ANTI-INFLAMMATORY PROPERTIES OF OKLAHOMA GRAPES

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CHAPTER I

INTRODUCTION

Many debilitating chronic conditions such as cardiovascular disease (CVD) [1], obesity [2], diabetes [3] and cancer [4] have been linked to chronic inflammation. CVD continues to be the number one cause of death for Americans [5]. In 2009, an estimated 81.1 million Americans were diagnosed with one or more forms of CVD and 9,000 people died each day from some form of the disease [6]. Obesity and diabetes are also prevalent conditions in our society. Statistics for 2007 have shown that 32% of American adults over the age of 20 are obese and an estimated 7.8% of the population have diabetes [7]. The prevalence of cancer is also alarming with an estimated half million Americans will die from this terrifying disease in 2009 [8]. Because of the relationship of inflammation with these chronic conditions, reducing or preventing inflammation may be one way to combat these diseases.

Currently, pharmacological agents used to reduce inflammation include corticosteroids, specifically glucocorticoids, and nonsteroidal anti-inflammatory drugs (NSAIDs). Glucocorticoids increase the transcription for anti-inflammatory cytokines such as interleukin (IL)-10 and IL-1, and inhibit the expression of pro-inflammatory cytokines, inducible nitric oxide synthase, IL-2 receptors, and adhesion molecules [9]. An example of a glucocorticoid is prednisone which is used to treat conditions such as chronic obstructive pulmonary disease [10], rheumatoid arthritis [11], Crohn's disease [12], and prostate cancer [13]. NSAIDs, on the other hand, block the cyclooxygenase (COX) enzymes and reduce prostaglandin synthesis resulting in the reduction of

inflammation, pain, and fever [14]. Two commonly used NSAID's are Celebrex and aspirin. Unfortunately, these pharmacological anti-inflammatory agents are associated with several side effects. Aspirin is cardioprotective by decreasing platelet aggregation, but causes gastrointestinal distress [15]. Celebrex, on the other hand, may increase the risk of cardiovascular thrombotic events, myocardial infarction, and stroke [16].

Many people prefer a natural option as opposed to a pharmacological approach in preventing chronic conditions. Fortunately, a number of natural products are also associated with reduction of inflammation. For example, omega-3 fatty acids including eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), decrease the production of pro-inflammatory molecules such as eicosanoids, cytokines, and reactive oxygen species (ROS) [17]. They can also reduce the expression of adhesion molecules by inhibiting arachidonic acid metabolism and altering the activation of the nuclear factor –κB (NF-κB) [17]. Another natural approach to reduce inflammation by inhibiting COX-2 production is the use of common spices turmeric and ginger due to its active ingredient curcumin [18]. Proteolytic enzymes found in the intestine of the silkworm called serrapeptase [17], bromelain found in pineapples [19] and hypericin a chemical from St.John's Wort [20] are other remedies found to reduce inflammation.

Another remedy for reducing inflammation may be the utilization of active compounds found in fruits and vegetables. For example, vitamin C inhibits tumor necrosis factor (TNF)-α from activating NF-κB thereby preventing the production of the pro-inflammatory cytokines IL-2 [21]. Vitamin E, a vitamin found in vegetable oils, also reduces the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α

[22]. Phytochemicals such as genistein, quercetin, and resveratrol are chemicals produced in grapes which have also been shown to reduce inflammation [23].

As in plants, phytochemicals induce various physiological responses and perform specific function in the human body. Genistein protects plants against fungal disease [24] and in humans functions as an anti-inflammatory agent by inhibiting prostaglandin E2 and COX-2 activity [25]. Quercetin guards plants against stress by acting as an anti-oxidant and removing toxic peroxides from plant cells [26]. In the human body, quercetin acts similarly by reducing ROS and decrease the expression of vascular cell adhesion molecules [27]. Resveratrol, like genistein, protects plants against fungal infections [28] and in the human is effective in protecting against inflammation by the inhibition of NF-κB, COX-2, and numerous other pro-inflammatory molecules [29]. Increased scientific understanding of the benefit of fruits and vegetables and their impact on health has led to an interest in polyphenols and their importance to the human body.

Polyphenols are phytochemicals of plant origin that possess one or more phenol rings used as building blocks to create thousands of molecules that protect plants against disease, animals, insects, and also have a role in reproduction and pigmentation [30]. Consumption of foods rich in polyphenols have been found to have numerous health properties including the capabilities to reduce inflammation and prevent oxidation [31]. Because of these properties, polyphenols are being widely investigated for their role in reducing many chronic conditions [32].

When considering the role of nutrition in reducing the incidence of chronic disease, the 'French Paradox' has long been a phenomenon of interest [33]. The French consume a high fat diet; yet, their incidence of atherosclerosis is far below that of

Americans. Among the potential dietary contributions, it is believed that consumption of wine with their meal has helped prevent or reduce CVD among the French. Initially, researchers thought alcohol contributed to the cardioprotective effect of the inhibition of platelet aggregation. Research has now shown that the polyphenols of the grapes from which the wine was made contributes to benefit the heart [33].

Grapes contain hundreds of different phenolic compounds but the most common and highest concentrations include gallic acid, quercetin, caffeic acid, catechin, epicatechin, rutin, myristicin, resveratrol, anthocyanins, proanthocyanidins, flavonoids, and flavan-3-ols [34]. Phenolic content is greatly influenced by the variety of grape and the environmental conditions during the growing season such as temperature and the amount of precipitation [35]. Soil conditions, climate, water hardness, and disease are other factors contributing to the concentration of polyphenols [36]. Research has found that the harvest time of the grapes throughout the year [37] and the differences of the grapes within the same variety [38] can create different polyphenolic profiles for grapes of the same variety.

The increasing trend in Oklahoma towards grape production has sparked the need to understand the health benefits associated with grapes grown locally, including their anti-inflammatory properties. Increased knowledge of the health benefits of grapes may further help grape growers to promote their products and increase their consumption which will help Oklahoma's viticulture and agritourism business. The ***hypothesis*** to be tested is that the varieties of Oklahoma grapes with high concentrations of total phenolic compounds will have the greatest impact on decreasing markers of inflammation *in vitro*.

Specific Aims:

1. To determine the phenolic content, anti-oxidant capacity, and anti-inflammatory properties of juice from different varieties of grapes grown in Oklahoma.
2. To assess the *in vitro* anti-inflammatory properties of extract from Oklahoma grape varieties with high and low total phenolics content (five varieties each) using LPS-stimulated macrophage.
3. To compare the effect of the grape extract from an Oklahoma grape variety to that of resveratrol on the expression of inflammatory genes in LPS-stimulated macrophage.

Limitations

The study utilizes grapes grown only in Cimarron Valley Research Station (Perkins, OK) which is not necessarily representative of the state as far as the condition of the soil, precipitation and other environmental conditions or disease. These conditions are known to influence the composition and concentration of the phenolic compounds in grapes.

There is no way to be certain that the extraction process used removed 100% of the polyphenols found in the grapes, although, the results were reasonable when compared to data of similar varieties. Nonetheless, the same procedure was used for all varieties and sufficient for comparative purposes. Uniformity of the final product cannot be verified. Temperature control, vacuum pressure, spillage, and other experimental

variables are all factors that we attempted to control for uniformity of the extract. The stability of polyphenols during processing and storage cannot be controlled and is currently unknown.

This study used the total extract and not the individual phenolic compound rather than whole grapes in an attempt to determine the effects of the grape polyphenols rather than other components of the grape such as carbohydrates. This study is an *in vitro* study using murine macrophages and because of this should be interpreted with caution. Studies with animals and subsequently humans are necessary to confirm the findings of this study since the effects of digestion and metabolism are not accounted for in a cell culture system.

CHAPTER II

REVIEW OF LITERATURE

Inflammation and Chronic Disease

Inflammation is a normal process for protection against foreign antigens such as bacteria, virus, and parasites or for the repair of an injury [39]. The majority of foreign antigens are kept out of the body by physical barriers like the skin and mucosal membranes of the innate immune response. If the antigen gets past the physical barriers, macrophages and neutrophils migrate to the site of the antigen, engulf and phagocytize or destroy the antigen [40]. The antigen is then displayed on the cell surface creating an antigen-presenting cell. T-helper cells that can recognize the same antigen interact with the antigen-presenting macrophage to stimulate the secretion of interleukin (IL-2) which causes the proliferation of cytotoxic T cells and B cells. The functional properties of B cells and T cells enables adaptive immunity to be extremely powerful. In both, innate and adaptive immune function, numerous pro-inflammatory and anti-inflammatory molecules are produced to create a self-regulatory effect, thereby ending the immune response when the antigen is removed [40]. In some people, diseases or genetic disorders interfere with the self-regulation of the immune response and cause a condition called autoimmune disease.

Chronic inflammation has been implicated in the development of many chronic diseases such as cardiovascular disease (CVD) [1], obesity [2], diabetes [3], and cancer [4]. CVD alone affects 81.1 million people in the United States [6, 41] and this number continues to grow as the prevalence of obesity and diabeties continues to increase.

According to the Behavioral Risk Factor Surveillance System, only 15.9% individuals were reported as obese in 1995 in comparison to 26.6% of adults in 2008 [42]. As obesity increases, so do other chronic diseases associated with inflammation, such as diabetes, which is projected to affect 23.6 million people in the US in 2009. Eighteen million of these people will be diagnosed and receive treatment for diabetes while the other 5.7 million people will remain undiagnosed [43]. Cancer is also a disease associated with chronic inflammation from which an estimated one half million Americans died from in 2009 [8].

Chronic inflammation plays a major role in the onset of atherosclerosis or the development of fatty deposits in blood vessels as one of the primary causes of CVD [44]. Monocytes are called to the site by monocyte chemotactic protein 1 (MCP-1) released by damaged endothelial cells lining the blood vessels. The monocytes infiltrate the space below the endothelium (the intima), differentiate into macrophages, and release more cytokines and other pro-inflammatory molecules. Low density lipoproteins (LDL) also enter the intima where they undergo oxidation (oxLDL) and promote endothelial and smooth muscle cell activation, secretion of inflammatory mediators, and expression of adhesion molecules, a sequence of steps that culminates in leukocyte accumulation in the intima. Oxidized LDL is taken up into the macrophages via scavenger receptors resulting in the formation of foam cells, a prominent feature of atherosclerotic plaques [45-47].

Chronic inflammation is also associated with obesity [48] and is a factor in the onset and progression of diabetes 2 [3]. Obesity is associated with an increased risk of developing insulin resistance, type 2 diabetes, atherosclerosis, and other chronic diseases. Traditionally, adipose tissue was considered to passively store triacylglycerols and

release free fatty acids. Now, it is recognized to be an active endocrine organ able to secrete a large number of pro-inflammatory molecules. The white adipose tissue (WAT) represents the majority of adipose tissue in humans and is the primary site of energy storage. The WAT is made up primarily of adipose cells and the tissue matrix which contains preadipocytes, endothelial cells, fibroblasts, leukocytes, and macrophages. Adipose tissue contains numerous activated adipocytes, macrophages, and T-lymphocytes that produce increased amounts of adipokines, and pro-inflammatory cytokines [49]. Some pro-inflammatory molecules produced in the WAT [50] include adiponectin, IL-1β, IL-6, tumor necrosis factor (TNF-α), monocyte chemoattractant protein (MCP-1) and monocyte inflammatory protein (MIP). The combination of obesity with other risk factors such as hypercholesterolemia and hypertension is likely to heighten the systemic inflammation to enhance the progression and severity of CVD [49].

Adipocytes produce a variety of chemokines that modulate the movement of inflammatory cells into the adipose tissue, such as monocytes that are then transformed into macrophages. The macrophage are induced to produce the proinflammatory cytokines TNF-α and IL-6. The increase of adipocytes creates insulin resistance. The high level of circulating insulin increases the free fatty acid (FFA) release from adipocytes and enhances delivery to the liver. The liver increases hepatic production of very low-density lipoprotein and the enzymatic activity of lipoprotein lipase is decreased. This decreases the production of high density lipoproteins. Other types of FFAs activate toll-like receptor 4 (TLR4) to produce IL-6. Adipocytes are responsible for controlling the hormones adiponectin and leptin. Adiponectin enhances insulin sensitivity in muscle and liver and increases FFA oxidation in several tissues, including muscle fibers. It also

decreases serum FFA, glucose, and triacylglycerol concentration. Plasma adiponetin concentrations fall with increased obesity. Adiponectin concentrations are inversely correlated with insulin resistance and hyperinsulinemia, which lead to type 2 diabetes and metabolic syndrome. Plasma leptin concentrations are highly correlated with body mass index (BMI). However, in most obese individuals, leptin concentrations are already high because of the increased amount of leptin-secreting adipose tissue. It appears that with increased leptin concentration, the hormone induces target cells to become resistant to its actions. Some of the actions to reduce circulating FFA and triacylglycerol are due to increased fat oxidation. The increase in fat oxidation is mediated by activating the enzyme adenosine monophosphate (AMP)-activated protein kinase which increases glucose transport in muscle. AMP-activated protein kinase is also activated by exercise which also increases fat oxidation and reduces insulin resistane. Thus, the adipocyte hormones and exercise act via a similar signal transduction pathway to increase fat oxidation and promote insulin sensitivity [51].

Cancer associated inflammation is characterized by the induction of cell cycling for tissue growth and repair. The initiation, promotion and expansion of tumors may be influenced by numerous components that function in the inflammatory response [52].

Pro-Inflammatory Molecules

Cytokines and nitric oxide (NO) are pro-inflammatory molecules released from activated immune cells including macrophages and lymphocytes. These molecules have different signalling functions during inflammation to ochestrate an effective immune response against the antigen thereby protecting the body from disease. The cytokines IL-

6 and TNF-α, as well as NO will be the focus of this review due to their extensive role in the initiation of the inflammatory response.

Cytokines are polypeptides secreted by various cells of the immune system. They are involved in acute and chronic inflammation as mediators of the inflammatory response by autocrine and paracrine actions. The major classes of cytokines include interleukins (IL), tumor necrosis factor (TNF), colony stimulating factors (CSF), transforming growth factor (TGF) and interferons (IFN) [53]. Cytokines may function either as pro-inflammatory or anti-inflammatory agents.

Interleukin-6 (IL-6)

The interleukins (IL) were first described as signals for communication between white blood cells [54]. Interleukins are the primary messengers and directors of the immune system and can cause cellular proliferation, cell activation, inflammation, physiologic changes such as fever and pain, allergies and growth [54]. There are currently thirty-five identified interleukins, however, only IL-6 is focused on in this project.

IL-6 is a cytokine secreted by T-cells, macrophages, and other cells such as smooth muscle cells and osteoclast. IL-6 is involved in a wide variety of biological functions [54] including the stimulation of osteoblast formation. IL-6 was shown to be secreted from muscle and osteoclast during exercise [54]. Smooth muscle cells, T-cells and macrophage produce IL-6 primarily as a pro-inflammatory cytokine [55] that stimulates the immune response by forming a receptor complex consisting of a specific IL-6 binding protein (IL-6R) and a signal-transducing subunit (gp130). The IL-6/IL-6R

complex induces several intracellular signalling events including the activation of transcription factor [54]. The transcription factor, in turn, promotes the transcription of pro- and anti- inflammatory proteins encoded in the cellular DNA. Generally, in acute inflammation, IL-6 acts as an anti-inflammatory cytokine that inhibits TNF-α activity by preventing the activation of NF-κB thereby ending the acute inflammation response [56]. In chronic inflammation, IL-6 is acts as a pro-inflammatory molecule which induces the production of C-reactive protein (CRP) from the liver. The increase in circulating IL-6 and CRP are important markers of chronic inflammation [56].

Acute inflammation is characterized by the accumulation of neutrophils to the site of infection while chronic inflammation involves the accumulation of monocytes and their differentiation into macrophages [56]. IL-6 is important in this transition between acute and chronic inflammation. During the early stages of infection, IL-6 and IL-6R form a complex which activates endothelial cells to induce expression of adhesion molecules such as IL-8 (a chemoattractant for neutrophils) and MCP-1. Neutrophils attracted by IL-8 bring more IL-6R attached to their membrane. Pro-inflammatory cytokines induce the neutrophil to release the IL-6R which is then free to bind with IL-6. This new IL-6/IL-6R complex will ligate with gp130 on the endothelial cell membrane and increase IL-6 and MCP-1 secretion, but not IL-8, thereby causing the transition from neutrophil to monocyte recruitment [56].

Circulating levels of IL-6 are directly correlated with adiposity and insulin resistance [57]. Approximately 15-35% of IL-6 circulating in the blood originates from the adipose tissue [50]. While adipocytes are capable of producing IL-6, it is thought the other cells in the adipose tissue such as macrophages and endothelial cells are the major

contributors of IL-6 [58, 59]. The macrophages in the cell matrix are responsible for contributing approximately 50% of the WAT derived IL-6 [60].

Several studies have been done on IL-6 in relation to chronic conditions [50, 54, 56, 58, 59, 61-68]. IL-6 has been found to be three times higher in obese individuals compared to lean individuals [62]. It has also been found to be required in the development of collagen and antigen induced arthritis [69-72]. In colorectal cancer, the IL-6 concentration in cancerous colon tissue was found to be approximately eight times higher than normal colon tissue [63]. IL-6 is significantly correlated with the staging of colorectal cancer [73]. However, the relationship between macrophage-derived and tumor-derived IL-6 has remained unclear until a study by Li and colleagues (2009). Their results indicated that macrophages in tumor infiltrates could release IL-6 creating a condition in which colon cancer cells secrete IL-6 [64].

IL-6 production differs between type 1 and type 2 diabetes. IL-6 is secreted from monocytes when adiponectin activates the NFκB pathway. Whereas circulating adiponectin is reduced in the sera of patients with type 2 diabetes and in patients with CVD [74], systemic adiponectin is elevated in type 1diabetes mellitus. Therefore, patients with type 1 diabetes should produce higher levels of IL-6. A study by Abke and colleagues [75] compared the circulating IL-6 and adiponectin from patients with type 1 diabetes to control patients to determine why they are more prone to cardiovascular disease. The type 1 diabetics had double the adiponectin of the controls, yet, when the IL-6 was measured type 1 diabetics had only half the concentration of the controls. This study indicates that adiponectin signal transduction pathways are impaired in type 1 diabetes monocytes decreasing the amount of IL-6 produced [75, 76].

Tumor Necrosis Factor-α (TNF-α)

TNF-α or cachectin is produced primarily by monocytes and macrophages [77]. Adipose tissue produces TNF-α in large quantities as with IL-6. Although, adipocytes produce some IL-6, adipocytes do not produce any TNF-α. TNF-α is produced by macrophages which are entrapped in the adipose matrix. TNF-α is involved in the initiation of three pathways of inflammation: activation of NF-κB, activation of the mitogen-activated protein kinases (MAPK) pathway, and induction of cell death signaling [78].

