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Cynthia Ann Norton

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**THE EFFECT OF WHOLE WILD BLUEBERRIES ON ENDOTHELIAL
FUNCTION OF THE SPRAGUE-DAWLEY RAT AS RELATED TO
CARDIOVASCULAR DISEASE**

By

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B.A. Westminster College, 1998

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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(in Food Science and Human Nutrition)

The Graduate School

The University of Maine

December, 2003

Advisory Committee:

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**THE EFFECT OF WHOLE WILD BLUEBERRIES ON ENDOTHELIAL
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An Abstract of the Thesis Presented
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Weanling male Sprague-Dawley rats were randomly fed three different diets (n=8 per group), a control diet (AIN '93) (**C**), a blueberry diet (**B**) for 13 weeks and a reverse diet (**R**)(C for 13 weeks, switched to B for 8 weeks). Aortae were excised, rings were prepared, and two intact and two denuded rings were immersed in tissue baths containing physiological salt solution (PSS) at 37°C, aerated with 95% O₂ and 5% CO₂ (pH 7.4). Following equilibration and pre-conditioning under 1.5gm preload, cumulative dose response curves were generated with six doses of the α -1 adrenergic receptor agonist L-Phenylephrine (L-Phe, 10⁻⁸ to 3 X10⁻⁶M). Relaxation was induced in the rings with Acetylcholine (Ach, 3 x 10⁻⁶M). Effective denudation was assessed by the absence of relaxation to Ach and the maximal contraction and relaxation force (Fmax) was determined. Intact arterial rings had a significantly lower Fmax than denuded rings (0.969gm vs. 3.076gm) (P<0.05). Mean Fmax of intact rings for C, B, and R groups

were 1.109, 0.873 and, 0.926gm (SEM=0.0463) respectively. A two-way ANOVA demonstrated that B and R groups had a lower Fmax than C group when contracted with L-Phe ($p<0.05$). There were no significant differences in Fmax means of denuded rings among diet groups ($p<0.05$). Our results indicate for the first time that whole wild blueberries function through the endothelium to influence the contractile machinery of the rat aorta in response to an α -1 adrenergic receptor agonist.

DEDICATION

It is with great pleasure that I dedicate this to my Father and friend, Gerald D. Norton.

I would like to acknowledge the incredible person he is, the role he has had in providing me with amazing educational opportunities, and his unconditional love and support.

Thank you for always helping me keep life in perspective.

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CHAPTER 1

INTRODUCTION

Cardiovascular Disease (CVD) has plagued developed countries as the primary cause of death for the past two decades accounting for nearly half of all deaths among the United States adult population (1). Approximately 60 million people in the United States have CVD with half of these individuals having no history of the disease (2, 3). CVD occurs as a result of continuous damage to the dynamic and delicate vascular endothelium that constitutes the inner layer of blood vessels (4, 5). The arterial integrity is determined by the condition of the endothelium. Potentially, it is the compilation of the various damaging factors that compromise the structural integrity of the artery (6-10). A dysfunctional vascular endothelium can be triggered by hypercholesterolemia, hyperhomocysteinemia, hypertension or smoking (4).

Blueberries, containing polyphenols and other antioxidants, have the highest antioxidant capacity of all fruits and vegetables. The possible roles of dietary flavonoids and antioxidants are currently being examined in a variety of *in vitro* studies. The results thus far have determined that polyphenols and antioxidants have beneficial effects on maintaining the integrity of the vascular endothelium *in vitro* (10, 12, 13). In addition, previous *in vitro* studies confirmed the beneficial effect of blueberry fractions on the biomechanical properties of the artery (13). Pilot studies recently conducted in Dr. Klimis-Zacas's laboratory determined that whole wild blueberries affect the mechanical properties of the arteries (11). The pilot study results determined that blueberries decrease the maximum force developed by the artery when challenged with the α -1 antagonist, phenylephrine. However, whole wild blueberries do not affect the cell

membrane receptors, as indicated by the vessel reactivity. As a result, the mechanisms by which blueberries affect the artery may involve the endothelium or smooth muscle cells. The role of whole wild blueberries as a functional food, with respect to their site and their mechanism of action on the endothelium, can only be evaluated and fully understood through an *in vivo* study.

This project is a dietary study examining the effect of whole wild blueberries on the biomechanical properties of the artery *in vivo* and its relationship to cardiovascular disease. Sprague-Dawley rats will be fed diets with and without blueberries. The passive and active properties of the vascular endothelium will be studied with the use of tissue force analyzers. Animals intact and denuded arteries will be studied from all diet groups. Denudation, removes the endothelial layer of cells from the artery. Thus, denudation, mimics the damage that occurs to an artery with CVD. This data will provide the first information on the role of whole blueberries as a functional food with respect to arterial integrity and CVD. This data will provide insight to blueberries' mechanism of action on the endothelium with respect to CVD. Ultimately, the results of this research will also indicate the possible implications whole wild blueberries may have on blood pressure regulation.

The objectives of this thesis are:

1. To evaluate the role of whole wild blueberries on the biomechanical properties (F_{max} and pD_2) of the Sprague-Dawley rat artery.
2. To determine the site of the action of whole wild blueberries on the artery (smooth muscle vs. endothelium)

CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular Disease

Even though the most recent medical advances and educational intervention tactics have decreased the overall rate of cardiovascular disease (CVD) cases, CVD continues to claim more lives in developed countries each year than any other disease (1). More than 2,600 Americans die each day from CVD (2). Consequently, CVD causes one death every thirty-three seconds (3). Approximately, one in five people have some form of cardiovascular disease (4). CVD will cost the United States an estimated \$351.8 billion in 2003 in health expenditures and lost productivity (5). The manifestations of CVD include angina pectoris, chronic heart failure, coronary artery disease, myocardial infarction, stroke, ischemic stroke and hypertension (6). CVD occurs as a result of chronic endothelial inflammation, the trademark of atherosclerosis. Chronic inflammation creates a dysfunctional endothelium and can be attributed to a variety of risk factors such as: smoking, high concentrations of oxidized low-density lipoprotein (LDL), diabetes, hypercholesterolemia, hyperhomocysteinemia, low levels of high-density lipoprotein (HDL) and hypertension (7, 8). Physical inactivity, poor nutrition and being overweight or obese are additional factors that can increase an individual's risk of CVD (6). However, only 30% of CVD cases can be related to traditional risk factors (2-4). Consequently, in order to decrease deaths related to CVD, a greater focus needs to be dedicated to the prevention of CVD.

2.2 Endothelium and the Vascular Smooth Muscle Cell

The etiology of cardiovascular disease is based on the structural integrity and health of the vessels of the cardiovascular system. The walls of an artery consist of the intima, media, and adventitia (9). The intima, the innermost layer of the artery wall, is contains a single layer of endothelial cells. The amorphous mucopolysaccharide ground substance containing elastin, collagen, and vascular smooth muscle cells (VSM) is referred to as the media layer. The VSM is composed of actin and myosin and undergoes tonic contractions initiated by mechanical, electrical and chemical stimuli. The adventitia, consisting of connective tissue, is the outermost layer surrounding the two inner layers (10, 9).

The endothelium functions as the modulator of cardiovascular health by balancing the vasoconstriction and vasodilatation of the artery. The vascular homeostasis achieved by modulatory role of the endothelium occurs through mediators that act to affect the musculature and vasculature of the artery (9). The vascular muscle activity of the aorta affects its permeability to various vasoactive substances by directly regulating the elasticity and distensibility of the aorta's vascular wall (11). The endothelium relays the message of vasoactive substances by serving as a communication medium between the blood and the VSMC. The vasoactive substances alter the vascular tone of the artery through myogenic mechanisms, endothelial factors, local hormones or chemical substances, and metabolic by-products. Consequently, signal transmission causes muscle contraction or muscle relaxation and can occur through numerous pathways involving nerve signals, blood-borne substances and locally generated substances (12).

Vasoactive substances functioning in the pathways act as vasoconstrictors or vasodilators. Vasoconstrictors typically bind to receptors on the VSMC or endothelium and can elicit a contraction. Angiotensin II, vasopressin, endothelin-1, norepinephrine and thromboxane A2 are examples of vasoconstrictors (13, 14, 9). Vasodilators include nitric oxide/endothelium derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor, C-type natriuretic peptide and kinins. Acetylcholine causes vasodilation through pathways involving NO, PGI₂, and EDRF production (15, 16). Vasorelaxation is stimulated by the release of the EDRF/NO and can also be stimulated by ATP, histamine, bradykinin, and other agents (10, 17). Vasodilators function to protect the integrity of the artery by inhibiting VSMC growth, platelet aggregation, platelet thrombosis, monocyte adhesion, inflammation and adhesion (7, 18). The appropriate balance between vasoconstriction and vasodilation is maintained by a functional endothelium and allows for a healthy cardiovascular system. The effect of various vasoactive substances is illustrated in Table 2.1.

Table 2.1: The Effect of Vasoactive Substances on the Endothelium

Vasodilators	Vasoconstrictors
Nitric Oxide	Angiotensin II
Prostacyclin	Endothelin
Atrial Natriuretic Peptide	Vasopressin (Anti-diuretic hormone)
C-type Natriuretic Peptide	Cytokines
Kinins	Thrombin
Endothelium-derived	Oxygen Free Radicals
Hyperpolarizing factor	Shearing Forces

Adapted from Klabunde et al and Hsueh et al (7, 12)

The autonomic nervous system, composed of the sympathetic and parasympathetic systems, can regulate the flow of blood based on the degree of vascular tone present in the artery. The α -1 and α -2-adrenergic receptors, located in the aorta's VSMC, bind with agonists to cause vasoconstriction (10, 19). Vasoconstriction occurs via the sympathetic nervous system due to the activation of noradrenaline or norepinephrine and subsequent binding to α -adrenoreceptors (9). An agonist is a substance that interacts with receptor molecules to produce an effect identical to the endogenous signaling molecule. For instance, phenylephrine is an alpha-1 adrenergic agonists for norepinephrine that can elicit a contraction (20, 21). Whereas an antagonist, is a substance that binds to a receptor, competes with the hormonal signal and prevents the response of the hormonal signaling molecule (9).

Also located in the blood vessels are β -adrenergic receptors that inhibit contraction and cause vasorelaxation in the VSMC (9). Acetylcholine, released into circulation by the parasympathetic nervous system, causes vasoconstriction under resting vascular tone and vasodilatation under raised vascular tone (20). The effect of acetylcholine is termed biphasic because its effect is based on the level of vascular tone. Acetylcholine affects pathways of vasoactive substances including factors such as NO, PGI₂ and EDRF (16, 22). The degree of relaxation that occurs in the presence of agonists binding the β -adrenergic receptors is based upon the degree of contraction present in the artery. In other words, for relaxation to occur a certain level of contraction must be present in the artery. In addition, β -adrenergic receptors also experience desensitization that causes their effects to diminish with increased exposure to agonists

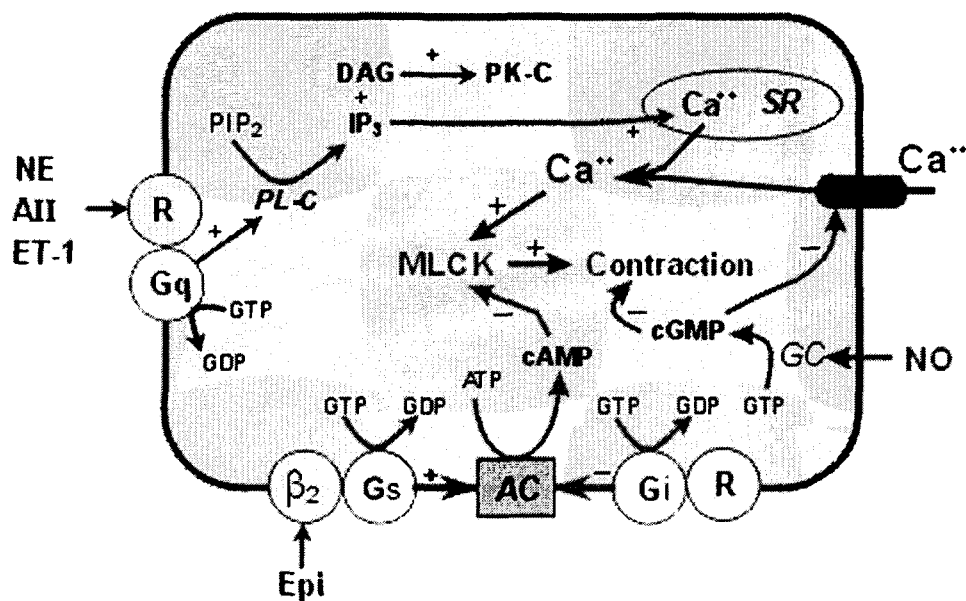
(10, 17, 21). Consequently, vascular tone of the artery determines the effect that the parasympathetic nervous system has on blood flow.

