by: the scavenging of ROS, the depression of inflammatory mediators (COX-2), and the role it plays on eNOS.\textsuperscript{45} Since eNOS facilitates vasodilation, gallotannins may be linked to decreased blood pressure.

Major aspects that affect blood pressure are related to oxidation.\textsuperscript{65} Reducing blood pressure greatly contributes to the atherosclerotic protection sumac offers. A previous study showed an increase in oxidative stress in rats fed a high sugar and high fat diet. Researchers concluded that this may be directly related to NO bioavailability and the significantly attenuated endothelial-dependent vasodilation observed.\textsuperscript{52,53}

\textit{Rhus coriaria L} has been shown to decrease superoxide, xanthine oxidase, and malondialdehyde.\textsuperscript{7,66,67} Superoxide and xanthine oxidase were combined and exposed to sumac powder, which resulted in the scavenging of superoxide and inhibition of xanthine oxidase, a mechanism to produce superoxide.\textsuperscript{66,67} Sumac extract inhibited xanthine oxidase seen by a decrease in the amount of uric acid formation since xanthine is normally converted to uric acid. Sumac also scavenged superoxide as evidenced by the reduction of nitroblue tetrazolium (NBT). The enzymatic assay of superoxide radicals showed that the xanthine-xanthine oxidase system generated superoxide radicals and was determined spectrophotometrically by monitoring the reduction of NBT.\textsuperscript{66,67} It was found
that a different, less common species of sumac, *R. hirta*, had greater antioxidant activity than vitamin C or green tea. Sumac extract (10g powdered sumac to 210mL methanol) mixed with DPPH also reduced superoxide.⁷ A later study found that sumac extract was capable of increasing SOD in rats. This study also found that postprandial blood glucose levels were improved by 24% and improved levels of HDL, TG, TC, and LDL were also observed in alloxan-induced diabetic Wistar rats. Rats were fed either 200 or 400 mg/kg BW of sumac where they averaged 0.2-0.25 kg body mass.⁶⁸

**Oleic acid composition in sumac**

Sumac reportedly contains oleic acid, a monounsaturated fatty acid.⁶² It has been shown to normalize fasting blood glucose and improve blood circulation by restoring vasodilation. Results from a four month-long study done in Ireland on 11 male adults with type 2 diabetes mellitus (T2DM), showed a decrease fasting blood glucose in subjects on the oleic acid-rich diet as well as a marked increase in flow-mediated dilation.⁶³ This was due to a decrease in HMG-CoA reductase.² The vasodilatory response is impaired in people with diabetes due to insulin resistance.⁵ This occurs as insulin exerts vasodilatory effects through upregulation of eNOS-derived NO. In another study, oleic acid was shown to prevent mitochondrial dysfunction, inflammation, and insulin resistance induced by palmitate exposure. The ability of oleic acid to decrease superoxide aids in its protective effect as well.⁶⁴ Sumac has also been found to contain nitrates which lead to the formation of NO. This is similarly responsible for helping to control blood pressure.²³
Assessment of Cardiovascular Function

It is imperative to correctly identify risk of cardiovascular events. This can be done by accurately measuring blood pressure as well as utilizing various blood pressure tests.

Central Blood Pressure

The ASCOT study showed that blood pressure is best measured by a central blood pressure test versus the traditional peripheral test. Researchers even found that peripheral blood pressure tests are not predictive of cardiovascular events, whereas central blood pressure tests were. The traditional peripheral test is conducted with a blood pressure cuff wrapped around the arm, inflated, and blood pressure is then read. The central blood pressure test is a little more extensive. Currently, there is a non-invasive method for conducting this test. A blood pressure cuff is also used along with SphygmoCor XCEL technology and aortic pressure is measured.

Flow Mediated Dilation

Alongside the central blood pressure test, an endothelial-dependent dilation of the brachial artery can be conducted, referred to as flow mediated dilation (FMD). FMD is a non-invasive alternative to measure endothelial function by the use of ultrasound. FMD measures the arterial diameter as blood flow increases and decreases. A sphygmomanometer cuff is applied to a person’s arm and inflated to put pressure on the arteries and prevent blood flow. It is then deflated to restore blood flow resulting in a hyperemic vasodilation response. When arteries are constricted, the flow of oxygen is decreased. Reactive hyperemia occurs at this point and is characterized by increased blood flow in order to deliver oxygen to tissues that have been deprived.
happening is that increased blood flow and shear stress on the vessel wall stimulates the release of NO which dilates the arteries. Vasodilation, as well as the blood flow are not as reactive, due to ischemia, in people with CVD. Therefore, an FMD test would be unfavorable in people with coronary artery disease. FMD results are also abnormal in people with diabetes.

**Beneficial Effects of Sumac**

In various studies, it has been identified that sumac plays a role in decreasing blood pressure and blood glucose.