In the first pathway, TNF-α binds to transmembrane receptors such as toll-like receptors, RANK, and TNF receptor (TNFR) activating the enzyme IκB kinase (IKK). Under normal conditions, IκB is bound to NF-κB in the cytosol. Phosphorylation of IκB caused it to be released from NF-κB. The IκB is then degraded by a proteosome and the liberated NF-κB is now free to move from the cytosol into the nucleus where it acts as a "rapid-acting" primary transcription factor and upregulates genes involved in cytokine production and T-cell development, maturation, and proliferation [78].

NF-κB activation is terminated when the newly synthesized IκB moves into the nucleus to bind the NF-κB and relocates it back into the cytoplasm via a nuclear export signal, thus, making NF-κB self-limiting [79]. NF-κB induces gene expression of a large number of immune molecules including cytokines (IL-2, IL-6, IL-8, IFN-γ, TNF-α), growth factors (granulocyte, monocyte, and granulocyte-monocyte colony-stimulating factors), immune receptors (T-cell receptor, MHC class I, IL-2 receptors), adhesion molecules (E-selectin, ICAM-1, VCAM-1), and inducible nitric oxide synthase (iNOS)

[80]. Furthermore, NF-κB plays an important role in both apoptosis and cellular proliferation [77].

The second pathway activated by TNF-α is the MAPK cascade. MAPK are serine/threonine specific protein kinases that respond to extracellular mitogens and regulate gene expression, mitosis, differentiation, and cell apoptosis [81]. MAPK positively regulates expression of many genes involved in inflammation, such as those coding for TNF-α, IL-1β, IL-6, IL-8, cyclooxygenase-2, and collagenase-1&3 [81]. A large number of cellular processes are mediated by the interacting network of MAPK from extracellular signals to their intracellular targets [82]. The MAPK cascades influence a variety of nuclear, membrane, cytosolic and cytoskeletal targets [82].

The induction of death signaling is a minor role of TNF-α in inflammation. Two TNF receptors possess death domains; TNF receptor 1 (TNFR1) and Fas receptor. These receptors are both transmembrane proteins which can activate the caspase cascade responsible for programmed death or apoptosis [83].

Nitric Oxide (NO)

NO is an inorganic molecule synthesized from the amino acid L-arginine by the enzyme, nitric oxide synthetase (NOS). There are at two types of this enzyme: constitutive and inducible. The constitutive enzymes (endothelial NOS (eNOS) and neuronal NOS (nNOS)) are cytosolic, calcium/calmodulin dependent, and release NO for short periods in response to receptor or physical stimulation. NO released by these enzymes are responsible for numerous physiological responses. NO has regulatory effects (vascular tone and permeability and cell adhesion), protective effects (anti-oxidant and

inhibits leukocyte adhesion), and deleterious effects (inhibits enzyme function, promotes DNA damage and induces lipid peroxidation) [84]. Inducible NOS (iNOS) is induced by the activation of macrophages, endothelial cells, and numerous other cells [85]. This enzyme differs from eNOS and nNOS in that it is calcium independent and releases NO for extended periods of time [86]. NO plays an active role in signaling as well as being a cytotoxic or regulatory effector molecule of the innate immune response. Because NO has an unpaired electron, it is highly reactive and readily binds to superoxide and forms a peroxynitrite which may damage cells [87]. Macrophages do not express iNOS unless they are activated by a cytokine or bacterial element such as LPS or IFN-γ. However, with reduced L-arginine availability iNOS produces superoxide and peroxynitrite to modulate macrophage function such as cytostatic or cytotoxic actions [88].

NO can easily diffuse across cell membranes and between cells making this the perfect intracellular and intercellular signaling molecule [89]. NO was first described by Furchgott and Zawadski as an endothelium-dependent relaxing factor for smooth muscles [90]. It has been found that NO functions in many more physiological processes that originally suspected. Besides acting as an inhibitor to angiotensin II, which is a vasoconstrictor, NO inhibits platelet and leukocyte adhesion to the vascular endothelium, scavenges superoxide anions, inhibits COX-2 production, and inhibits smooth muscle hyperplasia [85]. During chronic inflammation, circulating NO would be increased in an attempt to reduce the inflammation.

Pharmacological Options for Inflammation

There are many pharmacological options available for reducing inflammation. Two primary categories of anti-inflammatory drugs are the non-steroidal anti-inflammatory drugs (NSAIDs), which includes the cyclooxygenase inhibitor drugs, and the steroidal anti-inflammatory drugs that include glucocorticoids.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin are used primarily to treat inflammation, mild to moderate pain, and fever. Aspirin is used to prevent blood clots, strokes, and heart attacks in high-risk individuals [91]. Aspirin has also been shown to significantly decrease the progression of atherosclerosis in rabbits [92], apoE(-/-) LDLR(-/-) mice [93], diabetic patients [94], and healthy women and men [95].

NSAIDs are also included in many cold and flu preparations [96]. NSAIDs block the production of prostaglandins by inhibiting COX-1 and COX-2 which is responsible for inflammation, pain, fever, and platelet function. COX-1 is an enzyme which is normally present in a variety of tissues in the body, particularly the stomach and is also present in sites of inflammation. COX-1 produces some prostaglandins that protect the inner lining of the stomach. COX-2, on the other hand, is located specifically in areas of the body that commonly are involved in inflammation but not in the stomach. Common NSAIDs such as aspirin block both COX-1 and COX-2. Blocking the COX-1 enzyme reduces inflammation but can also damage the lining of the stomach [14]. COX-2 specific NSAIDs can reduce inflammation without the risk of injuring the stomach or intestines [96].

Steroidal anti-inflammatory drugs include the glucocorticoids such as hydrocortisone, prednisone and dexamethasone. Glucocorticoids have been shown to successfully reduce inflammation by repressing the production of pro-inflammatory cytokines, chemokines, adhesion molecules, tissue factor, enzymes, and nitric oxide. If not for the debilitating side effects, like osteoporosis, diabetes, cataracts, hypertension, thinning of the skin, and even arrested growth [97], it would be the perfect anti-inflammatory agent.

Glucocorticoid acts as ligand that binds with glucocorticoid receptors (GR) found in the cytosol. GR can regulate its target genes in either a positive or negative way. Positive regulation is mainly mediated by direct binding of ligand-activated homodimerized GR onto inducible enhancer elements in the gene promoter, called glucocorticoid response elements (GREs) [98, 99]. Negative regulation of the glucocorticoid dimer creates transcriptional interference with NF-κB which is responsible for translation of pro-inflammatory cytokines and other inflammatory molecules and activator protein-1 (AP-1). These molecules control a number of cellular processes including differentiation, proliferation, and apoptosis [97, 99].

Bioactive Components in Grapes

Grapes (*Vitis sp.*) are a common fruit found worldwide, although, they prefer a Mediteranean type climate. Grapes can be eaten fresh, dried as raisins, processed into grape juice, or fermented into wine. Nutritional analyses show that grapes are primarily sugar and water (81.3%) with very small amounts of fats, proteins, vitamins, and minerals [100] (**Table 1**). The most notable vitamin and mineral are vitamin K and

Table 1: Nutritional Analysis for Raw, American Type Grapes (USDA, 2005).

Nutrient	Unit	Amount/100g
Carbohydrate	g	17.2
Dietary Fiber	g	0.9
Fat	g	0.33
Protein	g	0.66
Vitamin A	IU	100
Vitamin C	mg	4
Vitamin E	mg	0.22
Vitamin K	mcg	14.57
Thiamin	mg	0.11
Riboflavin	mg	0.11
Niacin	mg	0.33
Folate	mcg	4.02
Calcium	mg	14
Iron	mg	0.33
Magnesium	mg	5
Phosphorous	mg	10
Potassium	mg	191.3
Sodium	mg	1.96
Manganese	mg	0.76

manganese which supply 17 and 33% of daily value, respectively [100]. Vitamin K has been shown to improve insulin sensitivity through suppression of inflammation [101] and manganese has been found to be beneficial in atherosclerosis, diabetes, obesity, and cancer by its superoxide dismutase activity [102-104].

In addition to the vitamin and mineral content of grapes, the health benefits of grapes are attributed to its phenolic content [105-114]. Phenolic compounds are chemicals produced in plants responsible for their color, odor, and protective qualities. Approximately one thousand polyphenols have been identified and many more remain unidentified [115]. The majority of polyphenols are usually linked with a sugar, most commonly glucose, but may also be linked with any monosaccharide, disaccharide, or polysaccharide.

Dietary polyphenols can be classified into flavonoids and phenolics acids. The flavonoids can be classified as flavonols (quercetin, kaempferol, myricetin), flavones (luteolin, apigenin), isoflavones (daidzein, genistein), flavanones (hesperetin, naringenin), flavanols which has the monomer class catechins (catechin, epicatechin, gallocatechin) and polymer class proanthocyanidins (procyanidins, prodelphinidins), and the anthocyanins (cyanidin, delphinidin). The phenolic acids are cinnamic acid (caffeic acid, ferulic acid, chlorogenic acid), benzoic acid (gallic acid), and ellagitannin (casuarictin, sanguine H6).

Some of the most extensively investigated polyphenols are resveratrol, flavonoids, anthocyanins, and procyanidins. Prior et al. [115, 116] has shown that there is a vast difference in the amount of polyphenols in different fruits and vegetables. For example, the total phenolic concentration of a grape may be 2000 μg/ml while that of an

apple is only 710 μg/ml [117]. A majority of the polyphenols have anti-oxidant properties which greatly aid in alleviating chronic inflammation. Polyphenols also function to inhibit the communication between cells and inhibit the binding of proteins to their receptors [32]. The phenolic composition of grapes varies with each part of the grape (**Table 2**) [111, 118]. For instance, the purple or red pigmentation in the skins is known to possess high concentrations of anthocyanin, resveratrol, quercetin, and flavonoids [118]. The grape seeds contain a high concentration of proanthocyanidins [119-121] while the pulp is rich in flavan-3-ols [122]. Once processed into wine, red wine is more abundant in phenolic compounds such as trans-resveratrol, quercetin, catechin and epicatechin [33, 105, 107] and white wine has primarily tyrosol, hydroxytyrosol [123] and caffeic acid [124].

Besides differences in the part of the grape, polyphenols differ from one variety to the next [125, 126]. The total phenolic and anthocyanin content of Cabernet Franc, Cabernet Sauvignon, and Merlot were reported to be 4246 and 587 μg/ml [126], 2348 μg/ml and 990.8 mg/kg, and 2329 μg/ml and 1048.8 mg/kg [125], respectively. An even greater difference is noted between the red and white varieties. For example, Chardonnay, a white grape variety, compared to Cabernet Franc, a red grape variety, has a phenolic concentration of 2011 compared to 4246 μg/ml. The anthocyanin content of Chardonnay was undetectable while the Cabernet Franc contained 587 μg/ml [126].

Growing conditions can also affect the phenolic content of grapes. Price and colleagues [127] reported that wines made from Pinot Noir clusters highly exposed to the sun had 60% higher anthocyanin concentration than wines from shaded clusters and 14% more than wines from moderately exposed clusters. Polyphenolic content can also be

Table 2: Polyphenol Composition of Grapes and Grape Products[111,117]

Product	Main Polyphenols
Grapes	Anthocyanidins (red grapes)
	Flavan-3-ols (white grapes)
Wine	Flavan-3-ols
	Flavan-3,4-diols
	Anthocyanins
	Flavones
	Tannins
	Resveratrol
Grape juice	Anthocyanins (red grape juice)
	Flavan-3-ols (white grape juice)
	Ellagic acid
Grape skin	Prodelphinidins
	Resveratrol
	Anthocyanins
Grape seeds	Procyanidins
	Proanthocyanidins
Grape pulp	Flavan-3-ols
Grape pomace	Anthocyanins
Polyphenol extract	Anthocyanidin-3-glucosides

different between growing seasons and harvest time [36, 37, 128]. For example, Mazza and colleagues [128] took samples of grapes every week from September to October for two consecutive years and assessed the phenolic content of the skin. The concentration of total phenolics, flavonoid, and anthocyanin were assessed for three varieties of grapes: Cabernet Franc, Merlot, and Pinot Noir. The flavonoid concentration remained constant between the two years, but there was up to 25% difference in the total phenolic concentration and up to 10% difference in anthocyanin concentration.

Anti-Inflammatory Properties of Grapes

Research on grapes reviewed in this section is divided whether it is an *in vitro* study, using animal models or done in humans. Further subdivision was also done according to the effects of grape products on platelet aggregation, oxidative stress, foam cell formation, inflammatory markers, and other effects. Grape products included in this literature review were juice, wine, extract, resveratrol, and proanthocyanidin.

***In vitro* Studies**

Oxidative Stress

The efficiency for preventing the oxidation of polyunsaturated fatty acids (PUFA) by resveratrol (3,4',5,trihydroxystilbene) and several flavonoids was compared to that of a wine extract containing proanthocyanidins [129]. Low-density lipoproteins (LDL) isolated from fresh pig blood was labelled with gold and fluorescein isothiocyanate (FITC) then oxidized in the presence or absence of a specific flavonoid (trans-resveratrol, quercetin, catechin, epicatechin, or trolox) or wine extract. Resveratrol was more

efficient than the flavonoids in preventing copper-induced and induced oxidation of PUFA. Moreover, resveratrol was more effective than flavonoids as a chelator of copper and less effective as a free-radical scavenger [129].

Inflammatory Markers

Shanmugam and colleagues [130] compared the extract from twenty-nine plants including grape seeds on NO and TNF-α production. RAW264.7 macrophages were activated with LPS and treated with the different plant extracts. Grape seed, along with bearberry, were among the most active in inhibiting both NO and TNF-α production [131]. Other plants with high polyphenolic content such as chamomile, echinacea, sage and valerian were unable to inhibit NO production indicating that polyphenolics have their own specific effects. These investigators [131] identified three structural criterion important in the anti-inflammatory activity of polyphenolics compounds (i) flavonoid aglycones are more potent than the corresponding glycosides, (ii) flavonoids with a 4'-OH substitution in the B-ring are more potent than those with a 3'-OH-4'-methoxy substitution, and (iii) flavonoids of the flavone type (with a C2=C3 double bond) are more potent than those of the flavanone type (with a at C2-C3 single bond).

The effect of Muscadine grapes skin extract on the release of cytokines by LPS-activated peripheral blood mononuclear cells, and the production of superoxide free radicals in phorbol myristate acetate-activated neutrophils was investigated by Greenspan and colleagues [132]. Muscadine grapes are native to the southeastern U.S. states and have been shown to be extremely high in anti-oxidant activity [133-135] yet, little research has been done with them. The phenolic and ellagic acid content of the

Muscadine grape skin extract used in the study by Greenspan and colleagues [132] was 2.1 mg/ml and 67 μg/ml, respectively. Diluted Muscadine skin extract inhibited TNF-α and IL-6 production by nearly 50%. The same dilution of the extract also inhibited superoxide production from neutrophils by 65%, an effect that is comparable to the positive control aspirin [132]. A more dilute solution of muscadine grape extract (1:400) inhibited IL-1β production by approximately 60% but TNF-α and IL-6 production were not affected. These results show that some component of Muscadine grape skin extract may have anti-inflammatory properties.

The effect of grape seed proanthocyanidin extract (monomer, dimers, trimers) on the production of pro-inflammatory molecules PGE_2 and NO was compared with different anti-inflammatory agents (aspirin, indomethacin, and dexamethasone) and pure phenolic fractions (catechin and epicatechin) [136]. Proanthocyanidins, also known as condensed tannins or oligomers or polymers of flavan-3-ol units are found in abundance in grape seeds [137]. NO and PGE_2 production were assessed in RAW 264.7 murine macrophages stimulated with LPS and IFN-γ to induce an inflammatory response. Proanthocyanidin extract inhibited NO and PGE_2 in a dose and time-dependent manner. The greatest inhibition of NO and PGE_2 occurred when the extract was incubated simultaneously with LPS and IFN-γ. Epicatechin and catechin did not exert any significant inhibition on NO production. Although PGE_2 was inhibited by the proanthocyanidins extract, the highest inhibition of PGE_2 was accomplished with the anti-inflammatory drugs, aspirin and indomethacin. They also found that increased polymerization of the proanthocyanidins increased the inhibitory effect [132]. These

findings indicate that not only does the type but also the polymerization of the phenolic compounds influence anti-inflammatory properties of grape proanthocyanidins.

Another study on grape seed proanthocyanidins extract investigated its effect on intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) expression in primary human umbilical vein endothelial cells induced by TNF-α [138]. Grape seed proanthocyanidin extract down regulated VCAM-1, but not ICAM-1 expression [138]. The regulation of VCAM-1 expression by grape seed proanthocyanidin extract was at the transcriptional or post-transcriptional level and not through NF-κB pathway indicating that another mechanism is involved in the regulation of transcription of VCAM-1.

Resveratrol, a phenolic compound found in grapes and grape products has been shown to have anti-inflammatory properties [139]. Holmes-McNary and colleagues [139] tested whether resveratrol could modulate NF-κB activity, a factor involved in the inflammatory process. Human monocyte (THP-1) and macrophage (U937) cell lines were treated with purified *trans*-resveratrol and stimulated with either TNF or LPS. Resveratrol was effective in blocking IKK activation hindering the DNA binding activity of NF-κB, thereby blocking production of inflammatory cytokines [139].

Based on the reported anti-inflammatory activity of resveratrol, Xiu Li Bi and colleagues [140] investigated the effect of resveratrol in inhibiting activation of proinflammatory molecules such as NO and TNF-α in microglia (the macrophage in the central nervous system). Murine microglial cell line N9 was treated with resveratrol and stimulated with LPS. NO and TNF-α production were both inhibited by resveratrol. LPS increased IκBα degradation while resveratrol inhibited the degradation of IκBα, resulting

in decreased production of NO and TNF-α [140]. This study demonstrates that resveratrol inhibits inflammation at the transcriptional level by reducing the degradation of IκBα.

Other Effects

Concord grape seed extract was used to determine its effect on relaxation of endothelium [141]. Rat aortic rings with intact endothelial lining were treated with 1μM phenylephrine causing the endothelium to contract. Several fractions of Concord grape seed extract were compared against acetylcholine in relaxing the endothelium. Fraction A (gallic acid), fraction B (catechin and epicatechin), and fraction C (epicatechin gallate and flavan-3-ol) displayed very little activity. Fractions D-G (containing various dimers, trimers, tetramers, and pentamers of proanthocyanidin) displayed significantly more activity. This experiment demonstrates that proanthocyanidins are the primary component of grape seed extract responsible for endothelial relaxation [141].