Alpha and β receptors can be present in the same vessel and also share the same neurotransmitter, norepinephrine (9). The α and β effect of norepinephrine is based on receptor affinity. Typically the α receptor has the highest affinity for norepinephrine and causes a contraction. Beta-receptors generally have a higher affinity for epinephrine than norepinephrine and elicit a relaxation (17). There are a variety of biologically active substances that bind specifically to various cell receptors of the aorta initiating a complex cascade of events (23) capable of causing a relaxation or contraction. Ultimately, the contraction or relaxation induced by the binding of a neurotransmitter or agonists has on the artery's vasculature depends on several known and even unknown factors. The effect is dependent upon the vascular tone present in the artery, the vasoactive substance binding the receptor and the effect the cascade of events have on the calcium concentration in the VSMC (21).

Ultimately, neurotransmitters and vasoactive substances affect the concentration of intracellular calcium via signal transduction mechanisms and determine the VSMC contraction. Increases in intracellular calcium cause calcium to bind to calmodulin, a special calcium binding protein (9). The binding of calcium to calmodulin forms a calcium-calmodulin complex and causes the activation of the enzyme myosin light chain kinase (MLCK). With ATP, MLCK causes the phosphorylation of myosin light chain (MLC), resulting in the formation of a cross-bridge between myosin and actin and a VSM contraction (22). Calcium concentrations are regulated by the phosphatidylinositol

pathway, G-protein-coupled pathway, and nitric oxide-cGMP pathway (24). The complexity of the pathways influencing VSMC contraction is illustrated in Figure 2.1.

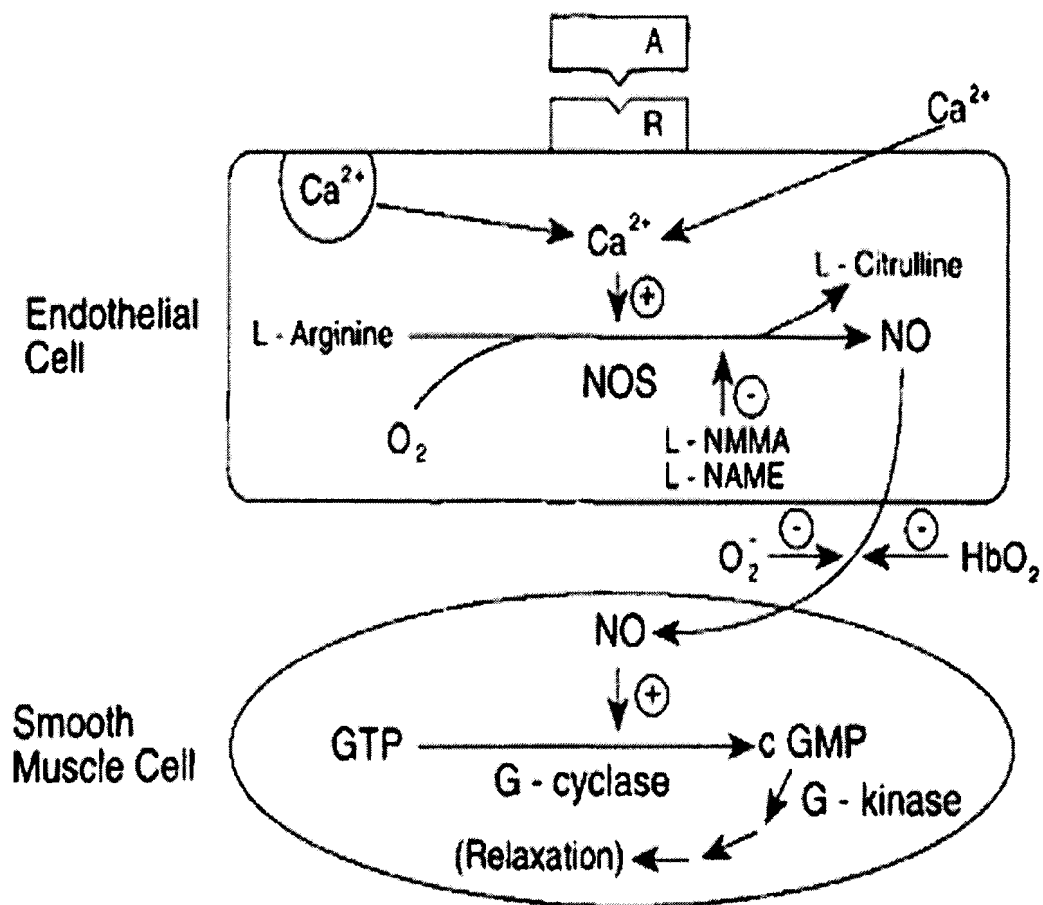
Figure 2.1: Signal Transduction Pathways Affecting Vascular Tone in the VSMC



NE: norepinephrine, AII: angiotensin II, ET-1: endothelin-I, PL-C: phospholipase C, PIP₂: phosphatidylinositol, IP₃: inositol triphosphate, SR: sarcoplasmic reticulum, DAG: diacylglycerol, PK-C: protein kinase C, β_2 : β receptor, Epi: epinephrine, Gs: G protein pathway stimulated, Gi: G protein pathway inhibited, AC: adenyl cyclase, NO: nitric oxide, GC: guanyl cyclase, MLCK: myosin like chain kinase, Ca: calcium, GTP: guanyl triphosphate, GDP: guanyl diphosphate, cAMP: cyclic adenosine monophosphate, ATP: adenosine triphosphate. Klabunde et al. 2002 (12)

Nitric Oxide (NO), the most important vasodilating substance, previously was referred to as endothelium derived relaxing factor (EDRF) (9). The role of nitric oxide is central to the modulatory function of the endothelium. The vascular protective functions of NO include; vasodilation, inhibiting sympathetic vasoconstriction, inhibiting platelet and leukocyte adhesion to the endothelium, inhibiting VSM proliferation, preventing thrombosis and scavenging superoxide anion to prevent inflammation (13, 16, 25). Nitric oxide is produced in the endothelial cell from the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS). Nitric oxide synthesis is presented in figure 2.2.

Figure 2.2: Nitric Oxide Pathway



Klabunde et al. 2002 (12)

Nitric oxide synthase has three isoforms, NOS-1, NOS-2, and NOS-3. Neural NOS (nNOS) or NOS-1 and endothelial NOS (eNOS) or NOS-3 are present during baseline physiological conditions (N20). Inducible NOS (iNOS) or NOS-2 is expressed during inflammatory physiological states (26, 27). Nitric oxide, produced in the endothelial cell, activates guanyl cyclase in the VSM to increase concentration of cyclic-GMP. The increased cGMP is capable of activating cGMP-dependent protein kinase. Activation of the protein kinase stimulates the following pathway; inhibition of calcium entry into the VSM, decreased calcium-calmodulin MLCK binding, decreased phosphorylation of myosin light chains, reduced contractile forces and vasodilation. The mechanism of NO- induced relaxation is portrayed in Figure 2.2 (9). Acetylcholine, bradykinin, substance-P, insulin, and histamine stimulate the production of NO by increasing the calcium concentration. Also, shear force created by blood flow can result in calcium release and cause eNOS to increase the production of NO. Inducible NOS is capable of increasing NO production in the presence of cytokines and bacterial endotoxins. Each of these mechanisms that increase NO production serve an important function in the cardiovascular system. The effect of NO is based on its production and bioavailability. The bioavailability can depend upon the presence of reactive oxygen species, intracellular availability of L-arginine, altered NOS activity and impaired receptor mediated release of NO (6, 12, 14). Along with the protective and regulatory role of NO, the cardiovascular system also possesses a series of defense mechanisms. The antioxidant defense mechanisms that function to prevent damage are composed of antioxidant enzymes and include; superoxide dismutase (SOD), catalase, glutathione

peroxidase and glutathione reductase (9). In addition, glutathione, α -tocopherol and ascorbic acid are free-radical scavengers that help to maintain arterial integrity (6, 28).

2.3 Cardiovascular Disease and Endothelial Dysfunction

The health of the cardiovascular system is determined by the condition of the endothelial cells. Various circulating factors have the potential to impair the modulatory role of the endothelial cells and cause vascular injury and disease. Consequently, cardiovascular disease is characterized by a dysfunctional endothelium. As the modulator of cardiovascular health, the endothelium controls platelet aggregation, fibrinolysis, vascular tone, growth, immune responses, smooth muscle cell proliferation and thrombus formation (9). Each of these components has the potential to cause inflammation and create a dysfunctional endothelium (13, 8). The endothelium functions to maintain vascular homeostasis and tone through paracrine mediators by regulating the vasodilatation and vasoconstriction of the artery. Paracrine mediators are vasoactive substances that only affect the target cells in close proximity to the cell releasing the mediators. A dysfunctional endothelium causes a disruption in the delicate balance of paracrine mediators regulating the vasoconstriction and vasodilatation (8). A disruption in the balance of paracrine mediators regulating the vascular endothelium, causes a loss of normal anti-platelet activity, vasodilator or anti-thrombotic activity, increases the potential the likelihood of vasospasm, thrombosis or further inflammatory damage (29). Consequently, the role of the endothelium is to balance the dilation and contraction of the artery in order to maintain the arterial integrity.

Inflammation, a multi-factorial process that causes a dysfunctional endothelium, is the main characteristic associated with the development and progression of atherosclerosis (8). Atherosclerosis is a form of CVD and is characterized by chronic inflammation. Cellular and molecular responses to the chronic inflammation cause the development and progression of arterial lesions. Initially, inflammatory stimuli cause the up-regulation of surface and soluble cell adhesion molecules and the release of cytokines (13). During inflammation platelets release pro-inflammatory mediators including thromboxane A₂ and serotonin (30). Early atherosclerotic events include the adhesion of platelets and monocytes to the endothelium. This process is referred to as the activation of the endothelium and is the initial event in the atherosclerotic process. Injury to the arterial wall from chronic inflammation results in the disruption of the physiological protective regulatory balance of the endothelium, another factor in the development of atherosclerosis. Growth factors are then released from platelets, endothelial cells, macrophages, and VSMC that further enhance the inflammatory process (31). Continued inflammation results in the enhanced release of hydrolytic enzymes, cytokines, chemokines, and growth factors from the activated endothelium. The release of these factors attracts circulating monocytes that then bind to adhesion molecules and migrate into the subendothelial space. Lipid-laden foam cells or fatty streaks develop from monocyte-derived macrophages that scavenge oxidized LDL and migrate into the subendothelial space. As the process is augmented, VSMCs migrate and extracellular debris, lipids, and cholesterol crystals are deposited around the lesion. The deposition of calcium is followed by necrosis of the foam and endothelial cells. The fibrous plaque

formed, is composed of the core lipid and necrotic tissue. Hemorrhage and thrombosis characterize the final stages of atherosclerosis (6, 8, 31).

Oxidative stress contributes to the atherosclerotic process and occurs when there is an imbalance between *in vivo* antioxidant defense mechanisms and the production and exposure of cells to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Superoxide, hydroxyl, peroxy, oxides of nitrogen, and peroxynitrite are all examples of reactive oxygen and nitrogen species that can cause oxidative stress (32). These free radical species can oxidize LDL particles (33). Thus, the atherosclerosis process can also be initiated and intensified by the oxidized LDL trapped within the lesion. The oxidative modification hypothesis and the research supporting this hypothesis provide the evidence that links CVD to the inflammation caused by oxidized LDL (34). A high concentration of plasma LDL particles results in the increase of both circulating monocytes adhering to the arterial wall and to a higher concentration of LDL particles entering the intima. Consequently, there is an increased chance of the LDL particle to become oxidized. *In vitro* studies discussed by Steinberg et al., state that foam cell formation occurs from macrophages/monocytes and smooth muscle cells that bind modified or ox-LDL (35). In addition, Tapiero et al. discussed *in vitro* studies that determined the simple incubation of LDL overnight with endothelial cells or smooth muscle cells created a foam cell, providing yet another mechanism by which LDL could function in foam cell formation (9, 35). Also, Noguchi et al. recently completed research indicating that ox-LDL not only damages the endothelium physically, but ox-LDL also induces a variety of pro-atherosclerotic genes (28). It is widely accepted that oxidized LDL play a critical role in the atherosclerotic process by exerting several harmful effects on the endothelium (36).