*Sumac-mediated reduction in blood pressure*

One study evaluated the cardioprotective activity of sumac leaves in isolated rabbit aorta rings. This study examined the effect of sumac on post-ischemic myocardial damage, vasodilation of isolated rabbit aorta, as well as the content of antioxidants and antioxidant activity with DPPH. The focus was on the presence of gallotannins and the ability of sumac to relax the aortic rings after an induced vasocontraction with norepinephrine both with and without endothelium. The dose of sumac used was 150-500 µg/mL. A reduction in TNF-alpha was only significant at 500 µg/mL. Because vasodilation was lost in endothelium-denuded aortic rings, sumac-mediated vasodilation is endothelium-dependent. Antioxidant activity was observed to be greater than that of gallic acid and ascorbic acid. According to this study, sumac may aid in cardiovascular protection by: preventing prostacyclin activation, activating eNOS, inhibiting TNF-alpha, and scavenging ROS. The mechanism proposed to reduce
inflammation is that in response to an ischemic event, the endothelium is triggered to generate prostacyclin in order to reduce the responsiveness to constriction.\textsuperscript{61}

Initial research has been conducted on the effect of \textit{Rhus coriaria L.} on blood pressure. This double blind randomized controlled trial found a reduction in peripheral blood pressure with 80 hypertensive human subjects after an eight-week trial where they supplemented 1000 mg of sumac powder per day. This is the only human trial that has measured the effect of sumac on blood pressure but did not use the gold standard central blood pressure test or FMD, as previously mentioned, which is dependent on NO.\textsuperscript{59} Thus further clinical trials should be conducted.

\textbf{Sumac-mediated Reduction in Blood Glucose}

Various studies have been conducted on subjects with T2DM with positive results on blood glucose in the sumac-fed groups. One study administered either 50, 100, or 250 mg/kg body weight (BW) of a sumac water extract to alloxan-induced diabetic rats and found improved blood glucose control as well as decreased amounts of malondialdehyde.\textsuperscript{28} A more recent study observed an improvement in blood glucose and HgbA1c as well as total cholesterol, triglycerides, high density lipoprotein (HDL) and LDL in streptozotocin-induced diabetic rats when given either 100, 250, or 500 mg/kg BW of a water extract of sumac for 21 days. The greatest improvement in blood glucose was seen in a dose of 250 mg/kg BW of sumac. Further, after dissection, researchers found a decreased amount of malondialdehyde and increased superoxide dismutase activity in the sumac-fed groups.\textsuperscript{60} A study conducted on people with T2DM were given 3,000 mg of sumac powder daily for 3 months. This study resulted in a significant decrease in blood glucose with sumac treatment.\textsuperscript{5} One study looked at alpha amylase
activity and sumac extract in a cell-free model. Researchers observed alpha amylase inhibition when glucose was exposed to sumac. Alpha amylase breaks down starch into simpler sugar molecules. In humans, this enzyme is not the cause of diabetes but, when inhibited, it is believed that there is better blood sugar control. They found that alpha amylase was inhibited 48% with a methanolic extract of sumac. There was an even greater inhibition, 87%, with an ethyl acetate extract. They found, with TLC analysis, that sumac contains tannins, flavonoids, and terpenoids and these may be the reason alpha amylase was inhibited. This is very relevant for people with T2DM if sumac can prevent alpha amylase activity and thus display better blood sugar control. Further, alpha glucosidases, which also alter carbohydrate digestion, were found to be decreased in a sumac intervention trial.

As mentioned, sumac also contains organic acids including gallic and phenolic acids which play a role in lowering blood glucose and cholesterol in diabetes.

**Sumac-mediated Improvements in the Lipid Panel**

Due to its immense antioxidant qualities, sumac may provide protective effects against atherosclerosis. This is seen in many studies where cholesterol is decreased. It is proposed that isoflavones effectively reduce total cholesterol through heightened LDL receptor activity and LDL catabolism in the liver.

A randomized clinical was conducted on 80 participants with a diagnosis of hyperlipidemia. They were fed 1000 mg sumac daily for 2 months and resulted in improvements in high density lipoprotein (HDL), a marker of cardiovascular health. A study conducted on hypercholesterolemic rats that were fed 100 or 200 mg/kg of sumac extract daily, resulted in decreased total cholesterol and triglycerides. With similar
results, another study was done on 72 obese adolescents with dyslipidemia. They were fed 1,500 mg of sumac powder daily. After one month, there was a decrease in total cholesterol, triglycerides, and low-density lipoprotein. Another study was conducted as a double blind randomized controlled trial where 41 subjects with type 2 diabetes mellitus were given 3,000 mg of sumac powder per day for three months. The results were: a decrease in blood glucose, ApoB, HbA1c, and increased ApoA-i. It was discussed that sumac lowered the ratio of ApoB/ApoA-i, which is a more important marker of atherosclerosis than low density lipoproteins and high-density lipoproteins.

Apolipoproteins are major factors in the development of heart disease because they are the structural parts of lipoproteins which transport lipids and cause atherogenesis. This ratio specifically tells you the amount of atherogenic versus anti-atherogenic lipoproteins (that’s low-density lipoprotein vs high density lipoprotein). The higher the value of this ratio leads to a higher outcome of atherogenesis.

**Sumac-mediated Reduction in Lipid Peroxidation**

One study found that sumac reduced lipid peroxidation by inhibiting linoleic acid in ferric thiocyanate almost in an equal fashion as BHT. Tannins and phenols reverse lipid peroxidation. It was found that sumac inhibited lipid peroxidation in an *in vitro* study in a similar capacity as tannins. One study analyzed the impact of a high fat diet combined with sumac supplementation on postprandial fat oxidative stress in rabbits (n=24). Results showed lower blood glucose levels, as well as lower LDL and total cholesterol.
CHAPTER 3
MATERIALS AND METHODS

Cell Culture Model and Reagents

Primary human aortic vascular smooth muscle cells (HAoVSMC) were purchased from Lonza (City, State). The donor cells were from a 22-year-old male, verified by positive gene expression for the sex-determining on region Y protein (SRY-1), a gene that enables the synthesis of the male sex-determining protein (Cells: CC-2571, Lot: 0000335663). Media were complemented with 5% fetal bovine serum (FBS)/DMEM/F12 plus growth factors. Cells were cultured in 100 mm plates until they reached a 70-80% confluency.