The effect of resveratrol on chemotaxis was investigated using human peripheral blood phagocytes and rat basophilic leukemia cell line (RBL-2H3) transfected with epitope-tagged human FPR (ETFR) cells. The cells were treated with resveratrol and incubated with various chemokines that are responsible for attracting phagocytic lymphocytes to the site of inflammation. Chemoattraction with all of the chemokines was inhibited as the concentration of resveratrol increased [142]. Resveratrol reduced phosphorylation of extracellular signal-regulated kinase (ERK1/2) and the activation of nuclear factor NF-κB induced by formylpeptide receptor agonists. These results suggest that resveratrol may inhibit the function of chemoattractant receptors contributing to the anti-inflammatory properties of resveratrol.

Animal Studies

Platelet Aggregation

A study by Shanmuganayagam and colleagues [143] was done to determine the effects of phenolic extract from grape seeds or grape skin alone or in combination with an enzyme blend in inhibiting platelet aggregation. Four classes of phenolic compounds (hydroxycinnamic acids, flavonols, anthocyanins, and polygalloyl polyflavan-3-ol) were found to be present in the grape skin extract and one (polygalloyl polyflavan-3-ol) in grape seed extract [143]. Seven dogs were given one of the following treatments: grape skin extract, grape seed extract, an enzyme blend, a combination of the two extracts, and the two extracts plus the enzyme blend for seven days followed by a seven day washout. Individually, the extracts and the enzyme blend had no effect on platelet aggregation. However, the grape extract with the enzyme blend worked synergistically to significantly inhibit platelet aggregation. Addition of the enzyme blend to the grape extract doubled the effectiveness in inhibiting platelet aggregation. Moreover, the combination of grape extract with the enzyme blend inhibited platelet aggregation for at least 24 hours after the final dose. Grape seed and grape skin extracts have little or no effect on platelet activity when used individually but elicit a greater anti-platelet effect when used in combination with an enzyme blend [143].

Another study by Shanmuganayagam and colleagues [144] investigated if the polyphenols of Concord grape juice would inhibit platelet aggregation in rabbits fed a hypercholesterolemic diet. Grape juice and a control of sugar water were given *ad libitum*

for 96 days. A significant decrease in platelet aggregation was observed in the grape juice treatment as well as significantly lower atheroma development, total serum cholesterol, and blood pressure [144].

The effect of red wine, white wine, or grape juice on platelet inhibition was investigated in dogs [145]. Cyclic flow reductions (CFRs) caused by platelet-mediated thrombi were measured as dogs were treated intraveneously and intragastrically with diluted red or white wine (2 and 4 ml/kg), or Concord grape juice (4, 6, 8, and 10 ml/kg) until the CFRs ended. Blood aggregation was compared before and after beverage administration using a whole blood aggregometer. Platelet aggregation was successfully diminished with red wine and grape juice but not with the white wine. A blood alcohol content (BAC) of 0.2 g/dl is usually required to inhibit platelet aggregation but the BAC of the dogs after administration of the wine was only 0.028 g/dl suggesting that other compounds present in red wine and grape juice, absent in white wine were responsible for the improved coronary blood flow [145]. Grape juice reduced platelet aggregation in a dose-dependent manner, although, approximately two and half times higher dose of grape juice was needed compared to the wine to inhibit platelet aggregation. A similar study by Ruf and colleagues [146] also demonstrated improved coronary blood flow in rats treated with dilute red wine for four months in comparison to white wine and ethanol [147].

A study by Osman and colleagues [148] compared the effect of three fruit juices (grapes, orange, and grapefruit) on platelet aggregation. Five monkeys were fed grape juice, orange juice, and grapefruit juice (5 mL/kg) for 7 days in a cross-over design. Grape juice reduced the amount of platelet aggregation while there was no difference in

the orange juice and grapefruit juice [148]. The investigators concluded that although orange and grapefruit juices contain many phenolics compounds, they do not contain the phenolic compounds responsible for reduction of platelet aggregation.

Lipid Profile and Oxidative Stress

A study by Rodrigo and colleagues [149] compared the effects of red wine to that of water, ethanol, or alcohol-free red wine on oxidative stress. Rats were given the corresponding treatments for ten weeks. Red wine or alcohol-free wine exhibited lower oxidative stress as indicated by the increased ferric reducing ability of plasma (FRAP) assay [149]. However, a study by Cestaro and colleagues (1996) showed that oxidative status does not appear seriously altered by ingestion of wine and polyphenols. The researchers suggest that the balanced diet and adequate intake of micronutrients were sufficient to counteract any oxidative damage [150].

The effects of grape products on oxidative stress, lipid profile, glucose, and blood pressure have also been investigated by Araya and colleagues [151]. They demonstrated that red wine rich in flavonols, ethanol (125 ml/l), and alcohol-free red wine increased blood HDL and triacylglycerols but it did not affect overall lipid profile compared to water. The red wine and alcohol-free red wine also increased FRAP values indicating greater anti-oxidative power [151].

Falchi et al [118] compared the effect of grape skin or flesh on oxidative stress. Rats were fed water as the control and grape skin or grape flesh for 30 days after which induced myocardial infarct size on the excised heart and malonaldehyde were assessed to determine oxidative stress. The flesh of grapes is equally cardioprotective as the skin as

indicated by the significantly improved aortic flow during reperfusion of the heart and the reduction of the infarct size in comparison to the control. The antioxidant potential of the skin and flesh of grapes are comparable with each other despite the fact that flesh does not possess any anthocyanin [118].

Cui and colleagues [111] investigated the dose-dependent effect of a standardized grape extract preparation on aortic flow, amount of myocardial infarction on the excised heart, and oxidative stress indicated by malonaldehyde content. Male Sprague-Dawley rats were given a standardized grape extract at 50, 100, or 200 mg/kg body weight per day for 3 weeks. Significant cardioprotection as indicated by greater aortic flow and a reduced amount of myocardial infarction and malonaldehyde concentration in the heart was induced by the 100 mg/kg and 200 mg/kg standardized grape extract. The lowest concentration of standardized grape extract (50 mg/kg) did not exhibit any cardioprotective effects [111].

Cui and colleagues [112] used the same model in determining whether white wine had any cardioprotective effect as indicated by malonaldehyde content. Extracts prepared from three different white wines from Italy (Verduzzo Friulano Isonzo, Tocai Friulano Collio, and Passito di Tocai Friulano Collio) were given to rats at either 50 mg/kg or 100 mg/kg dose. Comparison of the white wine with the control showed so significant effects [112]. The phenolic compounds found in red wine and grapes are more cardioprotective than white wine phenolic compounds.

The effects of grape seed polyphenol (GSP) on serum and hepatic lipid contents and on fecal steroid excretion were investigated by Nakamura and colleagues [152]. GSP was given orally at doses of 0.01-1.0 g/kg per day to normal rats and

hypercholesterolemic rats for 30 days. Their findings indicate that the GSP lowered the concentrations of serum and hepatic triglycerides rather than altering the metabolism of cholesterol [152].

Martin-Carron and colleagues [153] evaluated the effects of dietary fiber and polyphenol content from red and white grape peels and white grape seeds (100 g/kg) on lipid profile as well as on body weight, fat, and protein digestibility. Adult Wistar rats were fed cholesterol-free and cholesterol-added diets containing cellulose as a control or the test material for 42 days. Dietary fiber content of materials ranged from 540 to 590 g/kg. Intake of grape products increased stool weight and the amount of fat and protein excreted in feces, but did not affect growth or protein efficiency of the diet. All of the grape fractions lowered serum total cholesterol and LDL cholesterol concentrations in rats fed cholesterol diets [153].

The effect of grape pomace on serum and tissue cholesterol were compared to that of tomato and apple pomace [154]. Male Wistar rats were fed a cholesterol diet (0.3%) shortly after weaning continuing for 10 weeks with diets supplemented with the test material (5% in diet). None of the tested products affected serum cholesterol concentration. However, tomato and apple pomace significantly reduced cholesterol levels in the liver by 15% and 11%, respectively. All tested pomace reduced cholesterol content of the heart by approximately 20%. Triacylglycerol concentration was affected only by tomato pomace and solely in the heart. Cholesterol absorption measured by dual-isotope ratio method was not significantly affected by the pomace; however, all products strongly reduced the activity of HMG-CoA reductase in liver and increased the fractional catabolic rate of plasma cholesterol. All tested pomace reduced plasma levels of

conjugated dienes and tomato and grape pomace exhibited this effect also in the liver. All tested pomace reduced the activities of antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase in erythrocytes (by 34-56%) [154]. These results show that phenolic compounds from all three fruit pomaces are beneficial to the heart and related organs.

Inflammatory Markers

Li and colleagues [155] investigated whether grape seed proanthocyanidin can reduce swelling in croton oil-induced ear swelling in mice and carrageenan-induced hind paw edema in rats. Mice were injected with proanthocyanidins (10, 20, 40 mg/kg) or dextrose (2 mg/kg) thirty minutes before edema was induced. The edema extent was expressed as the weight difference between the inflamed and the control. The paws of the rats were treated in the same manner. Paw thickness was measured before and up to 12 hours after inflammation induction. The paw and exudate were harvested for measurement of pro-inflammatory molecules. Grape proanthocyanidins dose-dependently reduced swelling and inflammatory markers (NO, malondialdehyde, IL-1β, TNF-α, and PGE_2) in this animal model [121].

Terra and colleagues [156] used a rat model to investigate the effect of grape procyanidins in combination with high fat diet on inflammatory markers. The inflammatory markers, CRP, IL-6 and TNF-α increased with the high fat diet and diminished with grape procyanidins treatment. The anti-inflammatory cytokine, adiponectin, is decreased in the rats fed the high fat diet and significantly increased in the rats treated with procyanidins [156]. Procyanidin treatment may be beneficial in

increasing the adiponectin production and in reducing chronic inflammation in rats fed a high fat diet.

Another phenolic compound of grapes that has been investigated for their anti-inflammatory properties is piceatannol [157]. Piceatannol is a polyphenol found in grapes that acts as a protein kinase inhibitor which modifies multiple cellular targets including TNF-induced NF-κB inhibition [158]. Kim et al [157] investigated the effect of piceatannol in BALB/c mice given dextran sulfate sodium (DSS) in their water (control group received water only) to induce colitis. The DSS treated mice had higher plasma concentrations of NO and PGE_2 , however, treatment with the piceatannol reduced production of NO and PGE_2 in a dose-dependent manner. Similarly, DSS treatment significantly increased plasma concentrations of the inflammatory cytokines IL-1β, IL-6, and TNF-α and treatment with piceatannol (10mg/kg) inhibited cytokine production in this animal model. mRNA levels for iNOS and COX-2 were significantly induced by DSS, and again, reduced by treatment with piceatannol. This experiment demonstrates that individual components of grapes are potent anti-inflammatory agents [157].

Other Effects

The effects of two red wine polyphenol compounds on blood pressure were investigated by Diebolt and colleagues [159]. Two powder preparations of red wine polyphenol were given to rats intragastrically (20 mg/kg) for 7 days. Blood pressure dropped significantly on the 4th day after administration of both powders. This hemodynamic effect was associated with an enhanced endothelium-dependent relaxation and an induction of gene expression of inducible NO synthase and COX-2 within the

arterial wall [159]. In another study, red wine polyphenol powder fed to rats was shown to accelerate the decrease of blood pressure and improve structural and functional cardiovascular changes produced by chronic inhibition of NO synthesis created by administration of N-nitro-L-arginine methyl ester (L-NAME) [108].

Resveratrol has been shown to stimulate NO synthetase activity and reduce blood pressure in hypertensive female rats [160]. Resveratrol was shown to reduce blood glucose in rats with diabetes mellitus [161] and display cardioprotective effects [162].

Human Studies

Platelet Aggregation

The inhibitory effect of grape juice on platelet aggregation was studied by Keevil and colleagues [163]. Ten healthy participants were asked to consume 5-7.5 ml/kg per day of either purple grape, orange, or grapefruit juice in a randomized cross-over design. A 66% decrease in aggregation was observed after a week of drinking purple grape juice whereas there was no change after drinking orange or grapefruit juice [163]. The observed effect on platelet aggregation by the different juices may be due to differences in the content of phenolic compounds. Purple grape juice had a total polyphenolic concentration of almost three times that of orange or grapefruit juice (2.26, 0.75 and 0.86 g/L, respectively). This is a clear demonstration that the amount and class of polyphenols present in the fruit juice are responsible for the reduction of platelet aggregation.

Pace-Asciak and colleagues [164] investigated the effects of different grape beverages on platelet aggregation. Red wine, white wine, purple grape juice and purple grape juice supplemented with resveratrol were given to 24 healthy male participants for

a 2-week period. Red wine did not have an advantage over the white wine and the grape juice supplemented with resveratrol did not significantly reduce platelet aggregation in comparison to the commercial grape juice. These findings suggest that the dominant anti-aggregatory component in the beverages may have been dominated by the alcohol rather than the resveratrol [164].

Freedman and colleagues [165] also compared the effects of purple grape juice polyphenols on platelet aggregation, nitric oxide, and superoxide production. This study used 20 healthy participants who consumed 7 mL/kg per day of purple grape juice for 14 days. Purple grape juice significantly reduced platelet aggregation and superoxide production, but increased the release of NO [165]. These results show that purple grape juice is cardioprotective by inhibiting platelet aggregation due to its anti-oxidative potential.

Oxidative Properties

A study by O'Bryne and colleagues [166] compared the antioxidant properties of Concord grapes to α-tocopherol. Participants received either α-tocopherol (400 IU/day) or Concord grape juice (10 ml/day) for two weeks. Concord grape juice and α-tocopherol increased serum antioxidant capacity and protected LDL against oxidation. However, Concord grape juice decreased native plasma protein oxidation significantly more than did α-tocopherol. Concord grape juice was shown to protect against oxidative stress and reduce the risk of free radical damage in healthy adults by reducing the concentration of oxidized LDL involved in atherosclerosis [166].

Inflammatory Markers

Badia and colleagues [167] investigated the effect of two alcoholic beverages with high (red wine) or low (gin) polyphenolic content on TNF-α induced adhesion of human monocytes to endothelial cell. A randomized, crossover trial was conducted with eight healthy men who received 30 g ethanol/d as red wine or gin for 28 days. TNF-α induced adhesion of monocytes to endothelial cells was virtually abolished after red wine consumption but was only partially reduced after gin consumption [167]. Both beverages showed anti-inflammatory effects by reducing plasma fibrinogen and IL-1 levels. However, wine had the additional effect of decreasing high sensitivity C-reactive protein (hs-CRP), as well as monocyte and endothelial adhesion molecules [168]. While the alcohol is responsible for part of the anti-inflammatory effect, the polyphenols in the wine are much more effective in decreasing markers of inflammation.

A similar study investigated the effect of wine on lipid and inflammatory markers in healthy women [169]. In a cross-over study, thirty-five healthy women were given red wine or white wine with an equivalent of 20 g ethanol/d for 4 weeks. Serum HDL cholesterol increased while serum concentration of hsCRP, intercellular adhesion molecule-1, CD40L, and IL-6 decreased after either wine. Enhanced adhesion of monocytes to stimulated endothelial cells was reduced by 51% and 89% after consumption of white and red wine, respectively [169]. The findings of this study show that moderate wine consumption is associated with beneficial effects on various inflammatory pathways related to endothelial activation in healthy women.

The effect of wine was also investigated in men with angiographically-proven coronary artery disease [170]. Thirteen men consumed 2 to 3 glasses (4 mL/kg) of red

wine or white wine during a meal in a random cross-over design. Moderate wine intake acutely increased plasma levels of IL-6 in men with coronary artery disease. The researchers suggested that the increase in plasma IL-6 is a response to alcohol-induced oxidative stress in the liver [170]. There was no difference in the acute response between red wine and white wine. This suggests that the alcohol portion of the wine creates an inflammatory response in the liver of already compromised individuals but can be a preventative treatment for healthy individuals.

Castilla and colleagues [171, 172] conducted several studies on the effect of grape juice on the risk of cardiovascular disease in hemodialysis patients. In the first study, hemodialysis patients and healthy subjects were instructed to drink concentrated red grape juice (100 ml/d) daily for 14 days. The findings of their first study showed that red grape juice given to hemodialysis patients improved lipoprotein profile, reduced plasma concentrations of MCP-1 and oxidized LDL (oxLDL), and may favor a reduction in cardiovascular disease risk in this population [172]. In the second study, the effect on lipid profile of red grape juice was compared to that of vitamin E [171]. Thirty-two patients undergoing hemodialysis were divided into four groups: red grape juice (100 ml/day), vitamin E (800 IU), both, or the control (neither supplement nor placebo). Red grape juice, but not vitamin E, reduced plasma concentrations of total cholesterol and apo-b and increased HDL cholesterol concentrations. Both red grape juice and vitamin E reduced plasma concentrations of oxLDL and *ex vivo* neutrophil NADPH oxidase activity [171]. Regular ingestion of concentrated red grape juice by hemodialysis patients reduced neutrophil NADPH-oxidase activity and plasma concentrations of oxidized LDL and inflammatory biomarkers to a greater extent than vitamin E. This effect of red grape juice

consumption may favor a reduction in cardiovascular risk by alleviating atherosclerosis induced by oxidized LDL. Vitamin E is effective in reducing oxidative stress but did not influence the concentration of plasma cholesterol. However, red grape juice influenced both oxidative stress and plasma cholesterol making it more cardioprotective than vitamin E.

Renaud and colleagues [173] investigated the correlation of wine consumption and the rate of death from coronary artery disease. Thirty six thousand five hundred and eighty three middle-aged healthy men participated by completing an extensive questionnaire on alcohol use and a physical examination to determine the effect of beverage on coronary artery disease. Death due to coronary artery disease was monitored for over twenty years. Moderate wine drinkers had a significantly lower death rate than heavy wine drinkers and men that drank other types of alcoholic beverages [173].

Oklahoma Grapes

Grapes have been an important part of Oklahoma's history even before it was a state. *Vitis doaniana,* a wild grape named after winemaker Judge J. Doan, grew abundantly in the Indian Territory. In 1890, a year after the territory was opened to settlers, Edward Fairchild, a winemaker from New York, bought land near Oklahoma City for a vineyard and orchard [174]. Fairchild successfully grew Concord and Delaware grapes to make his wine until Oklahoma became a state in 1907. Oklahoma joined the union as a dry state thereby halting wine production [174]. Yet, in the 1910 census, Oklahoma reported over 4000 acres of vineyards, ranking it eighth in the nation in grape production. Thomas Volney Munson, a nurseryman, grape hybridizer, and ampelographer declared Oklahoma as a splendid grape-growing region [174]. Grape growing in

Oklahoma is becoming more widespread as researchers release hybrid varieties with desirable fruit characteristics: vine vigor, disease resistance, productivity, drought resistance, cold hardiness, and fruit acceptability for juice, jelly, table use, and wine [175].