Oxidized LDLs' cytotoxic effects include, increasing the production of growth factors, inhibiting the release of NO and increasing leukocyte adhesion to endothelial cells (36).

In humans, both observational and experimental studies have demonstrated an association between inflammation and endothelial dysfunction (8). Researchers have concluded that there are morphological and functional changes in the endothelial cells located in areas prone to atherosclerosis (37). Endothelial cells that undergo these changes have impaired dilation and have a diminished protective role against the atherosclerotic process (37). Goode et al. concluded through an *in vitro* study that human hypercholesterolemic peripheral arteries exhibit vascular abnormalities because of impaired endothelial function (38). Ishibashi et al. observed vascular abnormalities manifested by an impaired endothelium that affected forearm blood flow among patients with congestive heart failure (39). Numerous other studies have established two primary associations regarding the impaired functioning of the cardiovascular system. First, studies have confirmed an association between low-grade inflammation and atherogenesis (29). The second association is a relationship between acute systemic inflammation and increased risk for a cardiovascular event (29). Hingorani et al. determined a relationship in humans between endothelial dysfunction and risk of CVD, in the presence of a *Salmonella typhi* vaccine. The vaccine created a mild inflammatory event and the researchers observed impaired endothelium dependent vasodilation among the human subjects (29). These studies are only a few of the numerous studies that document the association between inflammation, endothelial dysfunction and risk of cardiovascular disease.

Along with impairment of endothelial function, hyperresponsiveness of the vascular smooth muscle can also lead to endothelial damage, vascular injury and disease. Hyperresponsiveness of the VSMC can potentially cause enhanced vasoconstriction. Atherosclerosis and hypertension enhance the contractile effects of the artery and tend to predispose a patient to myocardial ischemia (38, 39). In addition atherosclerosis and hypertension are risk factors for myocardial infarction. However, atherosclerosis and hypertension reduce endothelium dependent responses to acetylcholine, bradykinin, thrombin and platelet derived products causing enhanced contractile effects. Goode et al. determined that hypertension and atherosclerosis in animal and human subjects enhanced the contractile effects of serotonin (38). Hyperresponsiveness of the VSMC or impairment of the endothelium are capable of affecting the integrity of the artery and the endothelium derived relaxation. Denudation, removal of the intima layer of the endothelium, mimics the arterial damage that occurs during atherosclerosis and impairs the release of EDRF/NO.

EDRF/NO is produced by the endothelial cell and relaxes VSMC by activating guanylate cyclase to form cyclic GMP by protein phosphorylation. NO activity is responsible for vasodilation, lowering of blood pressure, inhibition of platelet aggregation, inhibition of platelet and leukocyte adhesion to the vascular endothelium, inhibition of VSMC proliferation and scavenging superoxide anions (12). Thus NO can function as a superoxide scavenger by reacting with superoxide anion to form biologically inactive nitrate. On the other hand, NO and superoxide can also form peroxynitrite radical which can form a hydroxyl radical (33). Peroxynitrite is capable of causing oxidative damage associated with the pathogenesis of inflammatory disease (40).

Thus, the biological activity of NO to function as an oxidant or oxidant scavenger is based on the intracellular formation of superoxide anion.

The role of NO is central in the regulation and the pathogenesis of cardiovascular diseases (41). The dysfunctional endothelium of atherosclerotic arteries impairs the synthesis, release and functions of EDRF/NO (9). Many diseases and conditions have been associated with decreasing the production of nitric oxide and impairing endothelium-dependent vasorelaxation. Hypertension, atherosclerosis, diabetes, obesity, dyslipidemias, heart failure, cigarette smoking, aging and vascular injury are diseases or conditions known to damage the endothelium and reduce the expression of eNOS (12, 42). Normal release of NO through the eNOS reaction is responsible for physiologic vasodilation. However, studies have determined that during septic shock, NO production through iNOS is possibly responsible for regulation of blood pressure and endogenous antioxidants (41). Numerous experiments have indicated that EDRF/NO is decreased in the beginning of the atherosclerotic process and decreased NO production has been observed to be a mechanism responsible for the endothelial dysfunction (9). Impairment of NO production can cause thrombosis from platelet aggregation and adhesion, inflammation from tissue damage by ROS, vascular stenosis or vasoconstriction (12). A correlation was shown by Cleland et al. between basal endothelial NO synthesis and low-grade inflammation (8). Thrombosis, myocardial infarction and reperfusion injury are cardiovascular complications that have been observed to have an impaired production and effect of NO (43). Nitric oxide production is also affected by pathologies damaging the endothelium, hypoxia, or trauma (44). Consequently, disruption in the NO function,

has been widely implicated in creating a dysfunctional endothelium and being a crucial factor in the progress of cardiovascular disease (43).

2.4 Blueberries

2.4.1 Composition of Blueberries

Lowbush blueberries, *Vaccinium angustifolium*, also referred to as whole wild blueberries, are commonly referred to as antioxidant powerhouses. The components of low bush blueberries are water, carbohydrates, eleven minerals and five vitamins (45). Bushway et al. determined that niacin, riboflavin, thiamin, vitamin C and vitamin A are the five vitamins found in blueberries (45). The minerals are calcium, potassium, magnesium, phosphorus, aluminum, boron, copper, iron, manganese, sodium, and zinc (45). Despite the antioxidant vitamin content of blueberries, Prior et al. concluded that the polyphenolic compounds of blueberries contribute substantially to their antioxidant capacity (46). Polyphenolic compounds consist of phenolic acids, their esters and flavonoids. Blueberries have been determined to have the highest oxygen radical absorbance capacity (ORAC) of all fruits and vegetables (47, 48). Prior et al. confirmed a direct correlation between the total ORAC and the total polyphenolic content of various blueberries (49). Kalt et al. reported that lowbush blueberries, *Vaccinium angustifolium*, have higher concentrations of anthocyanins, total phenolics and ORAC than the species of high bush “cultivated” blueberries (48).

Polyphenols function as reducing agents and can function to regenerate other antioxidants, such as vitamin E. The antioxidant capacity of polyphenols is provided by phenolic group’s ability to function as hydrogen donors. The polyphenolics possess an

aromatic ring bearing one or more hydroxyl substituents. Polyphenols also possess metal-chelating properties that possibly allow them to decrease iron and copper induced oxidations (50).

Among the flavonoids are the flavans, flavonols, proanthocyanidins, and anthocyanins. Kalt and McDonald determined that chlorogenic acid is the major phenolic acid in the low bush blueberry. Other major organic acids include citric (36%), malic (31%) and quinic (20%) (51). The major flavonols found in blueberries are kaempferol and quercetin (52). Proanthocyanins, higher molecular weight component of the blueberry, are a type of tannin found in blueberries that have been shown to inhibit the initiation stage of chemically induced carcinogenesis (53). Five major anthocyanins have been reported in the lowbush blueberry. The five anthocyanins, delphinidin, malvidin, petunidin, cyanidin and peonidin, are shown in figure 2.3 and table 2.2 (54). The anthocyanins in the lowbush blueberry are found as their 3-glucosides, 3-galactosides and 3-arabinosides. According to Prior et al., the total anthocyanin content of lowbush blueberries is 95.4 ± 2.6 mg/100g of blueberries (49). Based on a 1976 study conducted by Kuhnau et al., the estimated average U.S. American anthocyanin intake was 180 g in the winter and 215 g in the summer (55). Tsuda et al. explained the variation in flavonoid stating that human consumption of flavonoid ranges from 25 mg/day to 1mg/day (56). According to Marchand et al., flavonoid intake varies among different countries from 3 mg in Finland to 68 mg in Japan (56).

Figure 2.3: The Flavylum Cation. R1 and R2 are H, OH, or OCH₃; R3 is a glycosyl or H; and R4 is OH or a glycosyl. Excerpted from Kong et al. (57)

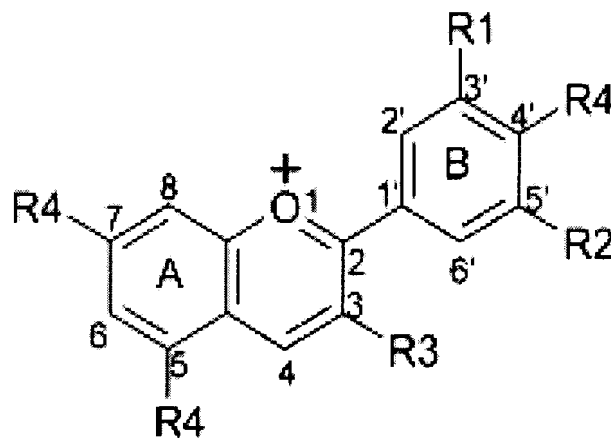


Table 2.2: Five Major Anthocyanidins Reported in the *Vaccinium angustifolium*

Name	Abbreviation	3	5	6	7	3'	4'	5'	Color
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Bluish-red
Malvidin	My	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Bluish-red

OH: hydroxyl, H: hydrogen, OMe: methyl

Excerpted from Kong et al. (57)

Although in the past, information on bioavailability has been referred as being scarce, researchers have recently made considerable contributions to the areas of flavonoid absorption, metabolism and excretion in humans and rats. Numerous researchers have observed the absorption of flavonoids from poly-phenolic rich foods across the gut barrier by obtaining measurements of the plasma antioxidant capacity before and after consumption (46, 57). Most recently, Kay et al. observed the ORAC and total antioxidant status (TAS) of eight men's plasma who had consumed 100 g of freeze-dried blueberries with a high fat meal in a single blind cross-over study. Kay et al. determined that the diet-induced ex vivo increase in serum antioxidant status, was directly associated with the observed *in vitro* antioxidant content of blueberries (58). The antioxidant activity observed is influenced by the absorption of the flavonoid. According to Heim et al. the absorption is affected by the mode of administration, antecedent diet, dosage amount, gender differences, and colonic microflora (59). For instance, iron and protein may bind flavonoid hydroxyl groups and hinder their absorption (60).

The structure of the flavonoid affects the absorption mechanism and the biological benefits of the metabolized flavonoid. Flavonoids naturally occurring in plants exist mostly as glycosylates rather than as aglycones, the form with the sugar moiety removed. However, until recently most research on flavonoid absorption was conducted on aglycones. The glycosylation of the sugar affects the absorption, metabolism and biological benefits of the flavonoid (52). Flavonoids containing sugars can be hydrolyzed at the brush border allowing the aglycones to diffuse across the cell membrane. Flavonoids with sugar moieties may also cross the enterocyte border via the sodium-dependent glucose transporter. Regardless of absorption, cleavage of the

glycosides by the cytosolic β -glycosidase can occur (52). Flavonoids are conjugated in the intestinal cells and bound to albumin for transport to the liver (30). In the liver, the flavonoid can be further conjugated with methylation or sulfation. The extent of conjugation is associated with the biological capacity of the flavonoid. For instance, in a review paper Nijveldt et al. hypothesized that the extent of conjugation is directly related to decreased toxicity, antioxidant activity and enzyme inhibiting capacity of the flavonoid (30). Mananch et al. demonstrated that continuously high intakes of the flavonoid quercetin in rats decreased absorption but increased the formation of the conjugated form, which has more antioxidant activity than the aglycone form (61). Paganga and Rice-Evans et al. confirmed that dietary flavonoids were absorbed as glycosides by identifying the presence of anthocyanins, rutin, and quercetin glycosides in human plasma through an *in vivo* study (62). The investigation confirmed that a polyphenolic plasma concentration required to attain 50% of maximal relaxation, 0.5-1.6 $\mu\text{mol/L}$, was detected in human subjects. Miyazawa et al. also confirmed the intestinal absorption, in humans, of orally administered anthocyanin extracts, cyanidin glucosides, and the subsequent increase in plasma concentration and antioxidant capacity (62). However, Wu et al. was unable to detect anthocyanins in the plasma of six women who consumed a 189 g of low bush blueberries as a meal. Wu et al. concluded that the anthocyanins were poorly absorbed and that only 0.004% of the 189g consumed was detected in the urine 6 h later (63). Tsuda et al. investigated the *in vivo* mechanism for the absorption and metabolism of 3-O- β -D-glucoside (C3G) in rats. After the oral administration of 0.9mmol/kg 3-O- β -D-glucoside body weight the following mechanism was purposed (56). First, C3G is hydrolyzed by β -glucosidase in the intestines and the aglycones of cyanidin (Cy), C3G,

is produced and degraded to protocatechuic acid (PC). Consequently, the free form C3G, observed in the previously discussed studies on rats and humans, may improve the antioxidant activity of this flavonoid (56). Numerous studies have confirmed the absorption of anthocyanin intact glycoside forms by detection in plasma and urine. Thus, more research is needed to understand the absorption, metabolism, biological benefiting mechanisms and excretion of dietary flavonoids in both humans and rats.