Cells were grown starting from passage 4 (P4), passaged 3 times (P5,6,7), and at P7, the cells were treated. The cells at P4 were grown in one plate. They were then split into 5 plates for P5. The cells were then split into 20 plates for P6. The cells were then split into 80 plates for P7. These plates were treated with one of the following: Vehicle 1 (BSA), Palmitate (100 µM), Sumac 10 µg/mL, Sumac 20 µg/mL, Sumac 40 µg/mL, Palmitate (100 µM) + Sumac 10 µg/mL, Palmitate (100 µM) + Sumac 20 µg/mL, or Palmitate (100 µM) + Sumac 40 µg/mL. Remaining plates were used for a different study.

Bursal et al. found that a dose of sumac of EC50 36 µg/mL was effective at scavenging superoxide. Based on their findings, the current study used doses of sumac both lesser and greater than 36 µg/mL.
Preparation of *Rhus coriaria* Water Extract

Bursal et al. found that the total reducing power of sumac water extract is more potent than an ethanol extract in scavenging DPPH and DMPD free radicals. The water extract was also found to contain more phenols and flavonoids. The lyophilized water extraction was prepared by Dr. Anne Jones (Chemistry Department at Arizona State University)- and Christina Forbes (Graduate Student at Arizona State University). Then added 25 g sumac (*R. coriaria* L.) to 250 mL distilled water, then boiled the mixture for 10 minutes with a magnetic stirrer, filtered it with filter paper, then the filtrates were frozen and lyophilized in a lyophilizer at 5 mm Hg pressure at -50 degrees Celsius (Labconco, Freezone 1). This created a lyophilized water extract of sumac. The lyophilized sumac was then stored at -20c. When used it was reconstituted with 10, 20, 40, 60, and 80 µg/ml in cell culture media or physiological saline solution for isolated blood vessel studies.

**Palmitate-induced cell stress**

To induce stress, HAoVSMC were treated with high fat exposure with 100 µM palmitate in 5% BSA vehicle. A stock palmitate (Pal) solution was made with 100 mM palmitate in 50% EtOH or 27.8 mg in 1 mL 50% EtOH. 1 mL of pal stock was added to 9 mL 5% BSA. Of this solution, 80 µL was added to each appropriate plate. The control was 100 µL of 50% EtOH in 900 µL 5% BSA in water. 80 µL was added to each appropriate plate. This concentration of palmitate was used based on the study done by Raman and Gonzales, 2018. 100 µM of palmitate was an effective dose to cause an increase in COX-2 expression and thus PGE2 production.
Cell culture prevention study

Sumac was added 30 minutes prior to the exposure of palmitate in order to dissolve the sumac in the media. Sumac and palmitate were then exposed for 18 hours using the following concentrations of sumac: 10, 20, and 40 µg/mL.

Cell culture reversal study

Sumac was introduced during the last three hours of treatment with the following concentrations of sumac: 40 ug/mL, 60 ug/mL, or 80 ug/mL. The cells were treated with palmitate for 18 hours and sumac was introduced during the last three hours of the palmitate treatment. Greater concentrations of sumac were used in the reversal study to evaluate the most effective dose of sumac.

Preparing for homogenization

Following the treatments, cells were lifted with trypsin. The suspension was collected and, via homogenization, the proteins were isolated with ice-cold lysis buffer and stored at -20°C. The buffer for homogenization contained 50 mM Tris Base, 150 mM NaCl, 0.04% SDS, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 25 mM β-glycerophosphate, 100 µM Na₃VO₄, and the protease inhibitors 1 mM DTT, 20 µM Pepstatin, 20 µM Leupeptin, and 0.1 μg/mL Aprotinin.

Whole Cell Lysate Western Blot Analysis

Western blotting is a technique used to probe for protein expression in cell lysates. When running a western blot test, a loading control needs to be used to accurately identify protein expression. When using a loading control antibody, it can be evaluated if
the samples were loaded equally in all wells. Further, it can be ensured that, during the western blot testing, proteins were transferred properly. Beta actin is an appropriate loading control since it has a much different molecular weight when compared to COX-2 (42 kDa and 75 kDa, respectively). It is a generally accepted loading control since the protein levels do not significantly vary during sample treatments. Further, it is appropriate because beta-actin is a protein found in humans. Typically it can be expressed at the same level within the body. There are 6 actin isoforms in humans in which play a role in cell motility, structure, and integrity. Beta actin is a non-muscle cytoskeletal actin.