In recent years, there has been an increase in grape production in Oklahoma. While approximately 170 acres of grapes were grown in Oklahoma in 1997, that number grew to 375 acres in 2002 and 525 acres in 2005. In 2007, the state boasted over fifty licensed wineries up from just four wineries six years earlier [176]. Small wineries also generate annual sales between $200,000 and $1.5 million, create employment, and spur the economic development of the surrounding area [177]. Yet, even though Oklahoma is a splendid grape growing area, development of hardier, disease-resistant varieties is vital to the industry.

Most grape varieties currently being grown in Oklahoma originated in Europe. Cross-breeding of grapes became necessary because *V. vinifera,* which originated in the Caspian Sea region, required a long growing season with relatively high summer temperatures, low humidity, a rain-free harvest period, and mild winter temperatures [178]. Not only was the growing season not suitable for other areas, but this European species was also susceptible to numerous afflictions such as insects, fungus, and viral and bacterial infections.

During the many failed attempts to introduce varieties of *V. vinifera* to North America, some of the *V. vinifera* naturally crossed with native varieties such as fox grape (*V. labrusca*), the riverbank grape (*V. riparia*), and the scuppernong (*V. rotundifolia*) to create varieties that would thrive on the North American continent [179]. The post oak

grape (*V.lincecumii*) is native to Oklahoma and surrounding states. This grape was hailed by Munson as being especially important for creating hybrid grape varieties [178]. Meanwhile, many of the world varieties of the European species must be grafted onto rootstocks of North American species to prevent devastation by the phylloxera root louse.

Rubaiyat is one of many varieties of grapes developed by Herman Hinrichs at Oklahoma State University. This variety was developed in 1952 as a cross between Seibel 5437 and Bailey. The overall genetic constitution of Rubaiyat is 37.5% *V. lincecumii*, 31.25% *V. vinifera*, 18.75% *V. labrusca*, 6.25% *V. rupestris*, and 6.25% *V. riparia*. Some characteristics of Rubaiyat include its medium vigor, medium cluster size, large berry size, disease resistance, cold hardiness, and usefulness as a teinturier (add color in blends)[178].

The increasing trend in Oklahoma towards grape production has sparked the need to understand the health benefits associated with grapes grown locally, including their anti-inflammatory properties. Increased knowledge of the health benefits of grapes may further help growers to promote their products and increase their consumption which will help Oklahoma's viticulture and agritourism business.

In this study we will investigate the concentration of total phenolic, flavonoids, and anthocyanins found in thirty-three varieties of grapes grown at the Cimarron Valley Research Station in Perkins, Oklahoma. The oxidative potential will then be measured to determine if the grape juice has anti-oxidative properties and *in vitro* investigation with LPS-induced RAW264.7 murine macrophage to see the effects of treatment with the juice on NO production. From this data, ten varieties were chosen for further analysis as extract. The concentration of extract for the maximum inhibition of NO will be

determined with LPS-induced macrophages. And finally, one variety with high total phenolic content and greatest inhibitory effects was chosen to compare against Resveratrol in production and expression of COX-2, IL-6, iNOS, and TNF-α which are markers of inflammation. The results indicate the cardioprotective properties of grapes by the ability to reduce NO, TNF-α, and IL-6 production and the reduced expression of TNF-α, iNOS, IL-6, and COX-2.

CHAPTER III

METHODOLOGY

Specific Aim 1: *To determine the phenolic content, anti-oxidant capacity, and anti-inflammatory properties of juice from different varieties of grapes grown in Oklahoma.*

Grape Juice Preparation

Thirty-three different varieties of grapes were obtained from the Cimarron Valley Research Station (Perkins, OK) at their respective harvest times in 2007 (**Table 3**). The vineyard utilized three different trellis systems; high cordon, Geneva double curtain, and vertical shoot positioning system. The trellis system allows for increased control of damage by insects and disease. More damage resulted from a spring freeze that occurred during budbreak. Varieties with early budbreak such as Cabernet Franc, Chardonnay, Viognier, Merlot, and Sangiovese had some damage however it was not significantly more than the other varieties. Another source of some damage was a phenoxy herbicide drift from an unknown source which damaged mostly the Sunbelt, Cimarron, Cynthiana, and Traminette [175]. Upon arrival to the laboratory, the grapes were refrigerated at 4ºC for less than 48 hours until processing. The grapes were washed and air dried at room temperature on a paper towel and the stems were removed before preparing the juice.

Grape juice was prepared two ways: smashed with a flask in a Buchner funnel or pureed in an Oster blender. Grape juice was then filtered twice in a Buchner funnel with No.4 Whatman paper filter, divided into several aliquots, and stored at -20ºC until analysis. The remaining whole grapes were also frozen at -20ºC for use if necessary.

Table 3: Harvest Time of Grapes used in this Study[1]

Variety	*Harvest Time*
Cabernet Franc	September 27, 2007
Cabernet Sauvignon	September 27, 2007
Chambourcin	August 28, 2007
Chardonel	August 17, 2007
Chardonnay	September 13, 2007
Cimmaron	August 23, 2007
Corot Noir	Not Available
Cynthiana	September 4, 2007
Frontenac	August 13, 2007
Gamay	August 28, 2007
GG 9318	October 9, 2007
GG 9330	October 9, 2007
GG 9336	October 9, 2007
GG 9356	October 9, 2007
H1 #249	September 5, 2007
H2 #211	September 5, 2007
H5 #125	September 5, 2007
Merlot	September 13, 2007
Montepulciano	October 4, 2007
Petit Verdot	September 27, 2007
Pinot Gris	September 13, 2007
Rubaiyat	October 4, 2007
Ruby Cabernet	September 27, 2007
Sangiovese	September 13, 2007
Sauvignon Blanc	August 28, 2007
Shiraz	September 13, 2007
Sunbelt	October 4, 2007
Traminette	August 28, 2007
Vignoles	August 17, 2007
Villard Blanc	Not Available
Viognier	August 23, 2007
White Riesling	August 28, 2007
Zinfandel	October 4, 2007

[1]Grapes were grown at Cimarron Valley Research Station (Perkins, OK).

Total Phenolic, Anthocyanin, and Flavonoid Content

The total phenolic content of the juices was determined using the Folin Ciocalteau assay [180]. The total phenolic assay utilizes the reaction between Folin-Ciocalteau's reagent and phenol to form a chromogen that can be measured spectrophotometrically. Gallic acid (Sigma Aldrich, St. Louis, MO) dissolved in 40% ethanol was used as the standard (0-1000 μM). An aliquot of each grape juice or standard solution was mixed with Millipore water and Folin-Ciocalteau reagent (Sigma Aldrich, St. Louis, MO). The reaction was neutralized with 20% sodium carbonate solution and Millipore water was added followed by incubation at room temperature for 2 hours. The absorbance was read at 760 nm using a Beckman spectrophotometer (Fullerton, CA). Total phenolic content was calculated from the standard curve and expressed as mg/L gallic acid equivalent.

Analysis of flavonoid content was based on the colorimetric assay of Kim and colleagues [181]. Catechin dissolved in water was used as the standard (0-200 mg/L). Grape juice or standard solution, Millipore water, 5% sodium nitrite solution, 10% aluminum chloride solution, and 1M NaOH were mixed in a test tube. The absorbance was read at 510 nm using a Beckman spectrophotometer (Fullerton, CA). Flavonoid concentration was calculated from the standard curve and results were expressed as milligram of catechin equivalents per liter grape juice.

Anthocyanin concentration was determined with a modified pH differential method also used by Lee and colleagues [182]. Potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5) were added to the grape juice. The grape juice to buffer ratio was 1:3 and 1:8 for white grapes and purple grapes, respectively. Absorbance was read at 510 and 700 nm using the Biotek microplate reader (Synergy,

Winooski, VT). Total monomeric anthocyanin content was calculated according to the equation:

Anthocyanin concentration (mg/L) = ΔA x MW x DF x 1000/ (ε x c)

where ΔA, absorbance = [($A_{510} - A_{700}$) pH1 – ($A_{510} - A_{700}$) pH4.5]; MW = molecular weight of anthocyanin (449.2 g/mol) ; DF = dilution factor (1:3 for white grapes, 1:8 for purple/red grapes); ε = molar absorptivity of cyanidin-3-glucoside (26,900 L/cm mol); and c = cell pathlength (1 cm).

Ferric Reducing Ability (FRA) Assay

Anti-oxidant capacity was measured by the FRA assay [183]. The working FRA reagent was prepared with 300 mM acetate buffer, 10 mM 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ), and 20 mM $FeCl_3$ in the ratio of 10:1:1. Ferrous sulfate solution (0-500 μM) was used as standard. Each sample or standard solution and FRA reagent were added and mixed in a 96-well plate. Absorbance was read at 593 nm every 2 minutes starting at zero up to twenty minutes of mixing time. FRA was calculated according to the equation [183]:

FRA value (μM) = {(0-10min. ΔA_{593nm} sample)/(0-10min.ΔA_{593nm} std)} FRA_{std} (μM)

where ΔA is the change in absorbance.

Cell Propagation and Treatment

RAW264.7 murine macrophages from American Type Culture Collection (Manassas, VA) were grown in Dubelcco's Modified Eagles Media (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), and 1% penicillin/streptomycin (Sigma

Aldrich, St. Louis, MO). The cells were incubated at 37ºC with 5% CO_2. After 24 hours, the media was replaced with fresh media. The flask was checked with a microscope for confluent growth after 48 hours and split into other flasks as needed.

Treatment of the cells with grape juice was performed in a 96-well plate containing approximately 2.0 X 10^5 cells/well. The plates were incubated at 37ºC with 5% CO_2 for 24 hours. Media containing different concentrations of grape juice (0, 1, 5, and 10%) were added to the cells which were then incubated at 37ºC with 5% CO_2 for another 24 hours. Media containing juice was removed and cells were washed with phosphate buffered saline. The cells were then given new media containing 500 ng/ml of *Escherichia coli* lipopolysaccharide (LPS, Serotype 0127:B8, Sigma, St. Louis, MO). After 24 hours of LPS treatment, media was collected for assessment of nitric oxide and the viability of the cells was also assessed. All experiments were repeated at least three times.

Cell Viability

Cell viability was assessed using a commercially available resazurin kit (Sigma Aldrich, St. Louis, MO). Resazurin detects cell viability by the conversion of a nonfluorescent dye to a highly red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth. Continued cell growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the redox indicator dye to change from the oxidized (nonfluorescent, purple color) form to the reduced (fluorescent, red color) form. Resazurin solution in cell media (5%) was added into each well and the plate was

incubated at 37°C with 5% CO_2 for 4 hours. Absorbance was read at 520 nm using a Biotek microplate reader (Synergy, Minooski, VT). Cell viability was expressed as a percentage of the control.

Assessment of Nitric Oxide

Nitric oxide was measured with the Promega Griess reagent system (Madison, WI). This nitrate kit utilizes a two step process for measurement of total nitrite concentration in a sample. The first step is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of Griess reagents (sulfanilamide and N-(1-Napthyl) ethylenediamine (NED)) which converts nitrite into a deep purple azo compound. The intensity of the color is directly proportional to the nitrite concentration.

Briefly, nitrate standard solution or samples (cell media collected after grape juice and LPS treatment) were pipetted into a 96-well plate. Sulfanilamide solution was added to each well and allowed to incubate at room temperature in the dark for 10 minutes. The NED solution was then added and allowed to incubate for 10 minutes at room temperature in the dark. The absorbance was read within 30 minutes at 520 nm using a Biotek microplate reader (Synergy, Winooski, VT). Nitric oxide was calculated from the nitrate standard curve.

Specific Aim 2: *To assess the in vitro anti-inflammatory properties of extract from Oklahoma grape varieties with high and low total phenolic content using LPS-stimulated RAW264.7 macrophage.*

Grape Extract Preparation and Analyses

Base on the results of Specific Aim 1, ten grape varieties (five varieties with relatively high total phenolic content and five varieties with relatively low total phenolic content) were chosen (**Table 4**). Grapes were pureed for one minute in an Oster blender with a solution of methanol, ethanol, and water (50:25:25 v:v:v). The puree was sonicated in ice for 30 minutes, and centrifuged for 30 minutes at 10,000 g at 4°C. The supernatant was poured through a 0.45μm filter with vacuum and evaporated (Rotovap, BÜCHI, Flawil, Switzerland) to the aqueous fraction. The aqueous fraction was filtered through a C18 (Waters Corp., Milford, MA) column in accordance with the product instructions to remove most of the carbohydrates. The resulting methanol fraction was evaporated (Rotovap, BÜCHI, Flawil, Switzerland) to dryness and the remaining extract was reconstituted with sterile water. The extract was aliquoted and frozen at -80°C until needed for cell culture. The total phenolic, flavonoid, and anthocyanin concentrations of the grape extract were determined using the Folin assay, and according to the methods of Kim, Lee and colleagues [181, 182] as described earlier.

Cell Treatment

RAW264.7 cells were plated on a 96-well plate at a seeding density of 2×10^5 cells/well. The cells were incubated for 24 hours at 37ºC with 5% CO_2, then the media was removed and the cells were washed with PBS. The cells were then treated with grape extract (0, 0.01, 0.1, and 1%) in complete media and incubated for 24 hours. The media was removed and the cells were washed with PBS. Finally, the cells were stimulated with

500 ng/ml LPS and incubated for another 24 hours. After LPS treatment, media was collected for nitric oxide analysis and the remaining cells were used to assess cell viability. All experiments were repeated at least three times. These methods were described earlier.

Table 4: Ten Grapes Varieties Chosen for *in vitro* Investigation of Anti-Inflammatory Properties of Grape Extract[1]

Varieties	*Total Phenolic (mg/L)*	*NO Production (μM)*
Cabernet Sauvignon	312.9 ± 30.9	40.7
Chardonney	294.6 ± 12.2	58.3
Cynthiana	524.6 ± 129.1	54.2
Pinot Gris	264.1 ± 29.4	29.4
Rubaiyat	689.1 ± 30.8	57.3
Ruby Cabernet	247.2 ± 29.1	39.9
Sauvignon Blanc	186 ± 42.3	34.5
Sunbelt	281.7 ± 86.7	64.4
Viognier	408.± 75.5	36.6
Zinfandel	1660 ±93.4	57.0

[1]Grape varieties were chosen based on total phenolic content (five varieties with relatively high total phenolic content and five varieties with relatively low total phenolic content) and effect on NO production using the grape juice (results of specific aim 1).

Specific Aim 3: *To compare the effect of Rubaiyat grape extract, a native Oklahoma grape variety, to that of resveratrol on expression of inflammatory genes in LPS-stimulated RAW264.7 macrophages.*

Cell Treatment

RAW 264.7 macrophages were plated into a six-well plate at a seeding density of 2 X 10^6 cells/well. After 24 hours, cells were induced with 500 ng/ml LPS and treated with 30 μM resveratrol (Sigma Aldrich, St. Louis, MO) , 0.1% Rubaiyat grape extract, or 1% Rubaiyat grape extract and a control of media only. The following day the supernatant was removed and analyzed for nitric oxide production (Greiss reagent, Promega) and the remainder stored at -80°C for cytokine analyses. The RNA was extracted from the cells for the assessment of expression of inflammatory genes.

RNA Extraction

RNA STAT-60™ (Iso-tex Diagnostics Inc, Friendswood, TX) was added to each well (1 ml/ 5 X 10^6 cells) to isolate the cellular RNA. The lysate was transferred to an RNase free centrifuge tube and stored for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform was then added (0.2 ml/ml of the RNA STAT-60™) to the homogenate, mixed, allowed to stand at room temperature for 2-3 minutes, and centrifuged at 12,000 g for 15 minutes at 4°C. The clear aqueous phase was carefully transferred to a new tube. Isopropanol (0.5 ml/ml STAT-60™) was added to precipitate the RNA, placed at -80°C for 1 hour, and centrifuged at

12,000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol, vortexed and centrifuged at 7,500 g for 5 minutes at 4°C. After centrifugation, the supernatant was poured off and the RNA pellet was air-dried. The pellet was dissolved in DEPC water and vortexed. The yield and quality of RNA was assessed with the nanodrop spectrophotometer (Nanodrop, Wilmington, DE).

Assessment of Inflammatory Gene Expression by Real Time-PCR

A working stock of reverse and forward primers for COX-2, IL-6, iNOS, TNF-α, and cyclophilin were prepared in DEPC water at a final concentration of 2.5 μM for each primer. The primer working stock, SYBR green, and DEPC-water were mixed to create the PCR Mastermix. DNA sample was pipetted into its respective well according to the prepared template and the PCR Mastermix was then added to each well. The plate was sealed well and spun at 3000 rpm for 5 minutes.

Detection of the reaction was performed on a 384-well plate in a 10 μL reaction mix containing unkown cDNA samples, forward and reverse primers, and SYBR Green I PCR reagents (Applied Biosystems, Foster City, CA, USA). The primers used for PCR are shown in **Table 5**. Amplifications of RNA were detected constantly by realtime quantitative PCR on 7900 HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA). The PCR amplification protocol is as follows: (i) initial attenuation at 95°C for 10 minutes; and (ii) three-segment amplification and quantification involving 40 cycles of 50°C for 120 s, 95°C for 10 min, 95°C for 9 s, 60°C for 60 s, 95°C for 9 s, and 60°C for 9 s. The data was expressed as relative abundance of RNA.

Table 5: Primer Sequences used for Real-time PCR.

Symbol	***Name***	***Sequence***
COX-2	Cyclooxygenase-2	QF 5'tgaagacgtcctccactcatg3’
		QR 5'cctgggatggcatcagttt3’
IL-6	Interleukin-6	QF 5'gaggataccactcccaacagacc3’
		QR 5'aagtgcatcatcgttgttcataca3’
iNOS	Inducible Nitric Oxide Synthase	QF 5'caggaggagagagatccgattta3’
		QR 5'gcattagcatggaagcaaaga3’
TNF-α	Tumor Necrosis Factor Alpha	QF 5`ctgaggtcaatctgcccaagtac3`
		QR 5`cttcacagagcaatgactccaaag3`
CYCLO	Cyclophilin	QF 5`tggagagcaccaagacagaca3`
		QR 5`tgccggagtcgacaatgat3`

ELISA Assays for TNF-α and IL-6

Enzyme linked immunosorbent assay (ELISA) kits from BD Biosciences (San Diego, CA) were used to determine concentrations of TNF-α and IL-6 in the media. The wells of microtiter plate were coated with anti-mouse TNF-α and IL-6 monoclonal antibodies before use. The plate was then coated with second biotinylated anti-mouse polyclonal antibody to capture the analytes in the samples. Unbound materials from samples were then washed off and horseradish peroxidase was applied to the immobilized biotinylated antibodies. The activity of horseradish peroxidase was monitored using 3, 3’,

5, 5'-tetramethylbenzidine as a substrate and absorbance at 450 nm was measured using a microplate reader (Biotek Synergy, Winooski, VT). The captured mouse TNF-α and IL-6 in the media is directly proportional to the increase in absorbance at 450nm/590nm.