The role of blueberries in disease prevention and treatment can be attributed to their ORAC value, among other beneficial components (47, 48). The metabolites of polyphenolic compounds produced during the digestion provide the blueberries with the high ORAC value. The high ORAC value allows the blueberries to protect the body from oxidative stress. According to Scalbert et al., the health benefits associated with blueberry consumption may correlate with the polyphenolic concentration and functions (46). Currently, research has indicated that blueberries have possible health benefit's in protecting against chronic diseases and include, preventing glaucoma, fibrocystic disease of the breast in humans, cancer, cerebrovascular conditions, diabetes, atherosclerosis, and cardiovascular disease (30). The anthocyanins, delphinidin and cyanidin, have also been identified as having other therapeutic effects acting as radiation-protective agents, vasotonic agents, chemoprotective agents, hepatoprotective agents and anti-inflammatory agents (47). Anthocyanins have also been postulated to function in the treatment of microcirculation disease and prevention of cholesterol-induced atherosclerosis in the rabbit (47).

The vast majority of the research conducted thus far has been on the polyphenolic extracts of blueberries rather than on the whole wild blueberry itself. The research

conducted on the extracts has been mostly *in vitro* (61, 68). Consequently, the role of whole wild blueberries in preventing various chronic diseases is accepted because of the high ORAC value of the blueberry's polyphenolic components. Blueberries are potential candidates in disease prevention and treatment, however, *in vivo* research is lacking and further studies are critical to understand their benefits on human health (48).

2.4.2 Flavonoids

Flavonoids have been documented to have beneficial effects as antioxidants, antiviral, anticancer, anti-inflammatory, anti-allergic and anti-atherogenesis components through *in vitro* experiments (30, 64). According to Schramm and German flavonoids may function to protect vascular health by the following mechanisms: free radical termination, protease inhibition, inhibition of the Maillard reactions, growth suppression of bacteria and prevention of leukocyte adhesion (65). Flavonoids have been proven to inhibit the atherosclerotic process in many animal studies (66). Aviram et al. examined the effect of pomegranate juice (PJ) on the atherosclerotic process in human subjects and atherosclerotic apolipoprotein e-deficient mice. PJ contains the following anthocyanins: cyanidin-3-glucoside, cyanidin-3, 5-diglucoside and delphinidin-3-glucoside. Aviram concluded that PJ decreased LDL susceptibility to aggregation in humans and in mice PJ reduced oxidation of LDL by 90%, reduced uptake of oxidized LDL by 20%, reduced the size of atherosclerotic lesions by 44% and the formation of foam cells. This study concluded that in atherosclerotic mice and humans PJ antioxidative properties provide antiatherogenic effects (67). Consequently, flavonoids have potential effects in improving vascular health by reducing platelet aggregation, improving capillary

permeability, reducing leukocyte adhesion, oxidative stress, and improving vasodilation of the arteries (68).

Bravo et al. summarized the possible protective mechanisms of flavonoids to reduce free radical formation, protect alpha tocopherol in LDL from oxidation, increase regeneration of oxidized alpha tocopherol and chelating metal ions (50, 59). Flavonoids are potent antioxidants that function as oxidizing species scavengers, neutralizing superoxide anion, hydroxyl radicals, peroxy radicals or as quenchers of a reactive oxygen species (59, 69, 70). Flavonoids react with free radicals to terminate the propagating chain reaction (71). The *in vitro* antioxidant capacity of flavonoids is directly related to the degree of hydroxylation. Another antioxidant mechanism determined by Arora et al. is the ability of flavonoids is to decrease membrane fluidity and stabilize membranes (72).

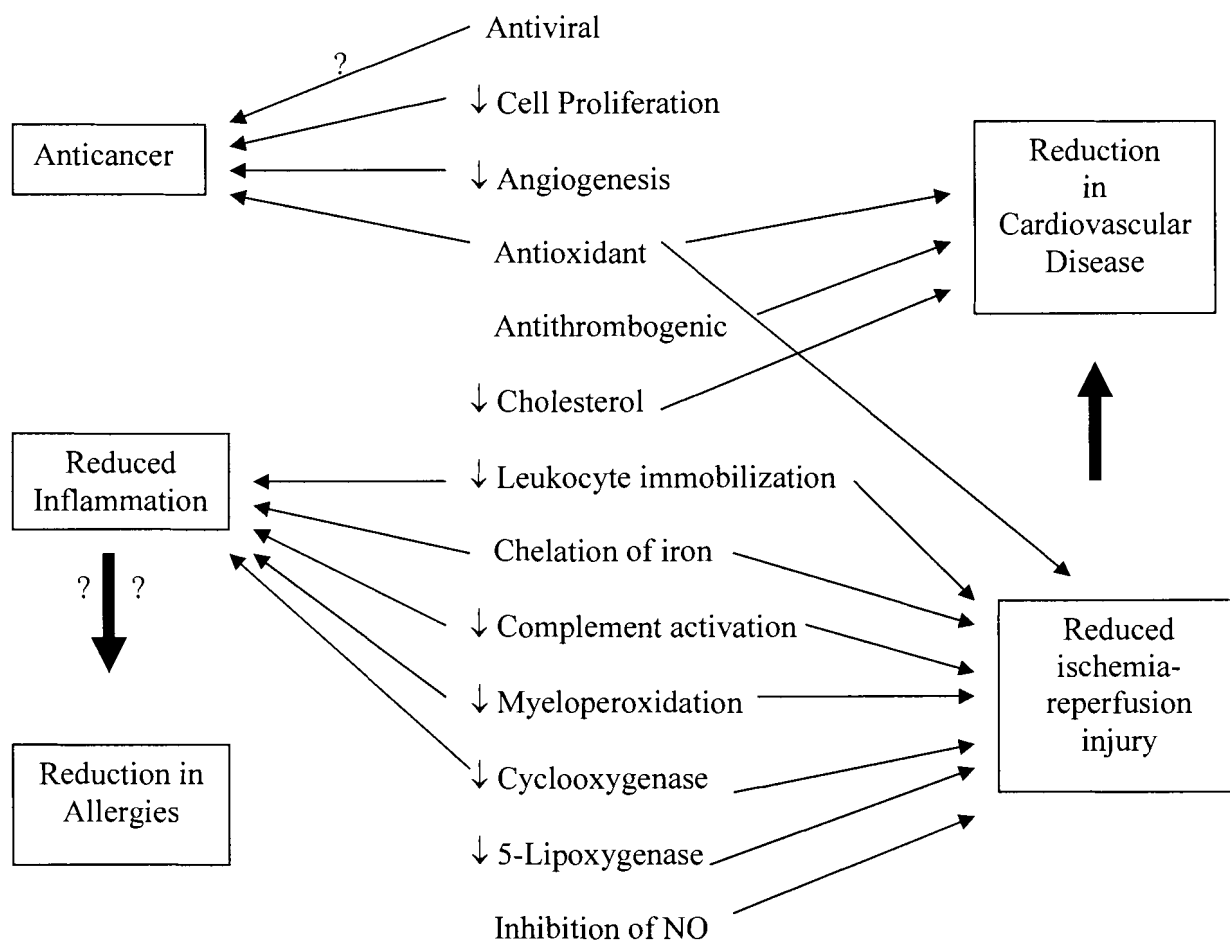
Flavonoids have been observed to function in twenty- four enzyme systems. VanHoorn et al. investigated the effect of flavonoids on xanthine oxidase, an enzyme that increases during periods of oxidative stress (64). During the re-oxidation of xanthine oxidase, both harmful superoxide radicals and hydrogen radicals are produced. VanHoorn et al. determined that flavonoids with hydroxyl groups located at the 5 and 7 position on the flavone backbone possessed the strongest xanthine oxidase inhibitory effect (64). Other effects flavonoids have on the enzyme systems include pathways involving; cell division, platelet aggregation, detoxification, inflammatory and immune responses (73, 74). The inhibitory effects of flavonoids are associated with enzymes involved in cell activation including, protein kinase C, protein tyrosine kinase and phospholipase A2 (75). Smith et al. conducted an assay used to detect cancer preventive action and determined

that flavonoids from wild blueberries inhibit the initiation stage of chemically induced carcinogenesis (53). There is also substantial experimental evidence regarding the action of flavonoids in various cancer-related biological pathways. The cancer-related pathways that flavonoids affect include; carcinogen bioactivation, cell signaling, cell cycle regulation, angiogenesis, oxidative stress and inflammation (74). Studies on a variety of flavonoid sources have determined that the flavonoid and antioxidant content in red wine, black tea, green tea, fruit juices and chocolate can function to inhibit LDL oxidation (66, 67, 68, 76, 77, 78 79).

The structure of flavonoids is directly associated with the ability of the flavonoid to function as an antioxidant or prooxidant. Galati et al. determined that polyphenolics containing a polyphenolic ring are more prooxidant than polyphenolics containing a catechol ring (80). According to Skibola et al. the result of excessive flavonoid intake causes the flavonoid to function as a mutagen, pro-oxidant and inhibitor of key enzymes (81). Further research is needed to elucidate the potentially toxic effects associated with excessive flavonoid intake and the intake levels that cause these effects. The antioxidant functions of flavonoids pertain to their ability to scavenge radicals, scavenge the high concentrations of nitric oxide created by macrophages via nitric-oxide synthase, and inhibit xanthine oxidase activity causing less oxidative damage (69).

Flavonoids have been implicated to have a preventive role in a number of inflammatory and disease processes including; asthma, cancer, cardiovascular disease, cataracts, diabetes, gastrointestinal inflammatory diseases, liver diseases, macular degeneration and periodontal disease (82). Figure 2.1 presents the hypothetical effects of flavonoids on disease mechanisms.

Figure 2.4: Hypothetical Mechanisms of the Role of Flavonoids on Diseases



Excerpted from Nijveldt et al. (69)

2.4.3 Anthocyanins

Anthocyanins, the most abundant flavonoid found in blueberries, are located within the outer layer of the blueberry and are responsible for the blue pigmentation of the blueberry. The Greek origin of the word anthocyanin is anthos, which means flower, and kyanos, which means blue. Anthocyanins, a phenolic phytochemical, are found throughout the plant kingdom and in the diet as crops, beans, fruits, and vegetables and in red wine (30, 40). Anthocyanins found in the lowbush blueberry, as presented in Figure 2.3 and table 2.2, include delphinidin, malvidin, petunidin, cyanidin, and peonidin (54). Although blueberries contain ascorbic acid, it is their anthocyanin content that is attributed to providing their high antioxidant capacity (53).