To perform the Western blot, a bicinchoninic acid assay (BCA) was used to determine the protein concentration of each sample. Protein, in the amount of 35 µg was loaded into each lane of the acrylamide gel (Table 1). The samples included sample buffer comprised of a 1:0.1 µL concentration of tris-glycine SDS and beta-mercaptoethanol. To perform the gel electrophoresis, an 8% acrylamide gel was used. The gel is comprised of an upper gel (stacking gel) plus a lower gel (separating gel). The gel is comprised of water, acrylamide, TEMED, and 10% APS in water, lower gel buffer, and upper gel buffer. The lower gel buffer contains trizma base (1.5 M concentration) and sodium dodecyl sulfate (SDS of 0.4% M concentration) and has a pH of 8.8. The upper gel buffer contains Tris HCL (0.5 M concentration) and SDS (0.4% M concentration) and has pH of 6.8. The charge of the tris base in the lower gel allows the protein to run through the upper gel in a straight line. The lower gel is where the proteins separate based on size and weight. To determine the size of the protein detected, a molecular marker (Bio-Rad) was loaded into lane 4. To begin the electrophoresis, the gel was run at 85 V for the first 25 minutes, and then increased to 120 V for 60 minutes in running buffer.
containing trizma base, glycine, and SDS with the following concentrations: 0.25 M, 1.9 M, and 1%, respectively. This allows the proteins to stack tightly as they run through the upper gel. The transferring process required the protein lysates to be transferred to a nitrocellulose membrane by running for 60 minutes at 0.25 A in transfer buffer containing tris-base and glycine with the following concentrations: 0.25 M and 1.9 M, respectively. The membrane was rinsed twice with enough tween-20 phosphate buffered saline (TPBS) to layer the bottom of the container used to block the membrane. TPBS stock solution was made with 50 mL 10X PBS (containing 40 g NaCl, 1 g KCl, 7.2 g Na₂HPO₄, and 1.2 g KH₂PO₄ in 500 mL of distilled water), 450 mL distilled water, and 500 µL Tween-20. Blocking buffer containing 3% skim milk was used to block the membranes for 60 minutes. Blocking the membrane is for the purpose of preventing the antibodies from sticking to the open spaces on the membrane. Primary antibodies were then exposed to the membranes overnight at 4°C. COX-2 (160126, Cayman Chemical, Ann Arbor, Michigan) and Beta-Actin (A5441, Sigma Aldrich, St. Louis, Missouri) were the two primary antibodies used. The beta-actin primary antibody was used to verify equal loading of protein. The primary antibodies were diluted in TPBS (see Table 2 for manufacturer information and dilutions). Then, the membranes were washed 5 times for 5 minutes each time and then exposed to the secondary antibodies for 60 minutes (refer to Table 2 for manufacturer and dilutions). The membranes were then washed again. An Odyssey Classic infrared imager (Li-Cor) was used to visualize the proteins. ImageStudio 3.0 software (Li-Cor) was used to analyze the data. The infrared imager detects fluorescence from the secondary antibody so that each protein of interest can be differentiated.
Table 1: The Western Blot Loading Sheet for Reversal Study:

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<th>(µg/µl) prepped sample</th>
<th>(µl) volume loaded</th>
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<td>16</td>
<td>32</td>
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<td>32</td>
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<tr>
<td>11</td>
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<td>1.167</td>
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<tr>
<td>12</td>
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<td>10.7</td>
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<td>1.167</td>
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<td>1.167</td>
<td>30</td>
<td>35</td>
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This table is per the Gonzales lab protocol for Western Blot loading
Table 2: List of antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody Information</th>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
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<tbody>
<tr>
<td><strong>Host</strong></td>
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<td>Goat Anti-Rabbit</td>
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<tr>
<td><strong>Vendor</strong></td>
<td>Cayman Chemical</td>
<td>Li-Cor</td>
</tr>
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<td><strong>Catalog</strong></td>
<td>160126</td>
<td>926-32211</td>
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<td><strong>Lot</strong></td>
<td>0431166-1</td>
<td>C60321-05</td>
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<tr>
<td><strong>Dilution</strong></td>
<td>1:500 in TPBS</td>
<td>1:15000 in blocking buffer</td>
</tr>
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Antibodies used for Western Plot Analysis. The dilution is uL:uL.

Endothelium-dependent Vasodilation

For 10 weeks, male Sprague-Dawley rats (6 weeks old) were fed a standard rodent chow (Harlan Teklad) or a high fat diet (HFD; Cat No. 12492, Research Diets Inc.) comprised of 60\% kcal from fat. Following the 10-week feeding protocol, the rats were given an overdose of sodium pentobarbital of 200 mg/kg body mass to euthanize them. In a Silastic-coated dissection dish, the mesenteric arcade was isolated and pinned out. This dish contained ice-cold HEPES-buffered saline solution (in mM: 134.4 NaCl, 6 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, 10 glucose, pH 7.4 with NaOH). Small resistance mesenteric arteries with an inner diameter averaging 121 μm were isolated. They were then cannulated with glass pipettes and, in a vessel chamber (Living Systems, CH-1), they were tied with silk ligatures. The vessel chamber contained HEPES-buffered saline.
solution and the chamber was transferred to an inverted microscope stage. The intraluminal artery pressures of all vessels were maintained at 60 mmHg. This was accomplished with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT). At a rate of 10 mL/min the isolated vessels were equilibrated for 15 minutes with warm (37°C) physiological saline solution (in mM: 129.8 NaCl, 5.4 KCl, 0.5 NaH₂PO₄, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose) and were aerated (21% O₂, 6% CO₂, balance N₂ gas mixture). Subsequently, in the superfusate, vessels were incubated for 30 minutes with this solution with or without sumac extract (80 µg/mL). Sumac was present throughout the dose-response curve. Then, increasing concentrations of phenylephrine were introduced to the vessels in the superfusate (10⁻⁹ to 10⁻⁵ M) in order to achieve 50% constriction of the resting inner diameter. Then the vessels were introduced to increasing concentrations of acetylcholine (10⁻⁹ to 10⁻⁵ M, 3 minutes per cumulative dose). Passive inner diameter was obtained by exposing vessels to a calcium-free physiological saline solution (calcium chelator 3mM EGTA) for 15 minutes following the acetylcholine dose response curve.
CHAPTER 4