Statistical analyses

Statistical analyses involved computation of least square means and standard error (SE) for each of the treatment groups using SAS version 9.1 (SAS Institute, Cary, NC). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. Differences were considered significant at $P < 0.05$

CHAPTER IV

FINDINGS

Specific Aim 1: *To determine the phenolic content, anti-oxidant capacity, and anti-inflammatory properties of juice from different varieties of grapes grown in Oklahoma*

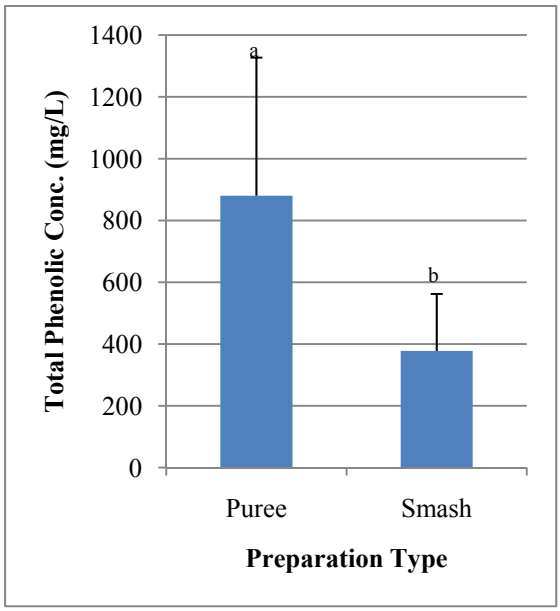
Total Phenolic, Flavonoid, Anthocyanin, and Anti-Oxidant Capacity of Grape Juice

Two methods of preparation of grape juice were used in the first part of the study – smashed and pureed. Total phenolic, flavonoid, anthocyanin, and anti-oxidant capacity (FRA) were all significantly less in the juice obtained by smashing the grapes compared to the pureed grapes (**Figure 1**). The total phenolic and flavonoid concentration of the pureed grapes was more than double the concentration from the smashed grapes (**Figures 1A and 1B**). A greater difference was noted in the anthocyanin and FRA concentrations of the juice from smashed grapes which was approximately only one-third the concentration of juice from pureed grapes (**Figures 1C and 1D**).

For most of the grape varieties except for Cynthiana, H2 #211, H5 #125, Shiraz, and Viognier, the total phenolic concentration of the juice obtained by pureeing was significantly higher than those obtained from smashing (**Table 6 and 7)**. This trend was also reflected in the flavonoid concentration where nearly 80% of the grape varieties had

Figure 1: Effect of Grape Juice Preparation on (**A**) Total Phenolic, (**B**) Flavonoid, (**C**) Anthocyanin, (**D**) Anti-Oxidant Capacity (FRA) from Combined Data of Thirty-three Oklahoma Grape Varieties

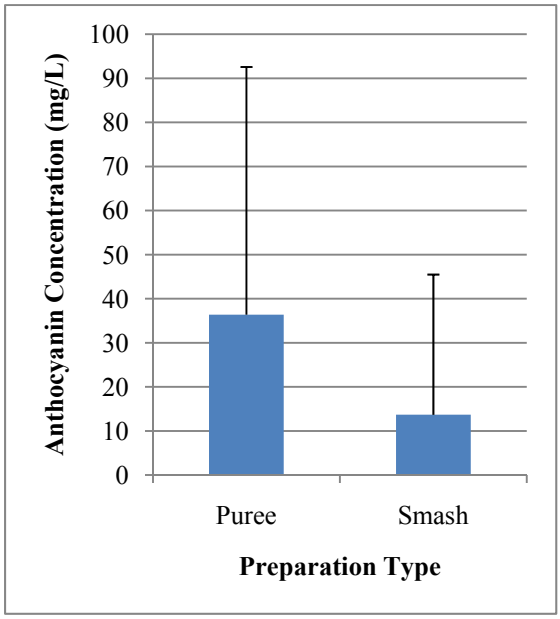
(A)



(C)

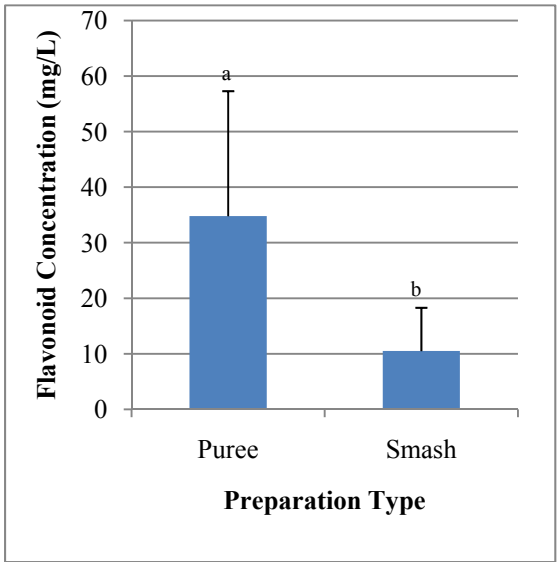
a

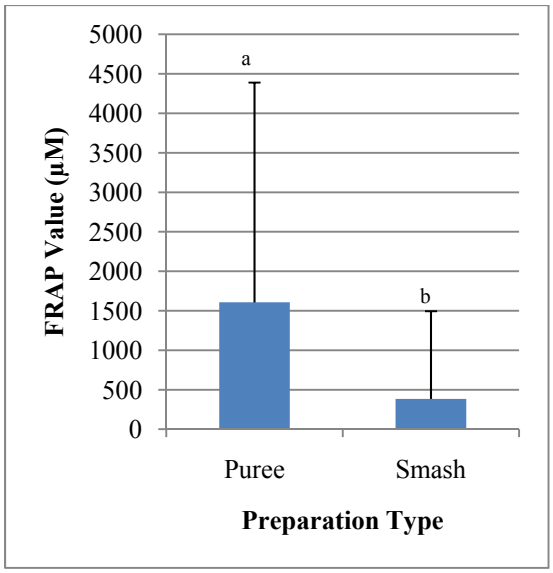
b



(B)

(D)





Bars are mean ± SD. Bars that do not share the same letters are significantly ($P < 0.05$) different.

significantly higher flavonoid concentration in pureed grapes in comparison to smashed grapes except for Chardonel, Frontenac, H5 #125, Pinot Gris, Vignoles, and Villard Blanc which displayed no significant difference in between juice preparations (**Table 6 and 7**). Only about half of the varieties showed a significant difference between preparations for anthocyanin and FRA concentration (**Table 6 and 7**). As expected, the white grapes (Chardonel, Chardonney, Sauvignon Blanc, Traminette, Vignoles, Villard Blanc, and Viognier) except for the White Riesling all had low to zero anthocyanin concentration and FRA values (**Table 6 and 7**). Grape varieties with high anthocyanin concentration (Rubaiyat, Sunbelt, and Frontenac except for Zinfandel) also had high FRA concentrations (**Table 6 and 7**).

When comparing smashed grape juice only, total phenolic concentration was significantly higher in Petit Verdot (794.8±91.5 mg/L) and Rubaiyat (689.1±30.8 mg/L), which are purple grapes, and H1 #249 (752.3±189.3 mg/L), a pink grape (**Table 7**). The grape variety with the lowest total phenolic concentration was Chardonel (162.4±72.5 mg/L) which is statistically similar to Pinot Gris (264.1±29.4 mg/L), Traminette (203.3±31.8 mg/L), White Riesling (202.4±6.4 mg/L), Villard Blanc (193.0±64.6 mg/L), and Sauvignon Blanc (186.0±42.3 mg/L), all white grapes and Cabernet Franc (250.8±33.1 mg/L), GG 9336 (225.2±6.4 mg/L) which are red grapes (**Table 7**). The grape varies with the highest flavonoid concentrations of the smashed preparation were Frontenac (30.1±4.7 mg/L) and Rubaiyat (30.1±6.4 mg/L) while the lowest flavonoid concentration was found in the Pinot Gris (0.5±0.9 mg/L) (**Table 7)**. Rubaiyat had the highest anthocyanin concentration (143.9±1.3 mg/L) while Sauvignon Blanc and Villard Blanc, both white grapes, had very little or no anthocyanin content (**Table 7**).

Table 6: Total Phenolic, Flavonoid, Anthocyanin, and Anti-Oxidant Capacity (FRA) of Oklahoma Grapes by Pureed Preparation[a]

Variety[b]	*Total Phenolic*[c] *(mg/L)*	*Flavonoid*[d] *(mg/L)*	*Anthocyanin*[e] *(mg/L)*	*FRA Value*[f] *(μM)*
Cabernet Franc	*805.6 ± 18.3	*51.6 ± 3.5	12.3 ± 6.0	*628.9 ± 291.1
Cabernet Sauvignon	*586.0 ± 24.0	*24.5 ± 3.7	9.1 ± 2.2	*217.8 ± 35.9
Chambourcin	*825.8 ± 162.9	*13.5 ± 2.9	53.9 ± 25.1	775.4 ± 451.7
Chardonel	*585.7 ± 16.5	14.3 ± 2.2	0.4 ± 0.3	5.7 ± 5.4
Chardonnay	*361.6 ± 2.8	*14.2 ± 1.4	0.4 ± 0.5	6.0 ± 7.2
Cimmaron	*952.4 ± 125.7	*57.1 ± 3.8	5.1 ± 4.9	277.0 ± 258.7
Corot Noir	*1500.9 ± 17.1	*109.0 ± 9.6	*81.8 ± 23.3	*8865.8 ± 2519.2
Cynthiana	744.7 ± 125.4	*22.2 ± 1.2	38.6 ± 15.8	846.5 ± 325.5
Frontenac	*1072.1 ± 51.5	34.0 ± 1.6	*149.0 ± 16.2	*5059.0 ± 573.4
Gamay	*681.5 ± 179.5	*24.6 ± 1.1	*10.7 ± 0.6	*264.0 ± 5.3
GG 9318	*643.4 ± 44.6	*77.7 ± 7.3	*70.0 ± 8.8	*5469.3 ± 1072.0
GG 9330	*689.2 ± 15.1	*43.2 ± 1.4	*66.2 ± 15.5	*2856.4 ± 677.4
GG 9336	*732.4 ± 35.0	*24.5 ± 1.5	*9.2 ± 2.2	*223.5 ± 44.0
GG 9356	*829.0 ± 96.5	*30.4 ± 1.1	*25.0 ± 1.4	*759.8 ± 50.9
H1 #249	*1843.8 ± 240.3	*38.8 ± 4.0	9.4 ± 9.3	384.1 ± 398.8
H2 #211	1172.0 ± 349.2	*20.7 ± 7.0	38.5 ± 18.8	*726.2 ± 301.5
H5 #125	1035.4 ± 286.6	18.2 ± 2.6	*16.7 ± 3.6	*309.6 ± 103.7
Merlot	*1025.9 ± 251.8	*37.1 ± 2.4	*18.3 ± 7.1	*685.7 ± 298.4
Montepulciano	*1086.7 ± 307.8	*35.0 ± 1.6	*29.8 ± 9.3	*1032.7 ± 281.5
Petit Verdot	*1817.7 ± 289.9	*63.3 ± 1.2	40.8 ± 24.8	*2571.8 ± 1549.7
Pinot Gris	*607.1 ± 97.3	17.5 ± 15.6	1.1 ± 1.0	22.9 ± 37.5
Rubaiyat	*1154.1 ± 37.0	*45.3 ± 4.6	*266.1 ± 42.1	*11939.2 ± 1208.6
Ruby Cabernet	*427.1 ± 96.8	20.5 ± 3.7	36.2 ± 13.4	*713.6 ± 210.9
Sangiovese	*433.3 ± 17.0	*29.9 ± 3.0	*24.7 ± 7.4	*726 ± 160.4
Sauvignon Blanc	*299.6 ± 47.5	*17.1 ± 0.4	*1.0 ± 1.0	17.8 ± 16.4
Shiraz	236.5 ± 17.9	*15.8 ± 1.4	*9.5 ± 2.9	*150.8 ± 49.5
Sunbelt	*1512.9 ± 128.7	*37.4 ± 2.8	*149.6 ± 12.8	*5571.7 ± 353.0
Traminette	*529.1 ± 105.8	*3.1 ± 0.3	0.4 ± 0.7	1.4 ± 2.4
Vignoles	*840.0 ± 223.8	23.9 ± 0.5	*0.0 ± 0.0	0.0 ± 0.0
Villard Blanc	*443.6 ± 16.0	13.6 ± 1.2	*0.0 ± 0.0	0.0 ± 0.0
Viognier	630.6 ± 129.9	*52.8 ± 0.1	*0.0 ± 0.0	0.0 ± 0.0
White Riesling	*1275.5 ± 170.2	*41.0 ± 1.9	*0.5 ± 3.8	106.9 ± 149.8
Zinfandel	*1660.6 ± 557.4	*77.3 ± 2.1	*2.1 ± 8.1	1810.2 ± 641.0

[a]Values or mean ± SD. Superscript letters are comparison of varieties. Values that do not share the same letter are significantly different ($P < 0.05$) from each other. Asterisk represent significant difference ($P < 0.05$) between the methods of preparation (smashed vs. pureed) for a particular grape variety.
[b]Obtained from Cimarron Valley Research Station (Perkins, OK) in 2007.
[c]Determined by the Folin-Ciocalteau assay
[d]Determined by the aluminum chloride assay
[e]Determined by the pH differential assay
[f]Determined by the FRAP assay

Table 7: Total Phenolic, Flavonoid, Anthocyanin, and Anti-Oxidant Capacity of Oklahoma Grapes by Smashed Preparation[a]

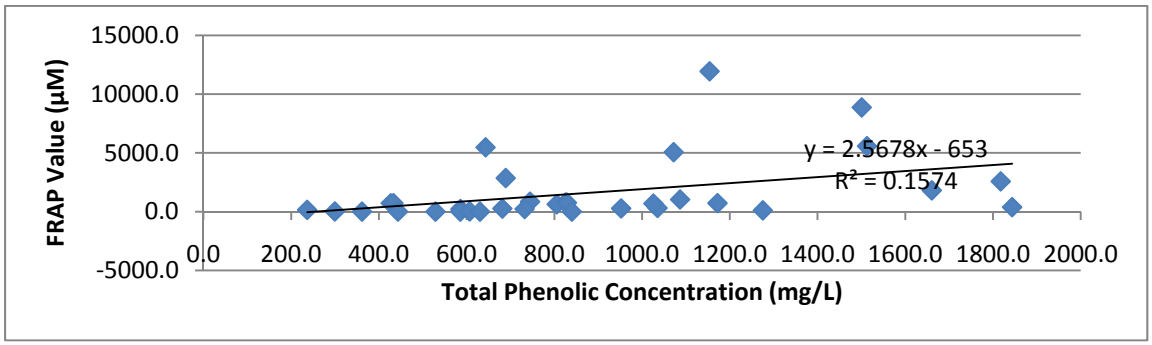
Variety[b]	*Total Phenolic*[c] *(mg/L)*	*Flavonoid*[d] *(mg/L)*	*Anthocyanin*[e] *(mg/L)*	*FRAP Value*[f] *(μM)*
Cabernet Franc	250.8 ± 33.1^{lmno}	9.2 ± 2.4^{ghi}	3.3 ± 0.7^{fgh}	30.7 ± 13.7^{c}
Cabernet Sauvignon	312.9 ± 30.9^{hijkl}	1.1 ± 1.9^{mn}	0.6 ± 0.6^{gh}	0.1 ± 0.1^{c}
Chambourcin	375.5 ± 47.0^{ghijk}	6.0 ± 2.7^{ijkl}	41.6 ± 5.6^{c}	254.0 ± 119.7^{c}
Chardonel	162.4 ± 72.5^{o}	14.9 ± 1.0^{cdef}	0.4 ± 0.3^{gh}	6.3 ± 5.3^{c}
Chardonnay	294.6 ± 12.2^{ijklmn}	7.7 ± 2.1^{hijk}	0.8 ± 0.5^{gh}	5.1 ± 3.0^{c}
Cimmaron	634.4 ± 15.7^{bcd}	13.5 ± 1.4^{ef}	0.4 ± 0.6^{gh}	4.7 ± 7.1^{c}
Corot Noir	439.1 ± 119.3^{efg}	4.7 ± 2.5^{jklm}	10.1 ± 3.2^{e}	42.2 ± 8.4^{c}
Cynthiana	524.6 ± 129.1^{def}	18.0 ± 1.1^{cd}	29.8 ± 6.4^{d}	538.2 ± 139.3^{c}
Frontenac	233.4 ± 23.3^{lmno}	30.1 ± 4.7^{a}	116.7 ± 11.7^{b}	3481.1 ± 237.4^{b}
Gamay	375.7 ± 41.2^{ghijk}	7.0 ± 2.2^{ijkl}	2.0 ± 0.8^{fgh}	15.2 ± 11.2^{c}
GG 9318	307.4 ± 40.1^{hijklm}	7.5 ± 1.1^{hijk}	5.6 ± 2.4^{efg}	40.1 ± 13.4^{c}
GG 9330	292.4 ± 35.0^{ijklmn}	6.1 ± 1.2^{ijkl}	1.6 ± 0.9^{fgh}	10.0 ± 6.9^{c}
GG 9336	225.2 ± 6.4^{lmno}	7.0 ± 1.5^{ijkl}	1.6 ± 0.5^{fgh}	11.0 ± 3.4^{c}
GG 9356	529.8 ± 50.3^{de}	22.6 ± 3.7^{b}	6.7 ± 3.6^{ef}	144.6 ± 65.2^{c}
H1 #249	752.3 ± 189.3^{ab}	11.9 ± 1.3^{fg}	2.5 ± 1.6^{fgh}	28.6 ± 15.4^{c}
H2 #211	628.6 ± 42.9^{cd}	7.6 ± 1.3^{hijk}	24.5 ± 3.7^{d}	186.7 ± 39.3^{c}
H5 #125	532.2 ± 177.3^{de}	18.5 ± 0.9^{c}	3.2 ± 2.5^{fgh}	58.6 ± 46.9^{c}
Merlot	567.4 ± 126.0^{d}	4.7 ± 1.2^{jklm}	1.2 ± 2.0^{gh}	4.3 ± 7.4^{c}
Montepulciano	385.6 ± 15.2^{ghij}	6.9 ± 1.4^{ijkl}	1.4 ± 0.9^{fgh}	9.4 ± 6.3^{c}
Petit Verdot	794.8 ± 91.5^{a}	11.4 ± 1.9^{fgh}	5.5 ± 5.0^{efgh}	60.3 ± 59.3^{c}
Pinot Gris	264.1 ± 29.4^{klmno}	0.5 ± 0.9^{n}	0.4 ± 0.3^{gh}	0.2 ± 0.3^{c}
Rubaiyat	689.1 ± 30.8^{abc}	30.1 ± 6.4^{a}	143.9 ± 1.3^{a}	4331.1 ± 874.8^{a}
Ruby Cabernet	247.2 ± 29.1^{lmno}	3.8 ± 3.3^{klmn}	3.8 ± 2.5^{fgh}	9.6 ± 11.0^{c}
Sangiovese	208.8 ± 43.1^{lmno}	8.0 ± 0.9^{ghij}	1.9 ± 2.0^{fgh}	13.7 ± 14.5^{c}
Sauvignon Blanc	186.0 ± 42.3^{no}	7.6 ± 0.3^{hijk}	0.1 ± 0.2^{h}	0.9 ± 1.5^{c}
Shiraz	269.7 ± 35.9^{jklmno}	11.4 ± 2.0^{fgh}	2.1 ± 0.6^{fgh}	23.6 ± 2.6^{c}
Sunbelt	281.7 ± 86.7^{jklmno}	14.4 ± 1.1^{def}	38.0 ± 7.3^{c}	543.9 ± 66.0^{c}
Traminette	203.3 ± 31.8^{lmno}	1.4 ± 0.1^{mn}	0.3 ± 0.5^{gh}	0.4 ± 0.6^{c}
Vignoles	408.4 ± 75.7^{fghi}	22.8 ± 3.8^{b}	0.2 ± 0.3^{gh}	4.1 ± 4.9^{c}
Villard Blanc	193.0 ± 64.6^{mno}	5.7 ± 5.1^{ijkl}	0.2 ± 0.2^{h}	0.5 ± 0.9^{c}
Viognier	424.7 ± 35.7^{efgh}	3.4 ± 1.7^{lmn}	0.7 ± 0.8^{gh}	2.9 ± 4.5^{c}
White Riesling	202.4 ± 6.4^{lmno}	3.2 ± 0.8^{lmn}	0.5 ± 0.2^{gh}	1.7 ± 0.4^{c}
Zinfandel	273.9 ± 93.4^{jklmno}	16.8 ± 1.9^{cde}	2.1 ± 0.8^{fgh}	2826.1 ± 2844.6^{b}