Tsuda et al. investigated the anti-oxidative, radical scavenging and lipid peroxidation inhibitory effects of three anthocyanins extracted from *Phaseolus vulgaris*. The three anthocyanins, pelargonidin 3-O- β -D-glucoside (P3G), cyanidin 3-O- β -D-glucoside (C3G), and delphinidin 3-O- β -D-glucoside (D3G) are three of the five anthocyanins found in lowbush blueberries. In addition, the aglycones, pelargonidin chloride (pel), cyanidin chloride (Cy) and delphindin chloride (Del) antioxidative activity were evaluated. The anti-oxidative activity was evaluated as reduced malondialdehyde by UVB radiation in a liposomal system. Each extract demonstrated strong antioxidative activity, but the radical scavenging and antioxidative activity were associated with each pigments structure (83). Other studies by Tsuda et al. documented that anthocyanins have antioxidant ability under physiological conditions during both *in vivo* and *in vitro* experiments (40). Tusda et al. concluded that the anthocyanin, cyanidin, is readily absorbed and metabolized in rats and was found to create a high serum antioxidant

capacity (40). Antioxidant activity of anthocyanins was determined based on effects on LDL oxidation and the lecithin liposome system *in vitro* by Gracia et al. (73). Gracia et al. determined that the ability of the anthocyanin extracts to inhibit oxidation increased as follows; pelargonidin < cyaniding < delphinidin < malvidin. The researchers determined that the antioxidant capacity of anthocyanins is affected by the metabolic system, the substrate and conditions used to catalyze the oxidation (73). Gabrielska et al. revealed that anthocyanin extracts of honeysuckle and chokeberry function reduced LDL oxidation *in vivo* (84). Frank et al. recently documented the effect of dietary anthocyanins ability to spare vitamin E in healthy rats *in vivo* (85). Other studies have determined the antioxidative activity of anthocyanins to be concentration and dose dependent (40, 84).

In 1976, Kuhnau et al. documented for the first time that anthocyanins can function in metal chelation (55). Further research by numerous investigators, has concluded that anthocyanins function as inhibitors of lipid peroxidation, as free radical scavengers or in biological systems through metal chelation or protein binding (73, 83). Sarma et al. identified that anthocyanins chelate metal ions to prevent the oxidation of ascorbic acid by forming an ascorbic metal anthocyanin complex (86).

Various studies have confirmed the biological effect of anthocyanin extracts. Joseph et al. discovered that anthocyanin extracts are important in neurological functioning. They determined in numerous studies that anthocyanin extracts can decrease the occurrence of the parameters commonly associated with several age-related neural dysfunction conditions and cognitive decline (87). Obi et al. concluded that anthocyanin extracts from *H. rosasinensis* administered to rats prevented carbon-tetrachloride induced liver damage (88). Anthocyanins cyanidin (Cy) and delphinidin (Del) have been found

to inhibit tyrosine kinase activity of epidermal growth-factor receptor and inhibit growth of human tumor cells in vitro by Meiers et al. (89). Other research has confirmed the effect of anthocyanins on anti-inflammatory activity, anti-convulsant activity, antioxidant activity, and anti-carcinogenic activity (73). Studies have documented the antioxidant capacity of anthocyanins to contribute to the following biological effects; decreased capillary permeability and fragility, reduced platelet aggregation and strengthening the collagen matrix of connective tissue (68).

2.5 Cardiovascular Disease and Blueberry Components

2.5.1 Dietary Intake and Cardiovascular Disease

Multiple mechanisms are capable of functioning to activate the vascular wall and thus compromise the arterial integrity of the vascular system. Epidemiological, observational, and experimental studies have produced a substantial amount of evidence linking the consumption of antioxidant containing fruits and vegetables to reduced risk of cardiovascular disease or improved cardiovascular health (32). According to Morton et al. the antioxidant content, including b-carotene, vitamin C, vitamin E and the polyphenolics, are responsible for reducing the risks of CVD and cancer (32). The Seven Countries Study, conducted by Hertog et al., evaluated the association between flavonoid intake and CHD among 16 cohorts, from seven different countries, in men between 65-84 years of age from 1960 to 1985 (90). Average flavonoid intake and mortality rates were inversely related with 90% of the variance explained by saturated fat intake and smoking. Hertog et al. investigated flavonoid intake and mortality associated with coronary heart disease (CHD) in one of the six cohorts from the Seven Countries Study, the Netherlands

cohort, in an epidemiological survey called “The Zutphen Elderly Study” (91). The sources of flavonoids were tea, onions, and apples with the daily average flavonoid intake being 25.9 mg. The findings of the Zutphen Elderly Study determined inverse relationships between flavonoid intake and mortality from CHD ($p=0.015$) and incidence of myocardial infarction ($p=0.08$). In addition, the result of the Zutphen Elderly Study, revealed a dose dependent association between flavonoid intake and CHD mortality (91). Knekt et al. determined a weak inverse relationship between flavonoid intake and CHD among 5130 Finnish men and women over a 20-year period (92). On the other hand, Rimm et al. in the U.S. Health Professionals Follow-Up Study identified a very weak inverse relationship between flavonoid intake and total CHD (93). However, this study that found a poor correlation between CHD and flavonoid intake only evaluated the presence of CHD, not mortality rates from CHD. In conclusion, although epidemiological and observational studies are not conclusive they indicate the potential benefits of flavonoid consumption with respect to CVD (V). More recently, epidemiological evidence regarding the intake of red wine, principally composed of polyphenolic compounds, has generated the concept of the “French Paradox” (94). The red wine consumption among the French population may be partially responsible for the reduced incidence of CHD among the French population. Hayek et al. determined that mice deficient in apolipoprotein E, which spontaneously develop atherosclerosis, had significantly reduced lesion development when fed red wine or quercetin (95). Yamakoshi et al. determined that proanthocyanidin extract from grape seeds inhibited atherosclerosis lesion development in cholesterol-fed rabbits (96).

Observational studies have identified flavonoids as having a protective effect against CVD. Later studies discussed the *in vitro* role of flavonoids and have revealed the protective antioxidant activity of phenolic compounds (32). However, researchers postulate that some cardiovascular benefit of polyphenolic consumption may be independent of polyphenolic antioxidant capacity and attributed to the endothelium-NO dependent relaxation through the NO-cGMP pathway. Regardless, current research efforts are being directed towards understanding the mechanisms and identifying both the individual dietary plant compounds and whole functional foods that may protect against CVD.

2.5.2 Adhesion and Aggregation

Flavonoids affect platelets, monocytes and VSMCs through a variety of mechanisms that directly influence the cardiovascular system (30). According to a review by Harborne et al., flavonoids have been demonstrated to affect cardiovascular health by inhibiting platelet adhesion to the endothelial wall, aggregation, and secretion (30). Up-regulation of inflammatory mediators, platelet aggregation and endothelial adhesion are critical steps in the atherosclerotic process. During inflammation, arachidonic acid is released and metabolized by platelets to form thromboxane A₂ which causes platelet aggregation and adhesion. Activated platelets release pro-inflammatory mediators and can inhibit the development of nitrous oxide and prostacyclin (69). Aggregation of platelets can cause stenosis, impede blood flow and result in a thrombosis (30).

Flavonoids have been implicated to affect multiple pathways and considered to have antithrombotic and antiaggregatory properties. Youdim et al. concluded that anthocyanins have implications in preventing vascular disease by protecting against oxidative insult based on a study that demonstrated that vascular endothelial cells can incorporate anthocyanins. Following a four hour incubation, anthocyanin extracts from the elderberry were incorporated into the membrane and cytosol of endothelial cells (97). In yet another study, Youdim et al. evaluated the antioxidant and anti-inflammatory effects of blueberry and cranberry anthocyanins and hydroxycinnamic acids in human endothelial cells. Berry phenols reduced up-regulation of inflammatory mediators and responses to inflammatory insults (tumor necrosis factor α) that result in the attraction of leukocytes and endothelial damage. Furthermore, berry phenols were protective at the endothelial cell membrane and cytosol against inflammatory insults, oxidative stress and may be protective components in the development of vascular diseases (98). Friesenecker et al. determined that flavonoids reduced the number of leukocytes adhering to the endothelium during an arterial injury, which reduces the inflammatory response (99). Tzeng et al. determined that flavonoids prevent thromboxane formation which results in reduced platelet aggregation and adhesion (100). Numerous studies have documented that flavonoids can also inhibit platelet aggregation by stimulating adenylate cyclase activity (71).

Chung et al. evaluated the effect of quercetin and kaempferol derivatives on platelet aggregation from various stimuli in the rabbit model. The results indicated that both quercetin and kaempferol derivatives function to inhibit the platelet aggregation *in vitro* (101). According to Melzig et al. kaempferol, a flavonoid found in blueberries, was

determined to prevent the action of deaminase, an enzyme responsible for adenosine inactivation, in rat aortic endothelial cells (102). Zaragoza et al. determined through an *in vitro* study examining the effects of the bilberry, that platelet aggregation was inhibited by a solution of 30 mg/ml of bilberry anthocyanins (103). Bilberry contains four of the five anthocyanins found in the whole wild blueberry. Freedman et al. evaluated the *in vivo* and *in vitro* effect of purple grape juice and purple grape juice derived flavonoids among 20 healthy male subjects with respect to cardiovascular disease and endothelial integrity. The researchers discovered that platelet aggregation was reduced with the oral supplementation and also during the *in vitro* trials. In addition, NO production was increased and superoxide production was decreased in both trials. Therefore, positive cardiovascular effects were observed both in *in vitro* and in *in vivo* studies with purple grape juice (104). Thrombin-induced platelet aggregation and platelet activity in thrombosis were decreased in rats fed grape polyphenolics (32). Numerous other *in vitro* studies determined that anthocyanins reduce capillary permeability, prevent platelet aggregation, and thrombus formation, which are all, associated with CVD (97, 98).

The antithrombotic and antiaggregatory activities of flavonoids are related to their structure. Flavonoids' ability to inhibit platelet aggregation, by inhibiting cAMP, is dependent upon the following: a double bond between C-2 and C-3, a 3-OH group, and a carbonyl group at C-4. According to research conducted by Robak et al., flavonoid glycosides and flavanone derivatives are not able to impair platelet aggregation. Chemical characteristics that impede the ability of a flavonoid to effect platelet

aggregation are: saturation of the double bond between C-2 and C-3, glycosylation at C-3 and polyhydroxylation. Consequently, the inhibitory effects of flavonoids are determined by the structural composition of the flavonoid (71).

2.5.3 Endothelium-dependent Vasorelaxation and Nitric Oxide

The vascular endothelium regulates vascular tone and blood flow through responses to vasoactive stimuli and shear stress (105). Vasorelaxation assists in preventing adhesion of platelets and reducing blood flow, which can affect the formation of a lesion. The release of endothelium derived substances, including NO and EDHF, produce endothelium dependent vasorelaxation. The reduced vasorelaxation observed with a dysfunctional endothelium in various vascular pathologies is attributed to a reduction in NO production and release (105). Thus, NO has been proven to play a pivotal role in endothelial dysfunction (105, 106, 107).

Flavonoids from various fruits, vegetables, nuts, teas and herbs have been implicated in inducing endothelium-dependent vasorelaxation through a variety of mechanisms (105). Andriambeloson et al. investigated the endothelial-NO dependent effect of anthocyanin enriched blueberry extracts *in vitro* on rat aortic rings. The results determined that the delphinidin, but not malvidin or cyanidin, enriched blueberry extract induced an endothelial-NO dependent vasorelaxation. With endothelial removal or suppression of NO synthesis via inhibitors, the relaxation mechanism was suppressed. Consequently Andriambeloson et al. concluded that, the mechanism utilized in the delphinidin-induced vasorelaxation was completely mediated by NO (105). Andriambeloson et al. then observed the *in vitro* vasodilatory effect of red wine

polyphenolic compounds (RWPC) on NO induced vasorelaxation in rat aortic rings. The RWPC induced endothelial-NO dependent vasorelaxation function by increasing the production and release of NO and cyclic-guanosine 3', 5-monophosphate (106).