STATISTICS

SigmaStat 3.0 software (Systat Software, San Jose, CA) was used for statistical analyses of endothelium-dependent vasodilation. Data is displayed as the mean +/- SEM and arcsine-transformed data were analyzed by Two-way RM Analysis of variance (ANOVA) with Tukey posthoc analyses. The data was expressed as a percent reversal of phenylephrine-induced tone. A p-value of <0.05 was considered statistically significant for all comparisons.
CHAPTER 5

RESULTS

Cell Culture Prevention Study

The Western analysis for the sumac prevention study (where sumac was exposed 30 minutes prior to palmitate) showed that palmitate, as predicted, increased COX-2 protein levels. However, sumac alone had no effect on COX-2 levels. Levels of COX-2 in cells treated with sumac plus palmitate, were not difference as compared to cells treated with palmitate alone (Figure 1). COX-2 protein expression appeared as doublets as COX-2 comes in at both 72 and 74 kDa, with 74 kDa being the active form of COX-2.

Figure 2: Representative blot of antiCOX-2 levels in the prevention experiments where human aortic vascular smooth muscle cells were exposed to vehicle, palmitate, sumac and palmitate plus sumac for 18 hours. COX-2 is shown in green. Beta-Actin is shown in red at the bottom. Molecular marker is shown in red on the left in the first lane. The lanes are identified as follows from left to right: Vehicle, Palmitate, Sumac 10 ug/mL, Sumac 20 ug/mL, Sumac 40 ug/mL, Palmitate (100 uM) + Sumac 10 ug/mL, Palmitate (100 uM) + Sumac 20 ug/mL, Palmitate (100 uM) + Sumac 40 ug/mL.
To quantitate COX-2 protein levels, the COX-2 signal intensity was normalized to beta actin (loading control) by taking the COX-2 signal intensity and dividing it by the beta actin signal intensity (Table 3). These values are plotted in Figure 2.

Table 3: Signal Intensity of prevention study for COX-2 and Beta Actin

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>COX-2 Signal Intensity</th>
<th>Beta Actin Signal Intensity</th>
<th>COX-2 Normalized to Beta Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>247000</td>
<td>4090000</td>
<td>0.06</td>
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<tr>
<td>Palmitate</td>
<td>130000</td>
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<td>Sumac 10 ug/mL</td>
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<td>3520000</td>
<td>0.07</td>
</tr>
<tr>
<td>Sumac 20 ug/mL</td>
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<td>0.06</td>
</tr>
<tr>
<td>Sumac 40 ug/mL</td>
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<td>Palmitate + Sumac 10 ug/mL</td>
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<td>3590000</td>
<td>0.37</td>
</tr>
<tr>
<td>Palmitate + Sumac 20 ug/mL</td>
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<td>4170000</td>
<td>0.24</td>
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<tr>
<td>Palmitate + Sumac 40µg/mL</td>
<td>1400000</td>
<td>3920000</td>
<td>0.35</td>
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</table>
Figure 3: COX-2 protein intensity of prevention study where human aortic vascular smooth muscle cells were exposed to vehicle, sumac, or palmitate alone and in palmitate plus sumac. This graph depicts the COX-2 protein intensity in each lane of the Western Blot. The lanes are identified as follows: Vehicle, Palmitate, Sumac 10 ug/mL, Sumac 20 ug/mL, Sumac 40 ug/mL, Palmitate (100 uM) + Sumac 10 ug/mL, Palmitate (100 uM) + Sumac 20 ug/mL, Palmitate (100 uM) + Sumac 40 ug/mL.

Cell Culture Reversal Study

The Western Blot analysis results for the sumac reversal study (where sumac was exposed for the final three hours of palmitate exposure) showed that cells without palmitate had similar levels of COX-2 as the cells with sumac plus palmitate. Increased concentrations of sumac, had higher levels of COX-2. Except the sumac 60 ug/mL
sample showed a decreased level of COX-2 protein expression. COX-2 protein expression appeared as doublets as COX-2 comes in at both 72 and 74 kDa.

**Figure 4:** COX-2 image of reversal study where human aortic vascular smooth muscle cells were exposed to palmitate for 18 hours and sumac was exposed for the final 3 hours of the palmitate exposure. COX-2 is shown in green. Beta-Actin is shown in red at the bottom. Molecular marker is shown in red on the left in the first lane. The lanes are identified as follows: Vehicle, Palmitate, Sumac 40 ug/mL, Sumac 60 ug/mL, Sumac 80 ug/mL, Palmitate (100 uM) + Sumac 40 ug/mL, Palmitate (100 uM) + Sumac 60 ug/mL, Palmitate (100 uM) + Sumac 80 ug/mL.