[a]Values or mean ± SD. Superscript letters are comparison of varieties. Values that do not share the same letter are significantly different ($P \leq 0.05$) from each other.
[b]Obtained from Cimarron Valley Research Station (Perkins, OK) in 2007.
[c]Determined by the Folin-Ciocalteau assay
[d]Determined by the aluminum chloride assay
[e]Determined by the pH differential assay
[f]Determined by the FRAP assay

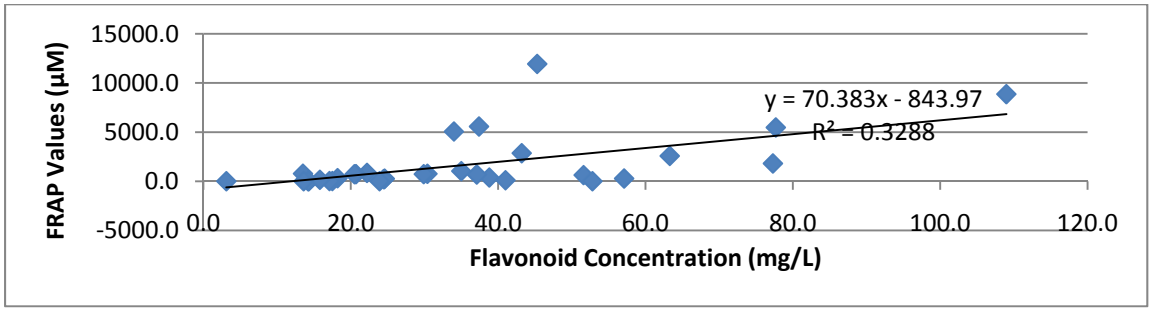
The highest FRA value was produced by Rubaiyat which was significantly higher than all the other varieties, especially the white grapes which displayed very little to zero anti-oxidant capacity (**Table 6**). There was a significant correlation between anthocyanin ($R^2 = 0.7870$, $P < 0.001$) and flavonoid ($R^2 = 0.6210$, $P < 0.001$) concentration with antioxidant capacity (**Figures 2B and 2C**). However, no correlation between total phenolic concentration and antioxidant potential ($R^2 = 0.07943$, $P = 0.434$) was observed (**Figure 2A**).

Figure 2: Correlation of (**A**) Total Phenolic, (**B**) Flavonoid, and (**C**) Anthocyanin Concentrations with Anti-oxidant Capacity (FRA) of all Grape Juices

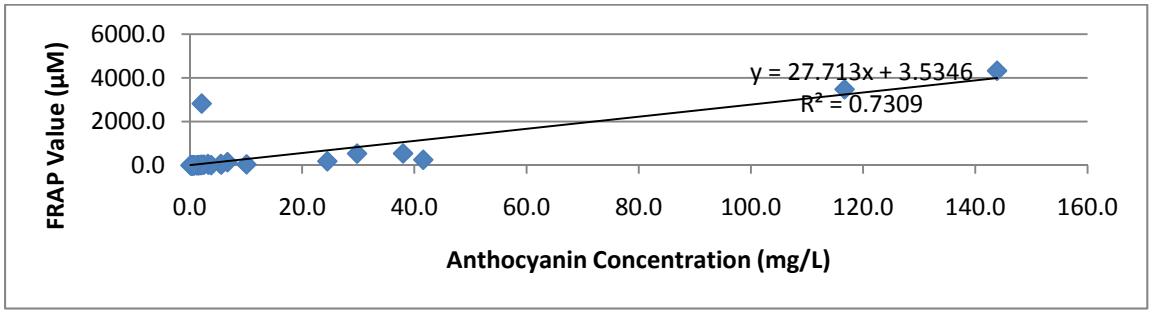
(**A**)



(**B**)



(**C**)



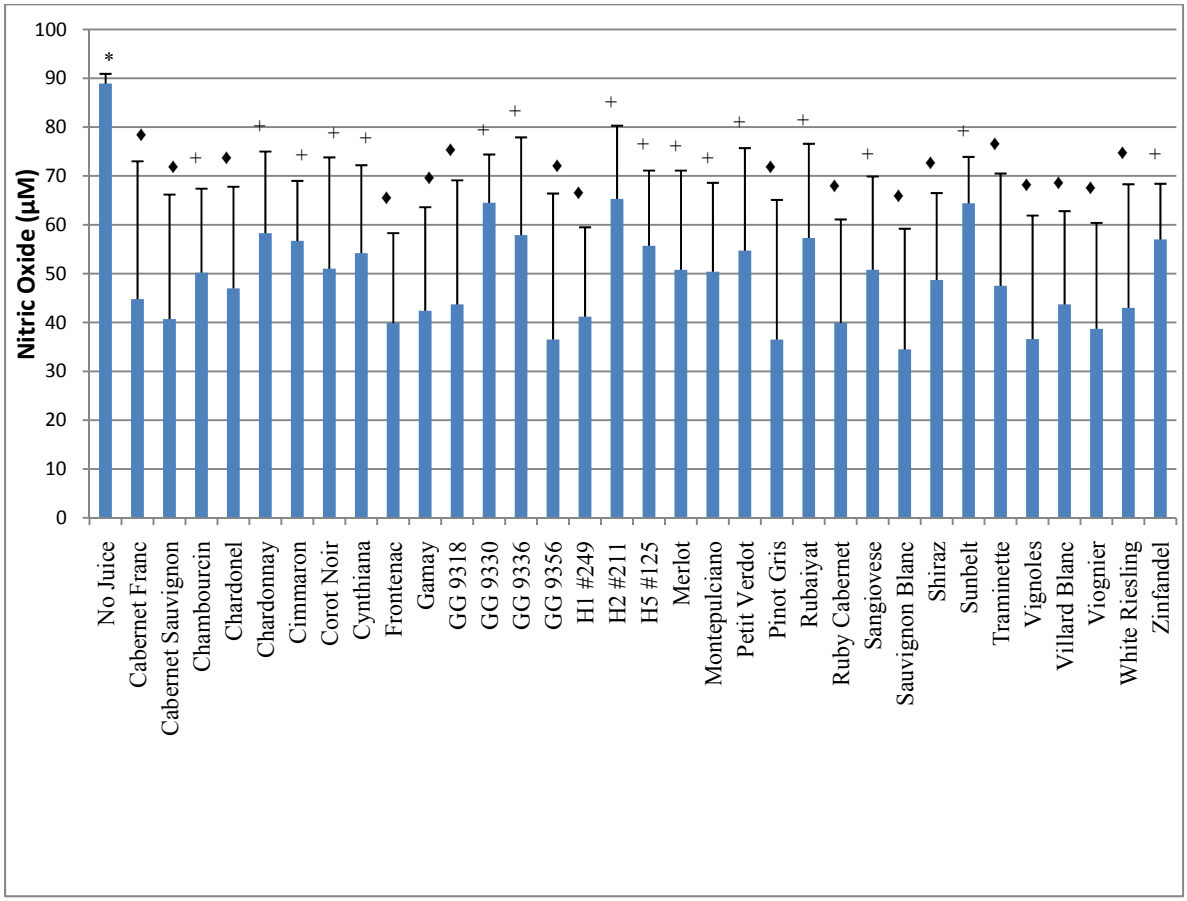
Effect of Grape Juice on Nitric Oxide Production and Cell Viability of LPS-stimulated RAW264.7 Macrophages

The grape juice prepared by smashing was used to determine its effect on NO production and cell viability of LPS-stimulated macrophages. The effect of each variety of juice on NO production was compared to those macrophages that were not treated with grape juice. All grape varieties significantly reduced NO production in LPS-stimulated macrophages compared to those not treated with grape juice (**Figure 3**). Over half of the varieties were statistically similar to Sauvignon Blanc, the grape variety with the greatest inhibition of NO production (**Figure 3**). H2 #211 is the grape variety that exhibited the least inhibition of NO production, yet, it was still significantly less than the NO produced by the untreated cells (**Figure 3**). The other half of the varieties were statistically similar to H1 #211 in inhibiting NO production (**Figure 3**).

There was a significant correlation ($R^2 = 0.113$, $P = 0.026$) with NO production and total phenolic content of all varieties of grape juice used for the treatment of LPS-stimulated macrophages (**Table 8**). However, there was no correlation between NO production and flavonoid and anthocyanin content of the grape juice (**Table 8**).

Treatment with LPS and grape juice did not cause cytotoxic effects in RAW264.7 macrophage (**Figure 4**). Pinot Gris had more than 100% viable cells (128.4% compared to the cells not treated with juice) and twenty-five percent of the varieties have similar effects with Pinot Gris (**Figure 4**) suggesting that the cells may have been stimulated to proliferation. Cimarron had the lowest viability (92.7% compared to cells not treated with juice) which was statistically similar to the remaining twenty-four varieties (**Figure 4**).

Figure 3: Effect of Juice from Thirty-three Oklahoma Grape Varieties on Nitric Oxide Production in LPS-stimulated RAW264.7 Macrophages[1]



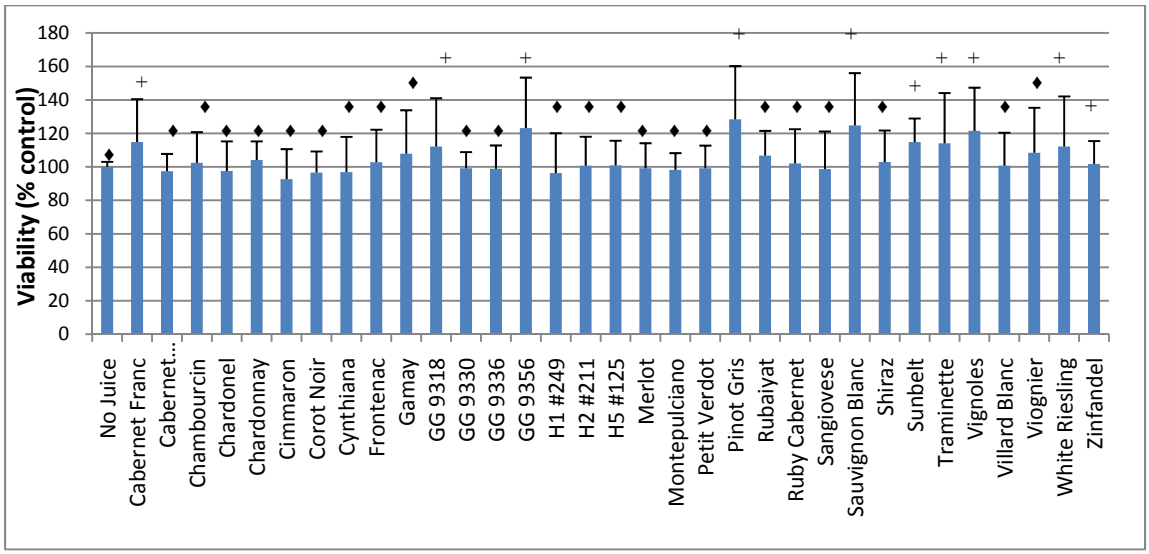
[1]Bars are mean ± SD; experiment was repeated at least three times.
*LPS-stimulated macrophage not receiving juice has significantly ($P < 0.05$) higher nitric oxide production compared to those receiving grape juice.
+ H2 #211 is the grape variety with the least inhibition of NO production. Other varieties sharing the symbol '+' are statistically similar to H2 #211 in inhibiting NO production.
♦ Sauvignon Blanc is the grape variety with the highest inhibition of NO production. Other varieties sharing the symbol '♦' are statistically similar to Sauvignon Blanc in inhibiting NO production.

Table 8: Correlation of Nitric Oxide (NO) Production from LPS-stimulated Macrophage with Total Phenolic, Flavonoid, and Anthocyanin Content from the Juice of Oklahoma Grapes

		Total Phenolic	Flavonoid	Anthocyanin
NO	Pearson Correlation	0.113	0.006	0.063
	P value	0.026*	0.457	0.139

*P < 0.05 is considered significant

Figure 4: Effects of Juice from Different Oklahoma Grape Varieties on Cell Viability of LPS-stimulated RAW264.7 Macrophage[1]

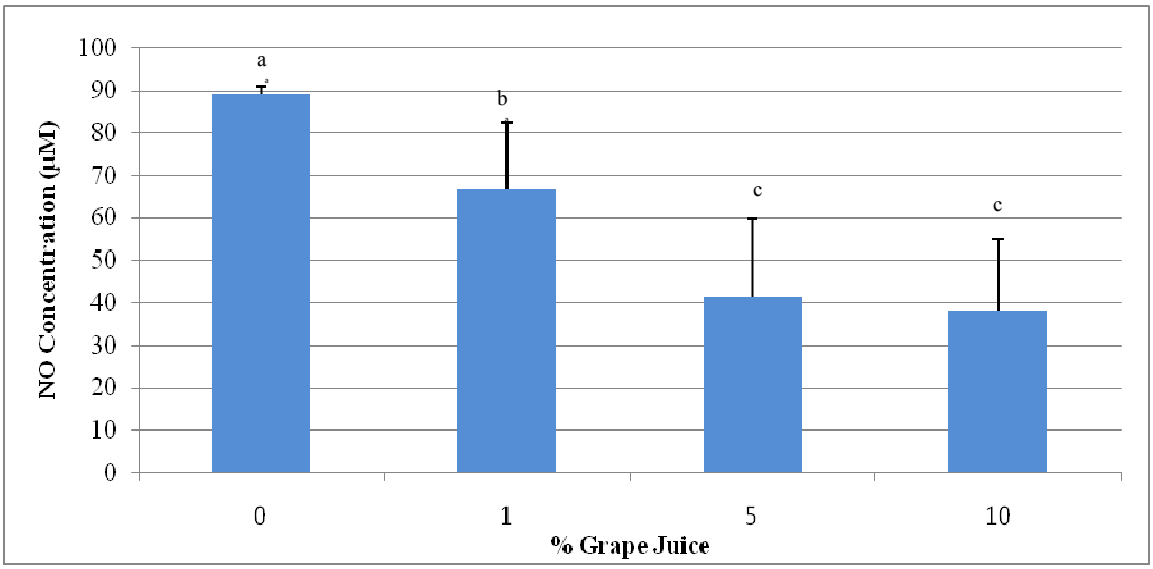


[1]Bars are mean ± SD; experiment was repeated at least three times.
+ Pinot Gris is the variety with highest cell viability (P<0.05). Bars that share the symbol '+' have statistically similar cell viability to Pinot Gris.
♦ Cimarron is the variety with the lowest cell viability (P<0.05). Bars that share the symbol '♦' have statistically similar cell viability to Cimarron.

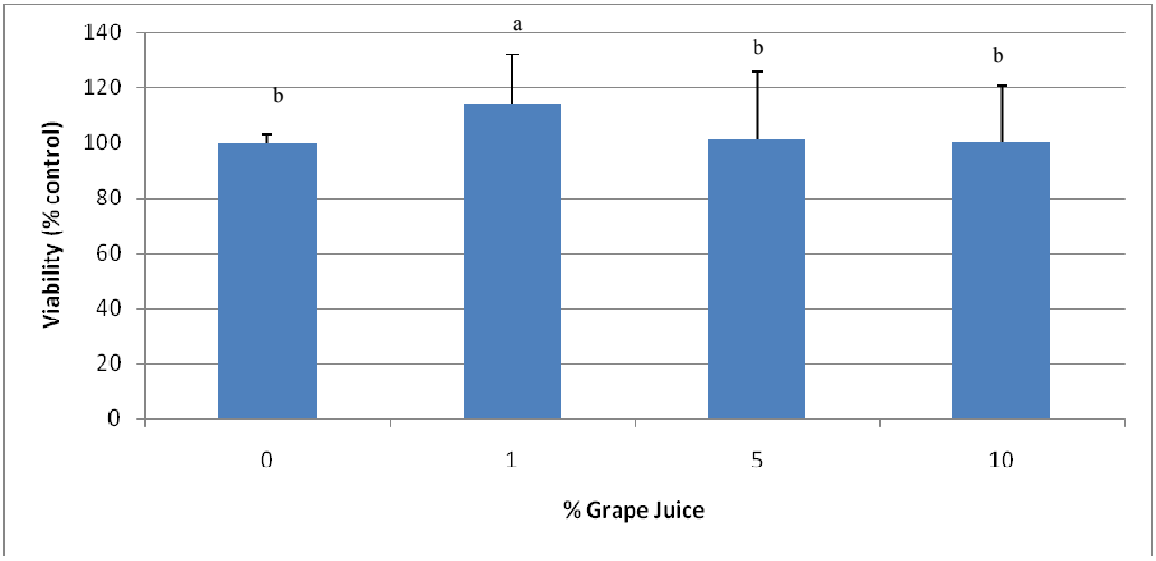
In addition to comparing different varieties, the effect of different concentrations (0, 1, 5 and 10%) of grape juice on NO production and cell viability were also assessed. Treatment with grape juice dose-dependently inhibited NO production (**Figure 5A**). Grape juice concentration starting at 1% significantly decreased the amount of NO produced in comparison to those not treated with grape juice. Meanwhile, the 5% and 10% juice were significantly more effective inhibitors of NO production with no difference in the inhibition of NO production between 5 and 10% juice (**Figure 5A**). The cell viability assay shows that there was no cytotoxic effects in any of the concentrations of juice used (**Figure 5B**). However, cells treated with 1% grape juice have significantly higher cell viability compared to the cells created with 5 and 10% grape juice.