Andriambeleson et al. postulated that although flavonoids have been observed to reduce the oxidation of lipids, the mechanism involved in RWPC induced vasorelaxation is possibly based on the inhibition of cyclic nucleotide phosphodiesterase rather than on the protection of NO from oxidative stress (106). Andriambeloson et al. also compared the *in vitro* endothelium-dependent vasorelaxant activity of the individual RWPC extracts, polymerized flavanols and anthocyanins of RWPC (105). Results of this study revealed the anthocyanin extracts were more potent in inducing endothelium-dependent relaxation than the oligomeric condensed tannins (105). In addition, the anthocyanin delphinidin, an anthocyanin found in blueberries, was shown to induce 89% vessel relaxation *in vitro*. On the hand, malvidin and cyanidin, two other anthocyanins found in blueberries, did not induce vasorelaxation *in vitro* (105, 106). The endothelium-dependent vasorelaxation effect observed with the anthocyanin extracts and the individual delphinidin extract was mediated entirely by NO and comparable to the effect observed with the original RWPC (106). Fitzpatrick et al. observed *in vitro* NO-endothelium-dependent vasorelaxation of the rat-precontracted aorta during exposure to RWPC, grape juice polyphenolics and grape skin polyphenolics (107). Muzutani et al. evaluated the *in vivo* effect of RWPC on blood pressure and the biomechanical properties of stroke-prone hypertensive rats. The rats were fed RWPC for 8 weeks and Muzutani et al. observed reduced blood pressure and improved arterial biomechanical properties (108). In conclusion, the anthocyanin extracts, particularly delphinidin, of both RWPC

and blueberries, elicit endothelial-NO dependent vasorelaxation *in vitro* in rat aortic rings (105). Furthermore, these studies indicate the endothelial-NO dependent relaxation of aortic rings is caused by an extracellular dependent calcium mechanism that activates NO synthase. The vascular relaxation effect observed with polyphenolic compounds, with respect to CVD, is most likely associated with the increases in NO production through the NO-cGMP pathway and independent of their anti-oxidant effect (32, 105, 106, 107). Lebeau et al. revealed encouraging results through an *in vivo* study in which rhamnoside quercetin rich diets, reduced myocardial post-ischemic damage in rats (109). Tsuda et al. fed rats cyanidin 3-O- β -D-glucoside (C3G) and observed an increased resistance to liver ischemia-reperfusion injury. The C3G increased the serum thiobarbituric acid-reactive substance (TBARS) indicating that C3G has *in vivo* antioxidant functions (110).

Djousse et al. examined the effect of red wine consumption with a high fat meal on the postprandial endothelial function of 13 healthy human subjects by evaluating flow-mediated dilation of the brachial artery. High fat meals may acutely impair endothelium-dependent vasodilation. The results did not indicate a significant difference in flow-mediated dilation with the consumption of red wine (111). Sanae et al. studied the effects of eight catechin derivatives on the vascular tone of intact and denuded rat thoracic aortas. The catechin derivatives were determined to inhibit the endothelium dependent relaxation by inhibiting NO activation and impairing the response of guanylate cyclase in VSMC. Consequently, Sanae et al. concluded that the catechin derivatives potentiated the contractile response to phenylephrine observed in intact rat thoracic aorta, and diminished the response in denuded rat thoracic aorta (112).

2.6 Cardiovascular Health and Whole Wild Blueberries

Blueberries, containing polyphenols and other antioxidants, have the highest antioxidant capacity of all fruits and vegetables (48, 49). Numerous studies have investigated the effect of polyphenolic extracts from flavonoid containing foods or the activity of individual anthocyanin extracts. However, the vast majority of the studies conducted thus far, has been *in vitro* or *ex vivo*, with only a few evaluating the effects of anthocyanin extracts *in vivo*. In addition, there are very few studies that have evaluated the role of whole wild lowbush blueberries (*Vaccinium Angustifolium*) in the etiology of cardiovascular disease. Research on flavonoids implicates that flavonoids found in blueberries could potentially improve vascular health by reducing platelet aggregation, improving capillary permeability, reducing leukocyte adhesion and oxidative stress and enhancing endothelial-NO dependent vasodilatation of the arteries (68).

Increased serum antioxidant status among human subjects following consumption of lowbush blueberries (*Vaccinium Angustifolium*) has been documented. Kay et al. investigated the effect of whole wild blueberries (*Vaccinium Angustifolium*) on postprandial antioxidant status in humans as related to chronic degenerative diseases. ORAC and total antioxidant status were used to evaluate the serum antioxidant status of eight middle-aged men following a high fat meal and a control supplement and then a high fat meal and a blueberry supplement. The blueberry supplement consisted of 100 g freeze-dried blueberry powder. The results revealed that the blueberry supplement caused a diet-induced increase in *ex vivo* serum antioxidant status (58). Kay et al. observed an *ex vivo* increase in antioxidant status among eight human subjects following

a one week supplementation with 100 grams/day freeze dried blueberry powder (58). Mazza et al. confirmed an increase in serum antioxidant status in five male subjects following consumption of 100 g of freeze-dried blueberry powder (*Vaccinium Angustifolium*) (113). Consequently, there is an association between the *in vitro* and dietary *ex vivo* antioxidant properties of blueberries. According to Aviram et al. serum antioxidant status and the development of chronic degenerative diseases are inversely related (67).

Blueberries and red wine are obviously not the only dietary sources containing anthocyanins. In fact, anthocyanins and polyphenolics found in numerous fruits and vegetables contribute to the concentrations of anthocyanins in human plasma. Studies have been conducted to compare anthocyanin plasma values in humans with the plasma concentration needed to induce relaxation. The effective concentration of plasma anthocyanins required to induce 50% of maximal endothelium dependent relaxation (EC_{50}), the EC_{50} concentration, in humans is 1-10 $\mu\text{mol/L}$. Paganga and Rice-Evans evaluated non-supplemented humans and found steady-state plasma concentrations of anthocyanins to be 0.5-1.6 $\mu\text{mol/L}$ (62). This observed anthocyanin plasma concentration is within the EC_{50} value and implies that human plasma concentrations can attain the amount needed to induce 50% of a maximal vessel relaxation. According to Cao et al., peak serum antioxidant status in humans occurs 60 to 70 minutes following consumption (114). Consequently, this evidence further enhances the possible therapeutic role of dietary anthocyanins particularly those found in whole wild lowbush blueberries (105).

Pilot studies in Dr. Klimis-Zacas's laboratory, as documented by Kalea et al. and Norton et al. determined the effect of whole wild blueberries on the biomechanical properties of the artery using the Sprague-Dawley rat model, as related to cardiovascular disease (11, 115). They determined that blueberries added to the diet of Sprague Dawley rats decreased the maximum contraction in response to alpha-1 adrenergic agonists in the rat aorta. This is the first time whole wild blueberries incorporated into animal diets showed an *in vivo* effect on the biomechanical properties of the arteries.

This research project is significant for two very unique reasons. One it is the first time the role of dietary whole wild blueberries and their effects on the mechanical properties of the arteries is being investigated. Hence, the study is one of the first *in vivo* studies conducted to examine the health benefits of incorporating blueberries into a daily diet. Second, this is one of the only nutritional projects worldwide utilizing the scientific technology of tissue force analyzers to unravel a nutritional problem. The tissue force analyzer is a highly sensitive and novel instrument being used in physiological experiments. Tissue force analyzers allow us to relate the physiological properties of arterial rings to their biomechanical properties. Consequently, this technology will allow the effects of blueberries on the mechanical properties of denuded and intact arterial rings to be studied as they relate to CVD.

2.7 Objectives

1. To evaluate the role of whole wild blueberries on the biomechanical properties (F_{max} and pD_2) of the arteries of the Sprague-Dawley rat.
2. To determine the site of the action of whole wild blueberries on the artery (smooth muscle vs. endothelium)

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal Models

The experimental protocols and procedures for handling rats were approved by The Institutional Animal Care and Use Committee of the University of Maine. Weanling male Sprague-Dawley rats were obtained from the Charles River Laboratories (Wilmington, MA). The animals were randomly assigned to one of three diet groups and housed in conditions previously described (Taylor et al., 1996). The animals were weighed weekly and provided with free access to food and tap water. The three diet groups were the blueberry (B), AIN-93 control (C) and reverse (R) diet group. The B and C group diet study were each fed for a period of 13 weeks. The R diet group consumed the AIN-93 control diet for 13 weeks and then the blueberry diet for a period of 8 weeks. Please refer to Table 3.1 for the Experimental Design of the Diet Study.

Table 3.1: Diet Groups

Diet Group Name	Diet Composition	Duration on Diet
Control Group	AIN-93 diet	13 weeks
Blueberry Group	AIN-93 diet plus blueberries	13 weeks
Reverse Group	AIN-93 diet AIN-93 diet plus blueberries	13 weeks 8 weeks

3.2 Diet Composition

Diet composition is presented in Table 3.2. The diets were composed of dextrose, egg white solids, vitamin mix, D-L-Methionine, biotin, mineral mix (1gm/kg), and corn oil. All purified ingredients were purchased from Harlan Teklad (Madison, WI), with the exception of the mineral mix that was purchased from ICN Biomedicals (Aurora, OH). Whole wild blueberries were purchased as a composite of blueberries from Wymans (Cherryfield, ME) and freeze dried with standard procedures by the American Lyophilizer Inc. (Bridgeport, PA). A blender was used to grind the freeze dried blueberries to a fine powder. Food intake among diet groups did not differ as determined by Kalea et al. (11).

Table 3.2: Diet Composition

Dietary Component	Control Diet	Blueberry Diet
Dextrose	691 g	611g
Egg white solids	200 g	200 g
Blueberries	0 g	80 g
Vitamin mix	10 g	10 g
D-L-Methionine	4 g	4 g
Biotin	0.002 g	0.002 g
Mineral mix (1g/kg Mn)	35 g	35 g
Corn Oil	60 g	60 g
Total	1000 g	1000 g

3.3 Drugs and Solutions for the Surgical Procedure

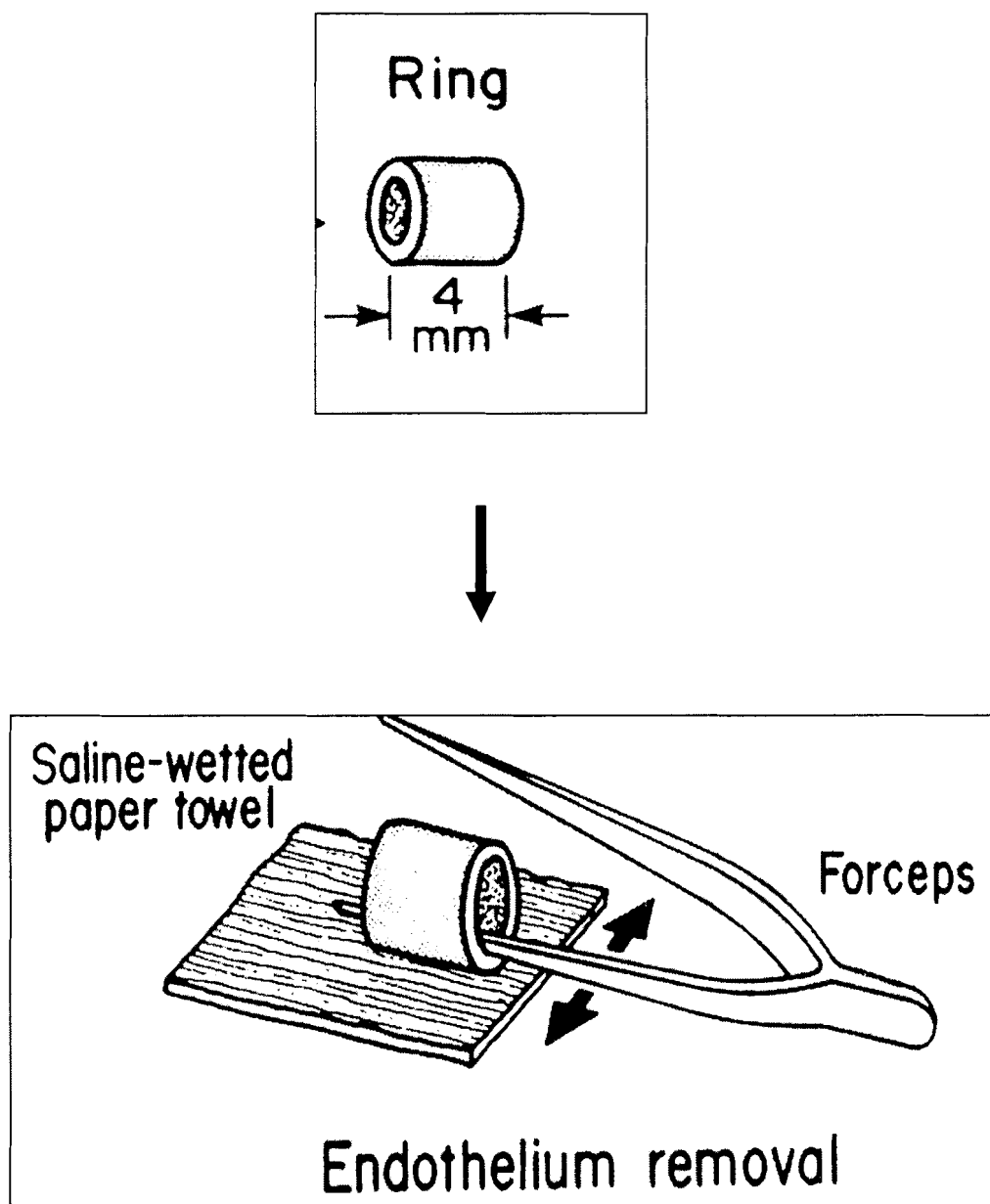
Salts for the Physiologic Salt Solution (PSS in mM: NaCl 118, KCl 4.7, CaCl 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 12.5, glucose 11.1), acetylcholine chloride and L-phenylephrine were purchased from Sigma (St. Louis, MO, USA).