**Table 4:** Signal intensity of reversal study for COX-2 and Beta Actin

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>COX-2 Signal Intensity</th>
<th>Beta Actin Signal Intensity</th>
<th>COX-2 Normalized to Beta Actin</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
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<td>Palmitate</td>
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<td>0.03</td>
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<tr>
<td>Sumac 40 ug/mL</td>
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<td>Sumac 60 ug/mL</td>
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<td>Condition</td>
<td>COX-2 Protein Intensity (Normalized to Beta Actin)</td>
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<tr>
<td>Sumac 80 µg/mL</td>
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<td>Palmitate + Sumac 80 µg/mL</td>
<td>251000</td>
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</table>

**Figure 5:** COX-2 protein intensity of reversal study where human aortic vascular smooth muscle cells were exposed to palmitate for 18 hours and sumac was exposed for the final 3 hours of the palmitate exposure. This graph depicts the COX-2 protein intensity in each lane of the Western Blot. The lanes are identified as follows: Vehicle, Palmitate, Sumac 40 µg/mL, Sumac 60 µg/mL, Sumac 80 µg/mL, Palmitate (100 uM) + Sumac 40 µg/mL, Palmitate (100 uM) + Sumac 60 µg/mL, Palmitate (100 uM) + Sumac 80 µg/mL.
**Endothelium-dependent Vasodilation**

Arteries isolated from rats fed a high fat diet had impaired vasodilation compared to arteries isolated from chow-fed rats (Figure 5). HFD arteries exposed to sumac had similar endothelium-dependent vasodilation responses as those not exposed to sumac. Although trends for improved vasodilation were observed as both 0.3 µM and 1 µM doses of acetylcholine tended to improve vasodilation (p=0.065 and 0.059, respectively). In contrast, isolated arteries from the chow fed rats developed irreversible vasodilation and were therefore not responsive to pre-constriction with phenylephrine.

![Figure 6: Percent reversal of phenylephrine-induced tone.](image)

**Figure 6**: Percent reversal of phenylephrine-induced tone. This graph portrays the percent reversal of phenylephrine-mediated vasoconstriction with incremental doses of acetylcholine in the superfusate of isolated rat arteries fed either a high fat diet (HFD), HFD + sumac, or chow. #p<0.05 HFD vs Chow. Data shown as mean ± SEM and arcsine transformed data was analyzed by two-way repeated measures ANOVA.
CHAPTER 6

LIMITATIONS AND DELIMITATIONS

The prevention and reversal studies used human coronary artery VSMC. The delimitation remains that the results of this study may differ from what may be seen in intact humans and may also vary depending on any comorbidities or sex. Limitations that exist involve the design of the prevention study as this scenario may not reflect what occurs in vivo. The reversal study is more relevant as inflammation was induced and there after treated with sumac. Further, the integrity of the sumac extract over time may have degraded, however, every effort was made to prevent freeze thaw and sumac was stored in -80 degrees Celsius. Finally, this was a pilot study and the Western Blot for the prevention and reversal studies were only performed once. Thus, reproducibility is unknown. This study used cells from one individual and thus significance cannot be determined. A more comprehensive kinetics study was not performed in order to test a wider range of sumac doses as well as different treatment times.

The limitation of the ex vivo research, involving isolated blood vessels from rats, is that it may not reflect what would occur in vivo. It is unknown if similar results will be seen in humans. Additional studies should be conducted to further investigate this. If sumac is fed to animals we may or may not see similar effects. We may find it likely that one needs to ingest the sumac in order to have an effect. This research has limitations in sample size. Thus, although there are trends, with increased sample sizes statistically significant correlations may not be observed.
CHAPTER 7

DISCUSSION AND CONCLUSION

The goal of this study was to test the hypothesis that sumac would reverse palmitate-induced inflammation in human coronary artery vascular smooth muscle cells and thus reverse vascular inflammation as determined by measuring COX-2 protein expression. We also tested the hypothesis that sumac would improve vasodilation in ex vivo arteries isolated from rats fed a high fat diet. Major findings of these studies were: 1) exposure of isolated VSMC to palmitate increased COX-2 expression, did not appear to be reversed by sumac, 2) sumac partially improved acetylcholine-mediated vasodilation of arteries from rats fed a high fat diet, and 3) sumac impaired acetylcholine-mediated vasodilation in arteries from rats fed a standard chow diet.

Effect of a high fat diet on the vasculature

Cardiovascular disease is in part characterized by inflammation and oxidative stress that occurs in the endothelial and smooth muscle cells within the vasculature leading to altered vasodilation and ultimately atherosclerosis. Palmitate has been found to activate TLR4 on VSMC which leads to an inflammatory cascade that triggers TNF-alpha, NF-kB, and a downstream mediator COX-2. This leads to increased inflammation and the generation of ROS. TNF-alpha promotes VSMC migration and initiation, adhesion molecule expression, activation of inflammatory cells, and increases in plasma triglycerides. Further, palmitate has been found to exert a dose-dependent increase in COX-2 expression. Increased COX-2 protein expression is problematic in that it produces proinflammatory prostaglandins.
The accumulation of ROS in the vasculature contributes to CVD by reducing the bioavailability of NO thereby promoting constriction of blood vessels. Chronic inhibition of NO can result in endothelial damage. A previous study showed that rats fed a diet high in saturated fat had increased oxidative stress as well as significantly attenuated endothelial-dependent vasodilation. This may be directly related to reduced NO bioavailability. In regards to NO bioavailability, superoxide has been shown in prior research to result in endothelial dysfunction likely due to scavenging of NO. This leads to the production of more harmful ROS such as peroxynitrite (ONOO-) and reduces the bioavailability of NO for vasodilatory activities. Studies have found that chronic diseases such as hyperlipidemia, hypertension, and hyperglycemia, result in eNOS uncoupling, further contributing to the loss of NO. ENOS is imperative for proper blood vessel functioning and prevent platelet aggregation an adhesion to the vascular cell walls as well as prevent VSMC proliferation and migration.