Figure 5: Effect of Different Concentration of Grape Juice from Thirty-three Oklahoma Grape Varieties on (**A**) Nitric Oxide Production and (**B**) Cell Viability of LPS- stimulated RAW264.7 Macrophage[1]

(A)



(B)



[1]Bars are mean ± SD. Experiment was repeated at least three times. Bars that do not share the same letters are significantly ($P < 0.05$) different.

Specific Aim 2: *To assess the in vitro anti-inflammatory properties of extract from Oklahoma grape varieties with high and low total phenolic content using LPS-stimulated RAW264.7 macrophage.*

Total Phenolic, Flavonoid, and Anthocyanin Concentration of Grape Extract

From the findings of specific aim 1, ten grape varieties were chosen to extract the polyphenols. The choice of these ten grape varieties was based on the inhibition of NO production and total phenolic concentration of the juice from the smashed grapes (results of specific aim 1). The ten grape varieties chosen were Sauvignon Blanc, Chardonnay, Voignier, Pinot Gris, and Cabernet Sauvignon (relatively low total phenolic content) and Cynthiana, Rubaiyat, Ruby Cabernet, Sunbelt, and Zinfandel relatively (relatively high total phenolic content).

Zinfandel extract had the highest concentration of total phenolic (280.9±2.7 mg/100g), anthocyanin (34.5±3.8 mg/100g), and flavonoid (106.4±7.2 mg/100g) (**Table 9**). Sauvignon Blanc extract had the lowest concentration of total phenolic (63.7± 1.1 mg/100g), flavonoids (25.6±0.2 mg/100g), and anthocyanin (0.1±0.1 mg/100g) (**Table 9**).

Table 9: Total Phenolic, Flavonoid, and Anthocyanin Concentration of Extract from Ten Oklahoma Grape Varieties[1]

Varieties	*Total Phenolic (mg/100g fresh grape)*	*Flavonoid (mg/100g fresh grape)*	*Anthocyanin (mg/100g fresh grape)*
Cabernet Sauvignon	141.5 ± 3.4[d]	52.8 ± 2.5[d]	4.5 ± 0.2[d]
Chardonney	93.3 ± 1.9[g]	40.6 ± 1.3[e]	0.0 ± 0.0[e]
Cynthiana	86.2 ± 0.1[h]	32.9 ± 0.5[fg]	30.7 ± 1.8[b]
Pinot Gris	147.0 ± 2.1[c]	64.0 ± 1.3[c]	0.4 ± 0.1[e]
Rubaiyat	104.8 ± 0.4[f]	31.0 ± 0.5[g]	24.0 ± 1.6[c]
Ruby Cabernet	197.9 ± 2.0[b]	77.8 ± 1.0[b]	22.4 ± 0.5[c]
Sauvignon Blanc	63.7 ± 1.1[i]	25.6 ± 0.2[h]	0.1 ± 0.1[e]
Sunbelt	101.8 ± 1.1[f]	35.6 ± 1.0[f]	4.0 ± 0.1[d]
Viognier	116.7 ± 0.9[e]	49.2 ± 2.0[d]	0.0 ± 0.0[e]
Zinfandel	280.9 ± 2.7[a]	106.4 ± 7.2[a]	34.5 ± 3.8[a]

[1]Values are mean ± SD; experiment was repeated at least three times. For each column, values that do not share the same letter are significantly different ($P < 0.05$) from each other.

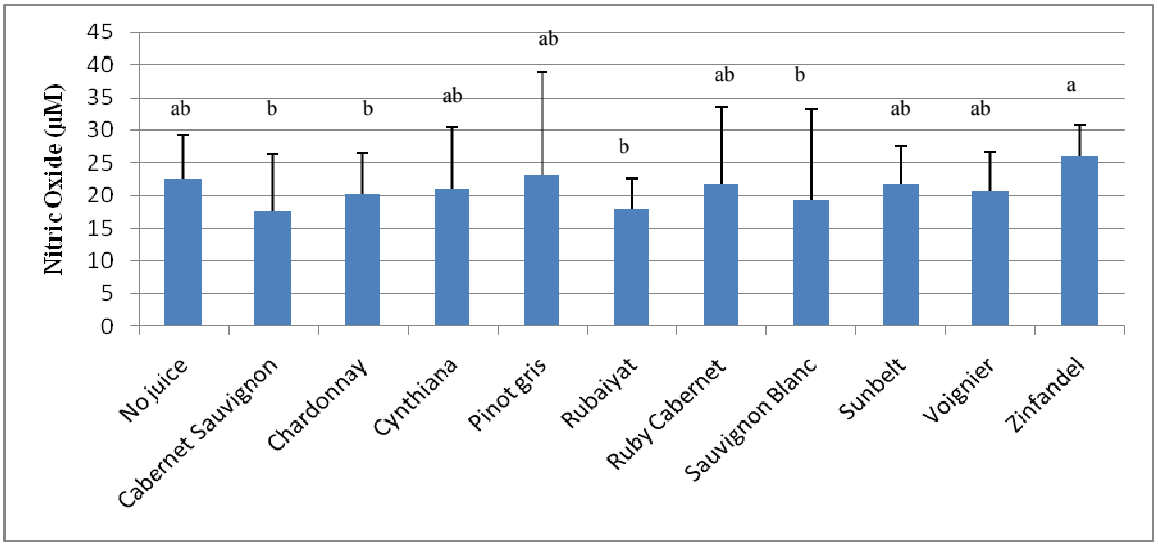
Effect of Grape Extract on Nitric Oxide Production and Cell Viability of LPS-stimulated RAW264.7 Macrophages

Grape extract from the ten different varieties did not significantly reduced NO production in LPS-stimulated macrophages (**Figure 6A**). Macrophage treated with Zinfandel extract produced significantly more NO than Cabernet Sauvignon, Chardonnay, Rubaiyat, and Sauvignon Blanc extract (**Figure 6A**). There were no statistical differences in the cell viability of the macrophages (**Figure 6B**).

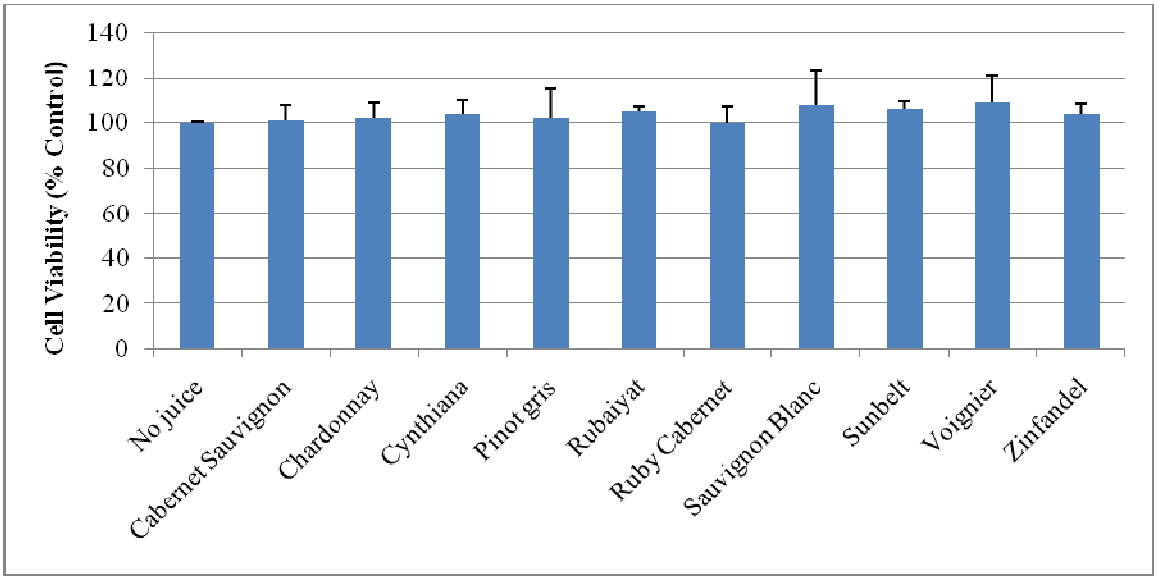
The LPS-stimulated RAW264.7 macrophages were also treated with different concentrations (0, 0.01, 0.1, and 1%) of grape extract. The 0.01% grape extract showed a significant increase of NO production while the 1% showed a significant decrease in NO production in comparison to those not given grape extract (**Figure 7A**). There was no difference in NO production between the 0 and the 0.1% grape extract (**Figure 7A**). The cell viability of the 0.01% and 0.1% concentrations were statistically greater than the 0 and the 1% extract (**Figure 7B**). NO production was significantly reduced from 0.01% to 1% in all but two varieties **(Figure 8)**.

Figure 6: Effects of Extract from Ten Oklahoma Grape Varieties on **(A)** Nitric Oxide Production and **(B)** Cell Viability in LPS-stimulated RAW264.7 Macrophage[1]

(A)



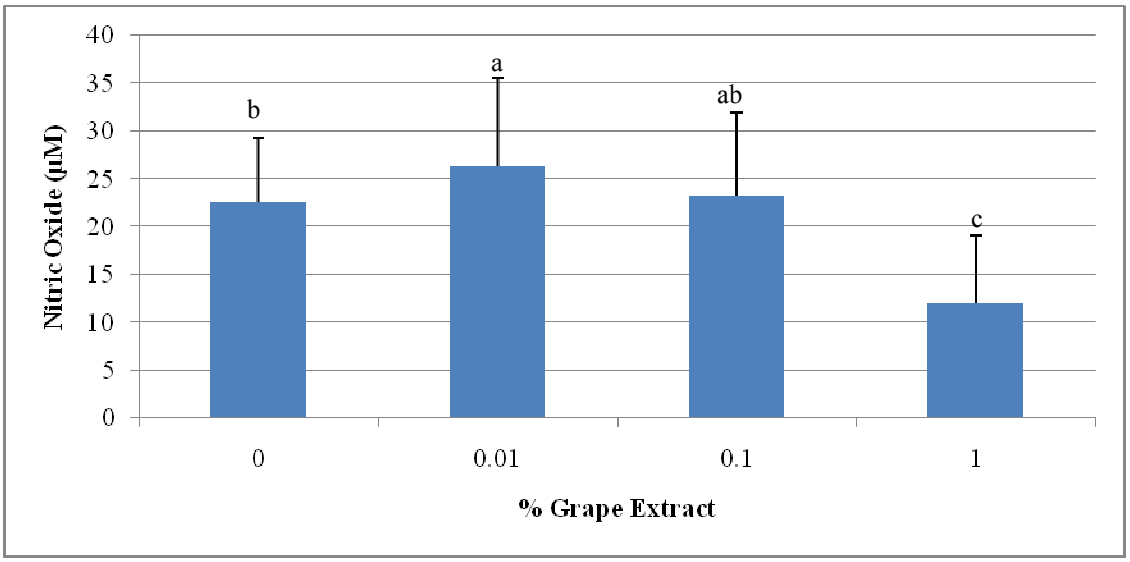
(B)



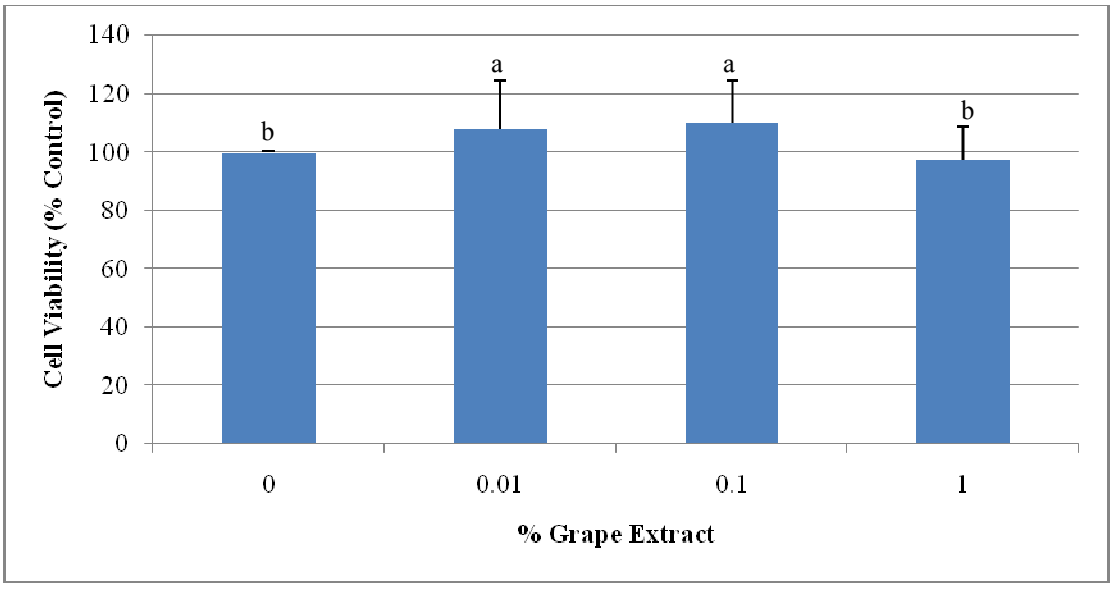
[1]Bars are mean ± SD; experiment was repeated at least three times. Bars that do not share the same letters are significantly different ($P < 0.05$) from each other.

Figure 7: Effect of Different Concentration of Grape Extract from Ten Oklahoma Grape Varieties on **(A)** Nitric Oxide Production and **(B)** Cell Viability of LPS-stimulated RAW264.7 Macrophage[1]

(A)

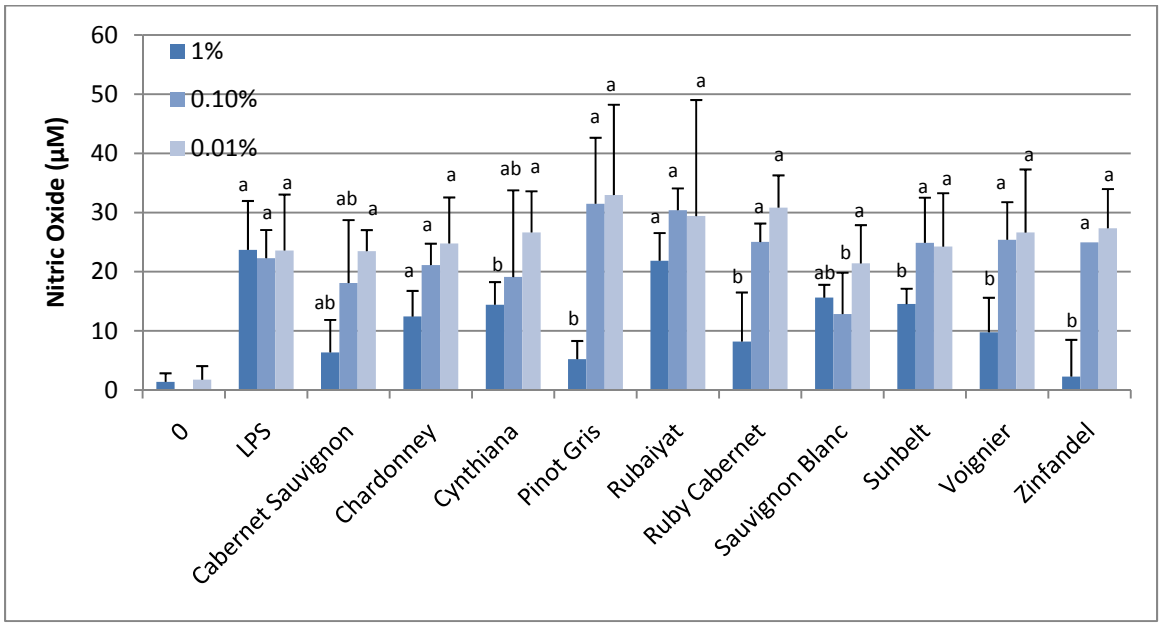


(B)



[1]Bars are mean ± SD; experiment was repeated at least three times. Bars that do not share the same letters are significantly different ($P < 0.05$) from each other.

Figure 8: Effect of Different Concentration of Grape Extract from Ten Oklahoma Grape Varieties on Nitric Oxide Production of LPS-stimulated RAW264.7 Macrophage[1]



[1]Bars are mean ± SD; experiment was repeated at least three times. For each variety, values that do not share the same letter are significantly different ($P < 0.05$) from each other.

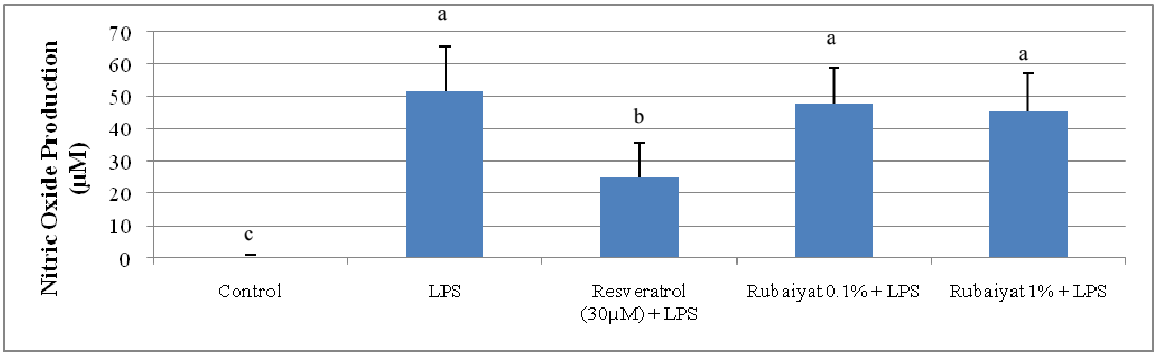
Specific Aim 3: *To compare the effect of Rubaiyat grape extract, a native Oklahoma grape variety, to that of resveratrol on expression of inflammatory genes in LPS-stimulated RAW264.7 macrophages.*

Nitric Oxide, TNF-α, and IL-6

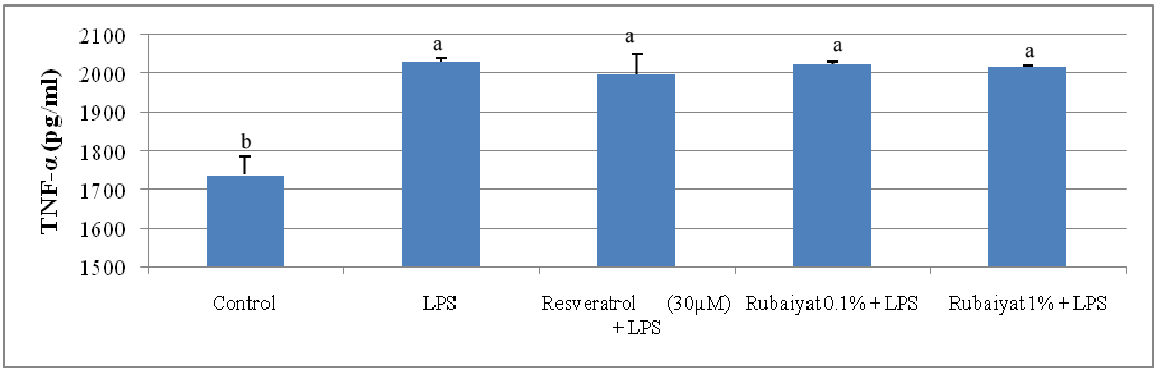
Treatment with LPS increased NO production in macrophages and resveratrol reduced NO production in these cells (**Figure 9A**). The two doses of Rubaiyat grape extract 0.1% and 1% did not reduce LPS-induced production of NO (**Figure 9A**).