3.4 Preparation and Mounting of Rat Aortic Rings

At the end of the feeding period, each animal was fasted for 10-12 hours prior to the beginning of each experiment. Each animal was sacrificed with the administration of 2 to 3 minutes of CO₂ anesthesia. Blood was removed via cardiac puncture and the thoracic aorta was carefully extracted and placed in PSS. The aorta was then measured in a silicon-coated petri dish containing a ruler fastened to the bottom. The adhering fat and connective tissue were carefully removed from the vessel. The shape, length and any abnormalities of the aorta were documented. The vessel was cut into four rings, each 2-3 mm in length using surgical scissors (George Tiemann & Co., Hauppauge, NY). With each animal, two of the rings were denuded and two were left intact for the experimental procedure.

Denudation was achieved in two of the four aortic rings by gently rubbing the intima of the aortic ring with curved forceps on a PSS saturated paper towel. Figure 3.1 illustrates the method utilized to achieve denudation. Denudation mimics the arterial injury that occurs in CVD. Furchgott and Zawadzki utilized the denudation method to discover the role, mechanism and factors influencing the pathway of EDRF/NO (116). These researchers determined that rabbit aortic vessels with an intact endothelium relaxed in the presence of acetylcholine.

Figure 3.1: Method Used for Denudation

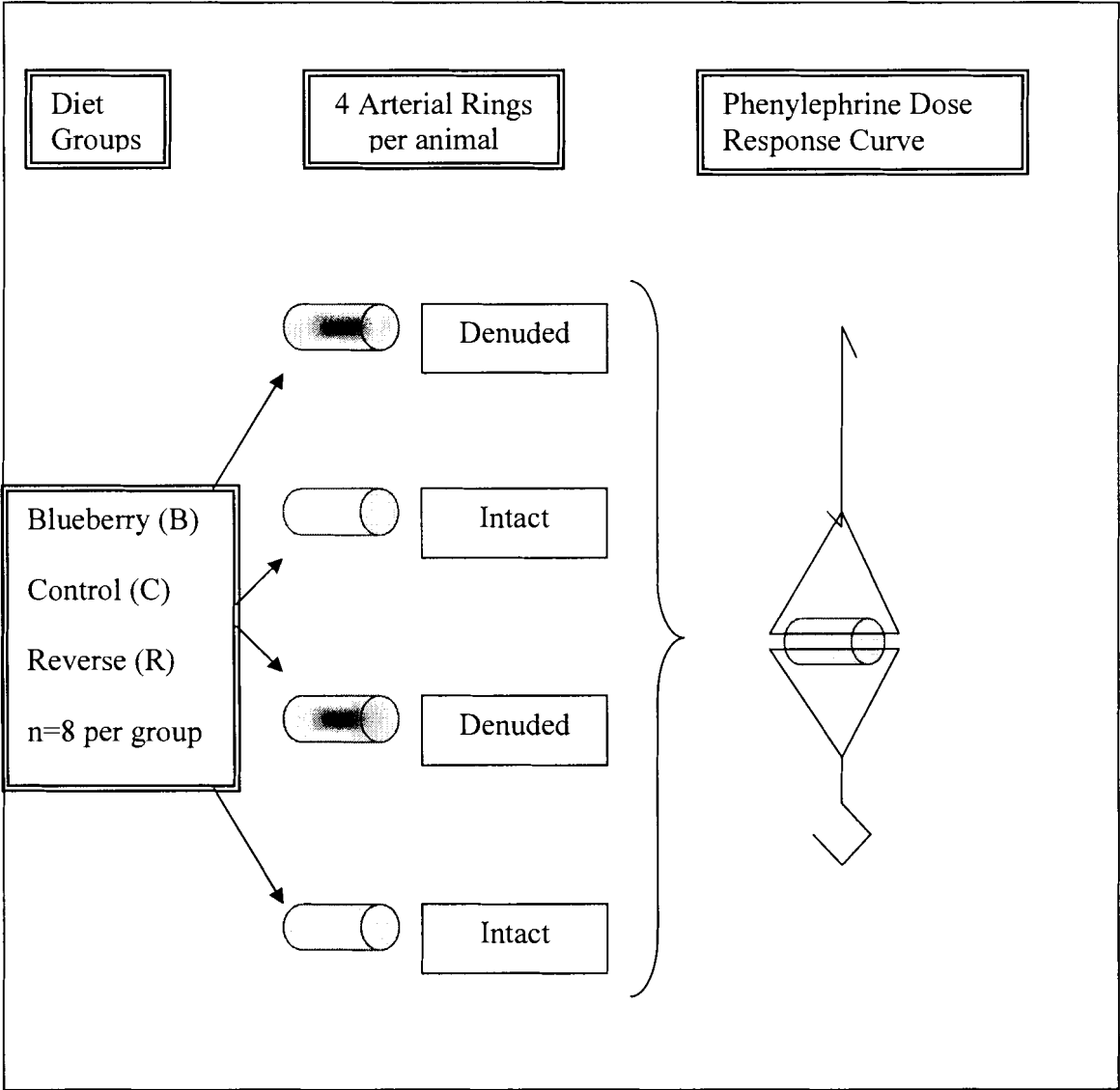


Excerpted from Luscher & Vanhoutte (9).

Whereas denuded aortic rings were not able to relax in the presence of EDRF/NO because the EDRF is released from the endothelium which is absent in denuded rings (116). In an intact endothelium, the contractile response of the α -adrenoreceptor is reduced because of the effect of NO to induce vasodilation (117). Disturbances in the production of EDRF/NO are commonly observed in atherosclerotic animal models because of the presence of a dysfunctional endothelium. Consequently, the role of whole blueberries as a functional food, with respect to CVD, can only be evaluated and fully understood through an *in vivo* study investigating both intact and denuded arterial rings.

The rings were passed through two stainless steel wires that were closed upon themselves to form two wire triangles (16, 117). The aortic rings were mounted in standard organ tissue baths containing PSS aerated with 95%O₂ /5% CO₂ (pH 7.45) and maintained at 37°C by a thermoregulator (16, 20). One of the triangles was connected to a wire attached to a microprocessor based Tissue Force Analyzer (Micro-Med, Louisville, KY) while the other wire was attached to a glass hook in the tissue bath. The order the rings were placed in tissue baths was random. All rings from each animal were mounted in organ baths within 75 minutes from the administration of the anesthesia. The experimental design in its entirety is diagrammed in Figure 3.2.

Figure 3.2: Experimental Design



3.5 Tissue Equilibration and Experimental Protocol

Following a 45 minute equilibration period under a 1.5 gram preload rings were preconditioned with the 17 μ l of acetylcholine (Ach) 10^{-3} and 17 μ l of L-Phenylephrine (Phe) 10^{-3} for a period of 10 minutes followed by five washouts. Contraction of the rings was accomplished with six progressively higher doses of the alpha-1-adrenergic receptor-agonist Phe (10^{-3} to 3×10^{-6}). Administration of acetylcholine (3×10^{-6}) induces relaxation and was utilized to validate the presence or absence of an endothelial layer among the rings. Following a 25 minute wash out period a second Phe dose response curve was generated as previously described (11).

The percent relaxation for each ring was assessed with the administration of the Ach dose. The exclusion criteria for the condition of the endothelium were determined by the percent relaxation observed in intact and denuded rings. Arterial rings with an intact endothelium demonstrated a relaxation between 85% and 100%. Intact arterial rings were excluded and considered to have a damaged endothelium if they relaxed less than 85%. Arterial rings without an endothelium did not show more than a 5% relaxation. Denuded arterial rings that exhibited an endothelium induced relaxation greater than 5% were excluded and determined to not have been successfully denuded (11, 16, 20, 117).

Tension changes in the artery assessed by the tissue force analyzers were measured by a personal computer with system integrated software to generate digitalized raw data (DMSI-210, Micro-Med, Louisville, KY). The digitalized raw data was used to generate individual dose response curves and the experimental parameters. The experimental parameters are presented in Table 3.4. Contraction and relaxation

generated tension changes were used to generate the maximum contraction force (Fmax) value. The Fmax is indicative of the myogenic behavior and mechanical properties of each aortic ring and was used to determine the effect of the diet on the contractile machinery of the artery. EC₅₀ is the concentration of the fraction required to inhibit 50% of the vessel response. The EC₅₀ was obtained by transforming the dose response curves to semi-log curves. The pD₂ value indicated the vessel sensitivity to each fraction of agonist added to the tissue bath. The pD₂ value was calculated as the negative log (base 10) of the EC₅₀ value and serves as an indicator of receptor agonists interactions (16, 117).

Table 3.3: Experimental Parameters

Experimental Parameter	Biological Interpretation	Assessment Method
Fmax	Maximum contraction and relaxation force of the vessel <i>Contractile machinery</i>	Dose response curve
pD ₂	Cell membrane receptor and vessel reactivity <i>Vessel reactivity</i>	Negative log of EC ₅₀
EC ₅₀	Ach dose required to inhibit 50% of vessel response	Semi-log transformations

3.6 Statistical Analysis

The Sigmastat Statistical Program Package (SAS Institute, Cary, NC) was used to conduct the statistical analysis. The values for Fmax and PD₂ were presented as the mean \pm SE. The Fmax and PD₂ value were each compared between diet groups with separate two-way analysis of variance (ANOVA) to detect the effect of diet. A three-way ANOVA was used to assess the differences between the intact and denuded rings with respect to different dietary treatments and the Student Newman Keuls (SNK) was used for multiple comparisons. Comparisons among animal body weights were conducted with standardized T-tests. Statistical significance was defined as a P value <0.05 (11, 16, 20).

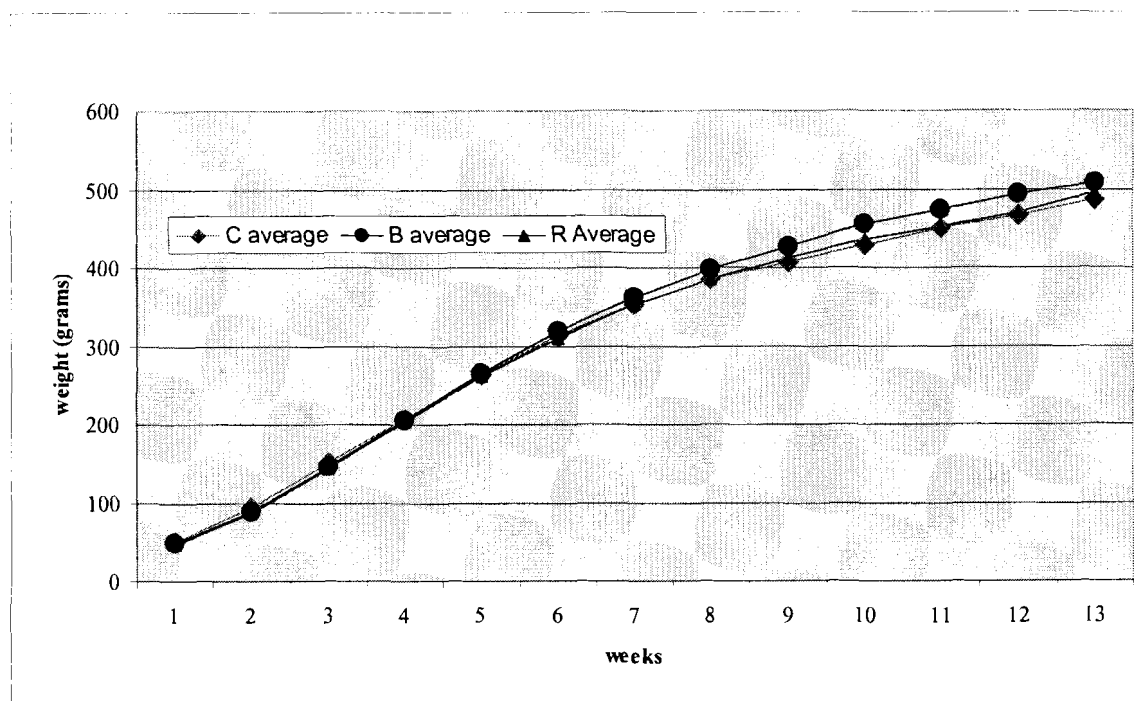
CHAPTER 4

RESULTS

4.1 Rat Growth and Weights

Figure 4.1 displays the rat growth curve. Animals on the B, C, and R diets all gained weight during the diet study. The rate of growth did not show a significant difference between the groups during the 13 week time period. The final mean body weights at the end of the 13 week period were 513 ± 11 g for the C group, 522 ± 7 g for the B group and 508 ± 9 g for the R group. The final mean body weights between the three groups did not differ significantly at the end of the 13 week period ($p < 0.05$).