Therefore, a diet high in fat such as palmitate, increases oxidative stress and inflammation leading to damage of the vasculature and thus CVD. Consistent with prior studies, the current study showed increased COX-2 protein expression in human vascular smooth muscle cells exposed to palmitate.

Effect of sumac on COX-2 protein expression

In regards to the effect of sumac on human cells, a prior study conducted on human breast cancer cells showed a reduction in TNF-alpha in cells treated with sumac for 24 hours. This study also concluded that sumac inhibits NF-kB transcriptional activity. However, there is a gap in the literature concerning human vascular cells and
sumac-mediated reductions in COX-2 protein expression. It was predicted that there would be an increase in COX-2 protein levels and sumac would reverse this response. Findings from the current study appear that sumac did not reverse palmitate-induced increases in COX-2 in human aortic VSMC. In the prevention study, cells exposed to both sumac plus palmitate, expressed similar levels of COX-2 as observed with palmitate only (Figure 1). However, COX-2 protein expression was slightly decreased in palmitate plus sumac 20 μg/mL. In the reversal study, cells without palmitate had similar levels of COX-2 as the cells with sumac plus palmitate. Further, with increased concentrations of sumac, there were higher levels of COX-2. In all, the results of the current cell culture study may differ from what may be seen in intact humans and may also vary depending on any comorbidities and sex. This was a pilot study and more data need to be collected. A more comprehensive kinetics study was not performed in order to test a wider range of sumac doses as well as different treatment times (efficacy and temporal studies). The case may be that a greater dose of sumac is necessary or the treatment time may not be effective. Finally, it is possible that the integrity of the sumac extract degraded over time.

The predicted outcome of this study was also formulated based on the antioxidant capacity of sumac as well as its association with improving the lipid panel. Sumac has been shown to inhibit ROS\(^7,39,66,67\) and prevent lipid peroxidation.\(^3\) The reduction in ROS was shown in a study on isolated human lymphocyte cells where the human subjects were fed a sumac supplement and their isolated cells were also exposed to sumac. It was found that with increasing doses of sumac, DNA comet formation decreased and thus the DNA was protected from oxidative damage.\(^39\) Specifically, sumac contains gallotannins which elicit an anti-inflammatory response as well as scavenge ROS, depress COX-2 levels,
upregulate eNOS, and in isolated rat VSMC, tannins extracted from sumac have been found to inhibit VSMC migration. A study conducted on sumac showed a depression in superoxide levels greater than what is seen with vitamin C and green tea. This study did not involve cells Other cell-free studies have also shown the antioxidant capacity of sumac through its ability to decrease superoxide levels. Various cell-free studies have shown that sumac has greater antioxidant activity than BHT, BHA, and alpha tocopherol. Total antioxidant status was increased in rabbits fed a sumac supplement for 3 months.

Studies have shown sumac contains many antioxidants. One study proposed that isoflavones, which are found in sumac, are capable of increasing LDL receptor activity and LDL catabolism in the liver. Many studies involving both human and animal trials showed an improved lipid panel with sumac supplementation. A few studies specifically showed a decrease in LDL with one human trial conducted. In one study conducted on alloxan-induced diabetic Wistar rats, improvements were seen in HDL, TG, TC, and LDL after being fed a 400 mg/kg dose of sumac. LDL was effectively decreased by 32% from baseline. In a similar study, significant decreases in LDL were seen with 100 mg/kg dose of sumac. The human trial conducted involved obese adolescents with dyslipidemia. They ingested 1,500 mg of sumac daily for one month and researchers observed a significant decrease in LDL.

Increased levels of LDL promote the movement of LDL into the blood vessels. LDL are lipid transporters in the blood that contain triglycerides, which contain fatty acids such as palmitate. LDLs can be oxidized and deposit in the blood vessel walls encouraging endothelial dysfunction. An immense inflammatory response follows
that initiates atherosclerosis. Therefore, with increased inflammation and the ability of sumac to decrease plasma LDL, it was expected that sumac would decrease COX-2 levels in palmitate-induced inflamed cells, in the current study.

**Effect of sumac on vasodilation**

Sumac may greatly contribute to improved blood pressure due to its ability to decrease superoxide and the fact that it contains tannins capable of upregulating eNOS,\(^7,45,66,67\) Most likely related to a mechanism based on its antioxidant capabilities (Figure 6), sumac may prevent superoxide from decreasing the bioavailability of NO in VSMCs, thus leading to vasodilation and preventing the creation of more harmful ROS or oxidants.

**Figure 7:** This sumac diagram portrays the proposed mechanism of sumac related to its antioxidant capabilities. Sumac may prevent superoxide from decreasing the bioavailability of nitric oxide, thus leading to vasodilation and preventing the creation of more harmful reactive oxygen species or oxidants.
Sumac also contains oleic acid, capable of increasing flow mediated dilation, as well as decreasing superoxide. Finally, sumac contains nitrates which lead to the formation of NO and help control blood pressure. However, these studies involved cell-free models and there are no published articles involving human models. One study was conducted on alloxan-induced diabetic Wistar rats where they were fed a sumac supplement and an increase in SOD was observed in red blood cells in vitro.