LPS-stimulated macrophages had increased production of TNF-α and neither Rubaiyat grape extract nor resveratrol reduced TNF-α production (**Figure 9B**). LPS-stimulated macrophages treated with Rubaiyat grape extract, particularly 0.1% dose, had the highest IL-6 concentration (**Figure 9C**). LPS-stimulated macrophages treated with resveratrol had similar IL-6 to the stimulated cells (control) (**Figure 9C**).

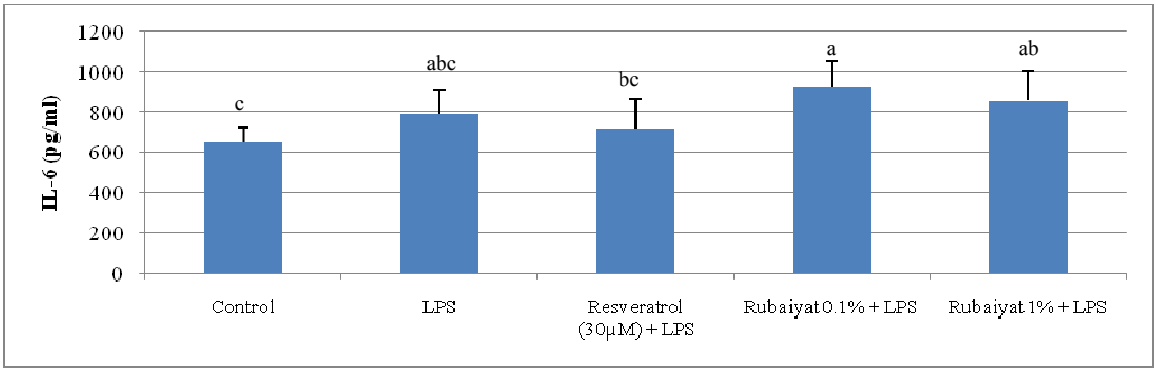
Figure 9: Effect of Resveratrol and Rubaiyat Grape Extract on (**A**) Nitric Oxide, (**B**) TNF-α and (**C**) IL-6 Production of LPS-stimulated RAW264.7 Macrophages[1]



(B)



(C)

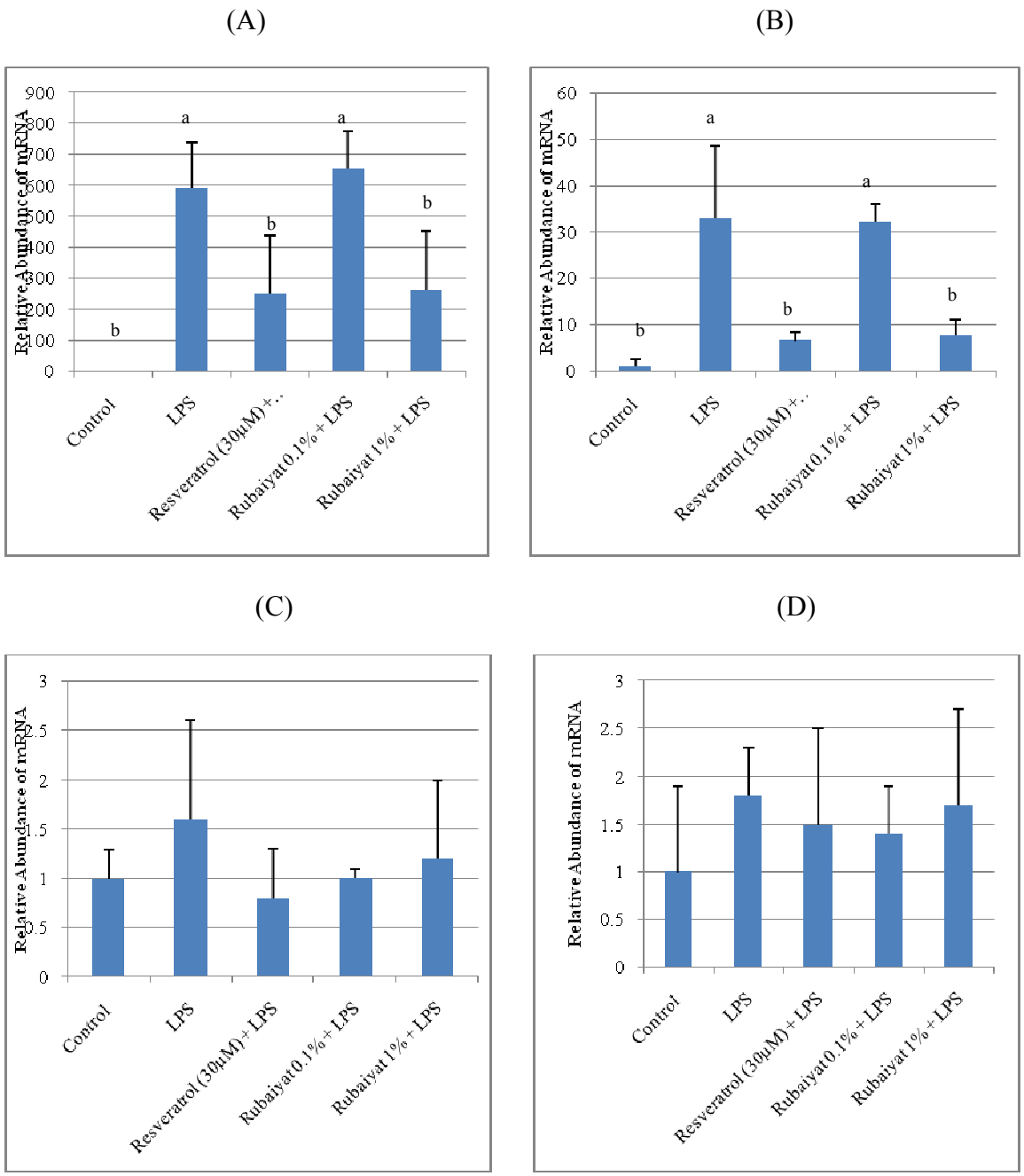


[1]Bars are mean ± SD; experiment was repeated at least three times. Bars that do not share the same letter are significantly ($P < 0.05$) different.

Real Time PCR

The expression of four pro-inflammatory genes, COX-2, IL-6, iNOS, and TNF-α were examined with real-time RT-PCR. LPS significantly increased gene expression of IL-6 and iNOS (**Figures 10A and 10B**). Resveratrol and the higher dose of Rubaiyat grape extract reduced the gene expression of IL-6 and iNOS to the level of the unstimulated macrophages (**Figures 10A and 10B**). The lower dose of Rubaiyat grape extract (0.1%) did not reduce IL-6 or iNOS expression (**Figures 10A and 10B**). There were no significant differences in the relative mRNA of COX-2 and TNF-α (**Figures 10C and 10D**).

Figure 10: Effect of Resveratrol and Rubaiyat Grape Extract on Relative Abundance of mRNA for (**A**) IL-6, (**B**) iNOS, (**C**) COX-2, (**D**) TNF-α in RAW264.7 Macrophage[1]



[1]Bars are mean ± SD; experiment was repeated at least three times. Bars that do not share the same letter are significantly ($P < 0.05$) different.

CHAPTER V

DISCUSSION

The primary objective of this study is to compare the phenolic content of Oklahoma grapes and determine anti-oxidant and anti- inflammatory properties. The ***hypothesis*** of the study is that the variety of Oklahoma grapes with high concentration of polyphenols will decrease markers of inflammation using murine macrophages.

Our findings show that the method of juice preparation has a significant effect on phenolic content. Over eighty percent of the varieties had significantly more total phenolic content by using the entire berry compared to just using the pulp. This indicates that the greater portions of phenolics are in the skin and seeds. Anthocyanin concentration was found to be highest in Rubaiyat grapes but reduced dramatically when only the juice of the pulp was assessed indicating that anthocyanin is present primarily in the skins of purple/red grapes. Anthocyanin concentrations ranged from as high as 266 mg/L to as low as 5 mg/L among varieties of purple grapes. The total phenolics of our purple grape juices tended to be comparable to Concord grape juice which has been reported around 1800 mg/L [144, 184, 185].

The antioxidant activity of the juice was found to have a strong correlation with anthocyanin but not with total phenolic. Orak and colleagues [125] found different results reporting the correlation between antioxidant activity and total phenolic stronger than

anthocyanin concentration. Kalt and colleagues [186], on the other hand, found a strong correlation between both total phenolic and anthocyanins with antioxidant activity [186]. This indicates that the antioxidant activity of grape cultivars is not directly associated with the presence of anthocyanin. Anthocyanin alone has been well documented as having antioxidant properties but a synergistic effect with anthocyanin and other phenolic compounds found in grapes may enhance the antioxidant activity in grape cultivars that do not have high concentrations of anthocyanins.

Grape juice made from pulp only was used to assess NO production in LPS-stimulated RAW264.7 murine macrophages. Sauvignon Blanc, a white grape, inhibited NO production the greatest accompanied by other white grapes Traminette, Viognier, and Vignoles. This indicates that there are phenolic compounds present in the pulp of different varieties of grapes capable of inhibiting NO production and it is not dependent on the amount of phenolic compounds. This agrees with the findings of Shanmugam et al [130] demonstrating that other plants with high polyphenolic content were unable to inhibit NO production indicating that only certain polyphenols or combination of polyphenols are responsible.

The total phenolics and anthocyanin content of purple grape extract have been reported to be between 817 and 3062 mg/L [125] and 40.3 to 990.8 mg/L [125], respectively. White grapes tend to have a much lower total phenolic concentration [126] and extremely low to no anthocyanin content. Ten grape varieties were chosen to further investigate the anti-inflammatory properties of grape extract. Our purple grape extracts

fell into the range of published data but the white grape extract of some varieties produced a much higher total phenolic than what was reported [126]. Only four of the extracts significantly inhibited NO production in LPS-stimulated RAW264.7 macrophages: two purple/red varieties (Cabernet Sauvignon and Rubaiyat) and two white varieties (Chardonnay and Sauvignon Blanc). When comparing the dose-dependent effect, we found that one percent extract was approximately 50% more effective than the control in reducing NO production. Our findings do not agree with our hypothesis that the higher total phenolic concentration will decrease NO production.

For the third aim of our study, we compared the effect of the Rubaiyat grape extract to that of resveratrol. Resveratrol has been shown to be a powerful antioxidant and exhibits many cardioprotective effects [129, 140, 162, 187-195]. Resveratrol has been documented to significantly inhibit NO production at a wide range of concentrations (3 – 30 μM) and also reported to be cytotoxic at 50 μM [196]. The effect of Rubaiyat grape extract was compared to resveratrol (30μM) on NO production and mRNA expression of TNF-α, IL-6, iNOS and COX-2. Rubaiyat grape extract was found to be most effective in inhibiting NO production starting at a concentration of 1% (30 μg/ml) or higher but cytotoxic at 10%. At this time, it cannot be determined if resveratrol present in Rubaiyat grapes is responsible for its effect on NO or other phenolic substances found in grapes such as procyanidin [156], proanthocyanidin [197-199] and anthocyanin [125, 200]. An analysis of all the phenolic substances present in Rubaiyat grapes would need to be done to determine which would be most effective.

Markers of inflammation assessed in this study were TNF-α, IL-6, iNOS, and COX-2. A compound that would inhibit the RNA expression of all four markers is desirable. Shanmugam and colleagues [131] found grape seed polyphenols to be active in inhibiting NO and TNF-α. Sung et al [201] investigated the preventative effect of a resveratrol tetramer against LPS induced inflammation and found that only iNOS displayed a dose dependent reduction in expression and no change in the TNF-α and IL-6 expression. A similar study conducted by Bi and colleagues [109] showed that besides a reduction in iNOS expression, there was also a reduction in TNF-α expression in microglia cells treated with resveratrol. As for COX-2 expression, grape polyphenols completely inhibited the increase in rats with inflammation due to ethanol treatment [202] and was also decreased by resveratrol [203]. In our study, iNOS and IL-6 expression were significantly decreased when treated with resveratrol and the 1% Rubaiyat extract, but TNF-α and COX-2 expression were not significantly different from the control. And although there was no reduction in the production of IL-6, there was a significant decrease of IL-6 expression. This could be accounted for as an accumulation of IL-6 in the media.

The resveratrol content was not measured in our study but other studies have found the concentration in purple grape juice to be as high as 0.5 mg/L [204]. Rubaiyat grapes grown in Oklahoma have a high total phenolic concentration with a very high anthocyanin concentration. Rubaiyat extract was compared to resveratrol which is known for its cardioprotective abilities. Upon assessment of the inflammatory gene

expression Rubaiyat and resveratrol affected each marker in the same way. IL-6 and iNOS were significantly inhibited while COX-2 and TNF-α were not significantly affected. At this point there is no way of knowing which phenolic substance is responsible for the cardioprotective properties of Rubaiyat grape extract. Each phenolic compound found in Rubaiyat grape extract would need to be analyzed. These findings support previous findings by Orak and colleagues [125] that it is not the concentration of polyphenols, but a specific phenolic or the synergy between phenolic compounds.

In summary, we found that a majority of phenolic compounds are found in the skin and seeds of most varieties of grapes with purple grapes having more total phenolics and anthocyanins than the white grapes. Yet a high total phenolic content did not necessarily represent a high anti-oxidant capacity. Some white grape varieties with low total phenolic content had a strong anti-oxidant capacity indicating that a phenolic compound found in some white grapes may also be a strong anti-oxidant. *In vitro* investigation of the grape extracts determined that both high and low total phenolic concentrations decreased NO production in RAW264.7 murine macrophage. Again, indicating that it is not the concentration of phenolics but the type of phenolic responsible for reducing inflammation.

There are several limitations to this study. First, this study utilizes grapes grown only in Cimarron Valley Research Station which is not entirely representative of the rest of the state as far as the condition of the soil, amount of rainfall, other environmental conditions or disease. Second, since this study only tested one year of harvest, the year to

year variation of growing conditions was not accounted for. Third, the appropriate storage conditions of all phenolic compounds found in grapes have not been determined. Many of the phenolic compounds may have been lost or altered during processing or storage at -20°C for a week to a month before analyses. These conditions can influence the polyphenol composition and concentration of the phenolic compounds in grapes. Nonetheless, the same procedure was used for all varieties and and should be sufficient for comparative purposes. Lastly, this study is an *in vitro* study using murine macrophages. The results should be interpreted with caution and cannot be related directly to humans. Studies with animals and subsequently with humans are necessary to confirm the findings of this study. The effects of digestion and metabolism are not accounted for in cell culture.

Based on the results of this study we reject the hypothesis that the variety of Oklahoma grapes with high concentration of polyphenols will decrease markers of inflammation using murine macrophages. We have found that it is the type of phenolic compound responsible and much investigation is required to determine which one or what group is responsible for the anti-inflammatory properties of grapes.

Further research on Oklahoma grape varieties would be beneficial since it has been shown that many phenolic compounds present in grapes have anti-oxidant properties and are capable of inhibiting markers of inflammation. More examination into the types of phenolic compounds present in the different varieties of grapes may aid in determining their functions in reducing inflammation.

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APPENDICES

VITA

Sandra Kay Peterson

Candidate for the Degree of

Master of Science

Thesis: ANTI-INFLAMMATORY PROPERTIES OF OKLAHOMA GRAPES

Major Field: Nutritional Sciences

Biographical:

Education:
Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May 2010.

Experience:
Research Analyst in Nutritional Sciences at Oklahoma State University
Laboratory Manager in Microbiology and Molecular Genetics at Oklahoma State University
Oklahoma National Guard

Name: Sandra K. Peterson 10065026 Date of Degree: May, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ANTI-INFLAMMATORY PROPERTIES OF OKLAHOMA GRAPES

Pages in Study: 108 Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study:

Many debilitating chronic conditions are linked to chronic inflammation. Recent studies have indicated that a relationship exists between phenolic compounds found in grapes and the reduction of chronic inflammation. The increasing trend in Oklahoma towards grape production has sparked the need to understand the health benefits associated with grapes grown locally, including their anti-inflammatory properties. Increased knowledge of the health benefits of grapes may help grape growers promote their products and increase their consumption which will help Oklahoma's viticulture and agritourism business. The hypothesis of the study is that the varieties of Oklahoma grapes with high concentrations of total phenolic compounds will have the greatest impact on decreasing markers of inflammation *in vitro*. The specific aims of the study were to: (1) determine the phenolic content, anti-oxidant capacity, and anti-inflammatory properties of juice from different varieties of grapes grown in Oklahoma; (2) assess the *in vitro* anti-inflammatory properties of extract from Oklahoma grape varieties with high and low total phenolics content (five varieties each) using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages; and (3) to compare the effect of the grape extract from an Oklahoma grape variety to that of resveratrol on the expression of inflammatory genes in LPS-stimulated macrophages.

The majority of phenolic compounds are found in the skin and seeds. Generally, purple grapes have more total phenolics and anthocyanins than the white grapes. A high total phenolic content did not necessarily represent a high anti-oxidant capacity. Some white grape varieties with low total phenolic content had a strong anti-oxidant capacity indicating that a phenolic compound found in some white grapes may also be a strong anti-oxidant. *In vitro* investigation of the grape extracts determined that both high and low total phenolic concentrations decreased NO production in LPS-stimulated macrophages, again, indicating that it is not the concentration of phenolics but the type of phenolic compound responsible for reducing inflammation. Resveratrol and the 1% dose of Rubaiyat grape extract reduced the gene expression of IL-6 and iNOS to the level of the unstimulated macrophage. Investigation on the types of phenolic compounds present in the different grape varieties may aid in determining the anti-inflammatory properties.

ADVISER'S APPROVAL: Dr. Edralin A. Lucas