In the current study HFD arteries exposed to sumac had similar endothelium-dependent vasodilation responses as those not exposed to sumac. However, there were trends for improved vasodilation observed with increasing doses of acetylcholine. Although, this does not necessarily mean there is a correlation. Based on previous studies where rats fed a diet high in saturated fat had significantly attenuated endothelial-dependent vasodilation, possibly directly related to NO bioavailability, it was expected that the HFD arteries in the current study would have impaired vasodilation. Further, it was expected that the HFD arteries exposed to sumac would have improved vasodilation due to the constituents found in sumac and its ability to decrease superoxide levels which may increase NO bioavailability for vasodilatory activities, however there is a gap in the literature involving superoxide levels in cell models exposed to sumac.

Conversely, the results of the CHOW fed rat arteries in the current study were unexpected. The chow fed rats developed irreversible relaxation. However, in vivo testing may render different results and what is seen in animals may differ from what is seen in humans. Further, it may be necessary to ingest sumac in order to have an effect. A study conducted on isolated rabbit aorta, induced with moderate ischemia followed by reperfusion and exposed to a range of sumac from 15 to 500 µg/mL prior to ischemia,
showed a significant improvement in endothelium-dependent vasodilation with a sumac concentration from 150 to 500 µg/mL. A significant decrease in vasodilation was observed in vascular tissue without endothelium suggesting that sumac-mediated vasorelaxation is endothelium dependent. Further, norepinephrine-precontracted aorta exposed to varying concentrations of sumac showed a dose-dependent vasodilatory response. In addition, with a dose of 500 µg/mL of sumac, a significant increase in PGI₂ was observed following reperfusion of the arteries. This suggests that sumac improved the endothelial vasodilation by increasing its ability to produce prostacyclin after an ischemic event. Further, a decrease in TNF-alpha was observed in the perfusate, indicating an anti-inflammatory capacity of sumac. A previous study conducted on human breast cancer cells treated with sumac showed a decrease in NO production and iNOS expression. Further, a study conducted on hypertensive human subjects fed a 1,000 mg supplement of sumac showed a decrease in peripheral blood pressure. The previous cell-free study conducted on sumac where decreased superoxide levels were observed also aided in the formulation of the hypothesis that sumac would improve endothelial dependent vasodilation.

It has been found that sumac contains gallic acid and nitrates that may play a major role in the ability of the CHOW fed rat vessels in the current study to be constricted. Gallic acid is a phenolic acid that protects the body against oxidative stress. It is considered a strong antioxidant for its ability to scavenge ROS. It is an organic acid that is found both free or as a part of a hydrolysable tannin, which is a gallic acid ester. Gallic acid can be cytotoxic in VSMC due to the potential for spontaneous autoxidation in the presence of oxygen where-by it produces superoxide and large quantities of H₂O₂.
Hydrogen peroxide contributes to a state of irreversible vasodilation due to cell damage. Further, the current study used phenylephrine to constrict the blood vessels in order to induce tone from which to measure vasodilation. Phenylephrine operates by activating cytochrome P450. It has been found that gallic acid esters inhibit biotransformation of drugs that are carried out by cytochrome P450, thus preventing vasoconstriction.\(^7\) Since sumac contains gallic acid, it was not possible to constrict the vessels from CHOW-fed animals, thus it was not possible to measure vasodilation. Therefore, sumac may be acting as an adrenergic antagonist. Adrenergic antagonists are typically used to treat high blood pressure (i.e. dobutamine and epinephrine).

In conclusion, the sumac prevention and reversal studies were pilot studies and may not reflect what occurs in vivo. Palmitate reacted as expected where COX-2 protein expression in the human VSMC increased. Sumac did not appear to prevent or reverse palmitate-induced increases in COX-2, however, this was a pilot study and different results may be seen when sumac is ingested. Further, Western blot analysis was only performed once, thus reproducibility is unknown.

The ex vivo rat vessel study showed trends for improved vasodilation in a high fat environment. Sumac contains numerous antioxidants that may prevent superoxide from decreasing the bioavailability of NO in VSMC, thus leading to vasodilation and preventing the creation of more harmful ROS. However, the chow fed rats developed irreversible vasodilation that may be related to the presence of gallic acid and nitrates within the sumac. Given the unexpected results of this study, sumac may have a different, possibly more favorable effect in vivo. It is possible that sumac may have an indirect effect on the vasculature in the body that targets blood pressure. This study was in vitro
and looked specifically at the direct effects of exposing isolated blood vessels and cells to sumac. We observed a toxic effect of sumac in arteries from chow-fed animals, although arteries from rats fed a high fat diet tended to have improved vasodilation. It is possible that sumac administration *in vivo* may target a pathway that causes vasodilation rather than directly targeting the blood vessel like this research study.

It is anticipated that future research could investigate the administration of sumac and its ability to improve NO-mediated vasodilation, and overall vascular health, through enhanced bioavailability of NO in participants consuming Western diets. The current cell culture reversal study can be expanded where greater doses of sumac are used to treat the palmitate-induced inflammation in human VSMC as well as exposing the sumac at different times. This will help find the perfect dose of sumac and time of treatment.

Western blot analysis can be expanded to test TNF-alpha expression. Cells should also be tested for the level of superoxide expression. Finally, Westerns should be run with at least an *n=*2 in order to use statistics to determine significance and draw conclusions.
REFERENCES


39. Chakraborty A, Ferk F, Simić T. DNA-protective effects of sumach (Rhus coriaria L.), a common spice: Results of human and animal studies. Mutat Res. 2009;661:10-17