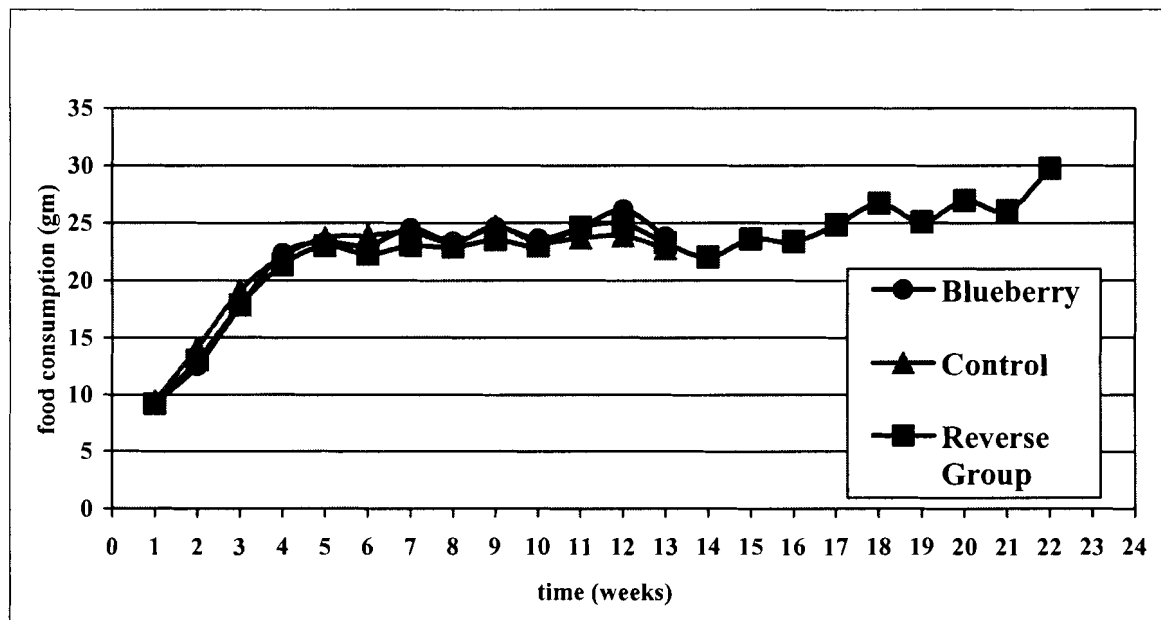
Figure 4.1: Rat Growth Curve



4.2 Food Consumption

The daily food intake among animals on a C, R and B diet was evaluated in the pilot study conducted in our laboratory during the previous year (11). Food intake per day did not differ significantly among diet groups over the course of the 13 week study. For the three groups, food intake steadily increased during the first five weeks and then steadily plateaued. No statistically significant differences were found in food intake among the three diet groups (See figure 4.2).

Figure 4.2: Effect of Whole Wild Blueberries on Food Intake of Sprague-Dawley Rats



4.3 Maximum Contraction Force (Fmax Value)

4.3.1 Denuded and Intact Arterial Rings Maximum Contraction Force

The mean maximum contraction force (Fmax) was evaluated in the denuded and intact arterial rings of all three diet groups and is presented in Table 4.1. The maximum contraction force that developed in response to phenylephrine was significantly different between the denuded and intact arterial rings. The intact arterial rings developed a significantly smaller contractile response, while the denuded arterial rings developed a larger contraction force ($p < 0.05$) in response to phenylephrine. Mean Fmax among the intact arterial rings of the three diet groups was 0.969 ± 0.00781 ; while among denuded arterial rings mean Fmax was 3.076 ± 0.00781 with statistically significant differences at $p < 0.05$.

Table 4.1: Effect of Denudation on the Mean Maximum Contraction Force (Fmax) of Arterial Rings

Arterial Ring Treatment	Fmax Value (grams)
Intact Arterial Rings	$0.969 \pm 0.00781^{(1)}$
Denuded Arterial Rings	$3.076 \pm 0.00781^{(2)}$

(1) (2) Means between groups not having the same number are statistically significant at $p < 0.05$.

4.3.2 Intact Arterial Rings Maximum Contraction Force

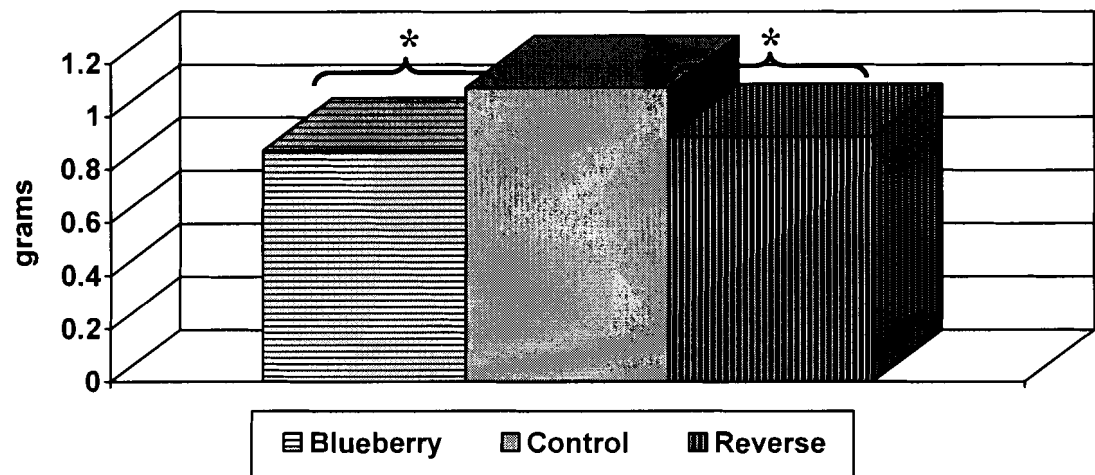
The effect of the diet on intact and denuded rings is presented in Table 4.2. The mean maximum contraction force that developed among the intact arterial rings of the control group was significantly larger than the mean maximum contraction force that developed among the intact rings of the blueberry and reverse groups ($p < 0.05$). The mean F_{max} observed among the control group's intact arterial rings (1.109 ± 0.0463) was significantly different than the mean F_{max} observed in the intact rings of both the reverse (0.926 ± 0.0463) and blueberry (0.873 ± 0.0463) groups ($p < 0.05$). Thus, the intact arterial rings of the blueberry and reverse groups developed a significantly smaller contraction force when challenged with the L-phenylphrine compared to the control group ($p < 0.05$). Additionally, the F_{max} of the blueberry group's intact arterial rings tended to be lower than the reverse group's intact arterial rings but did not reach statistical significance ($p < 0.05$). Figure 4.3 graphically depicts the mean maximum contraction force of the intact arterial rings.

Table 4.2: Effect of Whole Wild Blueberries on the Mean Maximum Contraction Force (F_{max}) of Intact Arterial Rings

Diet Group	F_{max} Value (grams)
Control Group	$1.109 \pm 0.0463^{(1)}$
Blueberry Group	$0.873 \pm 0.0463^{(2)}$
Reverse Group	$0.926 \pm 0.0463^{(2)}$

(1) (2) Means between groups not having the same number are statistically significant at $p < 0.05$.

Figure 4.3: Effect of Whole Wild Blueberries on the Mean Maximum Contraction Force (Fmax) of Intact Arterial Rings



*Statistically significant at $p < 0.05$.

4.3.3 Denuded Arterial Rings Maximum Contraction Force

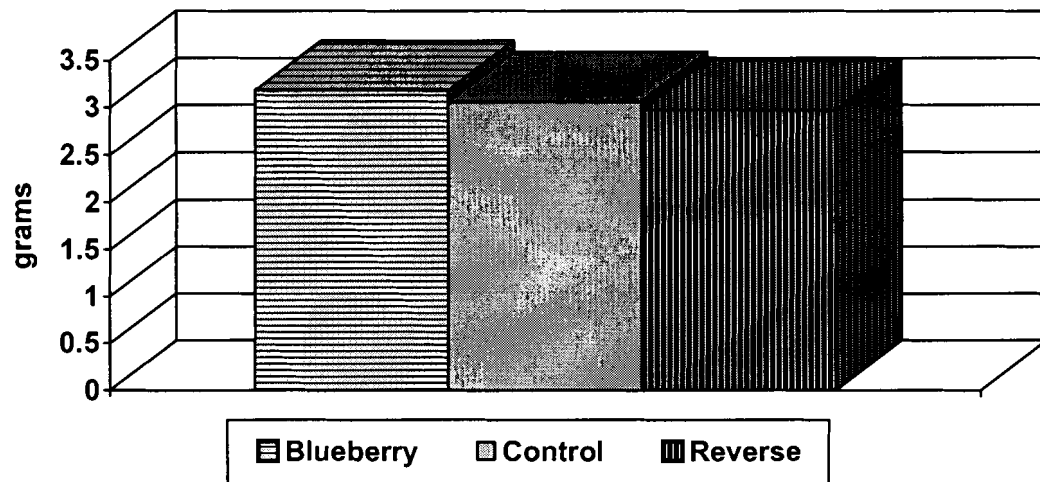
The mean Fmax of the denuded arterial rings from the blueberry, control, and reverse diet groups are presented in Table 4.3. The mean Fmax developed by the denuded arterial rings of the control, blueberry and reverse groups were 3.065 ± 0.0580 , 3.185 ± 0.0580 and 2.977 ± 0.0580 respectively. No statistically significant differences in Fmax among the denuded arterial rings were observed ($p < 0.05$). Figure 4.4 presents graphically the effect of diet on the Fmax of denuded arterial rings.

Table 4.3: Effect of Whole Wild Blueberries on the Mean Maximum Contraction Force (Fmax) of Denuded Arterial Rings

Diet Group	Fmax Value (grams)
Control Group	3.065 ± 0.0580
Blueberry Group	3.185 ± 0.0580
Reverse Group	2.977 ± 0.0580

Means are not statistically significant at $p < 0.05$.

Figure 4.4: Effect of Whole Wild Blueberries on the Mean Maximum Contraction Force (Fmax) of Denuded Arterial Rings



Not statistically significant at $p < 0.05$.

4.4 Vessel Sensitivity: pD_2 Values

4.4.1 Denuded and Intact Arterial Rings Vessel Sensitivity

The pD_2 value is a measure of vessel sensitivity to the alpha-adrenergic receptor for each phenylephrine dose. Vessel sensitivity indicates the membrane receptor conformational changes that occurred for each phenylephrine dose. Table 4.4 presents the pD_2 values for the intact and denuded arterial rings. The pD_2 value for the denuded arterial rings (7.471 ± 0.00451) was significantly higher than the pD_2 value for the intact arterial rings (6.776 ± 0.00451) at $p < 0.05$.

Table 4.4: Effect of Denudation on the Mean pD_2 of Arterial Rings

Arterial Ring Treatment	Vessel Sensitivity: pD_2 Value
Intact Arterial Rings	$6.776 \pm 0.00451^{(1)}$
Denuded Arterial Rings	$7.471 \pm 0.00451^{(2)}$

(1) (2) Means between groups not having the same number are statistically significant at $p < 0.05$.

4.4.2 Intact Arterial Rings Vessel Sensitivity

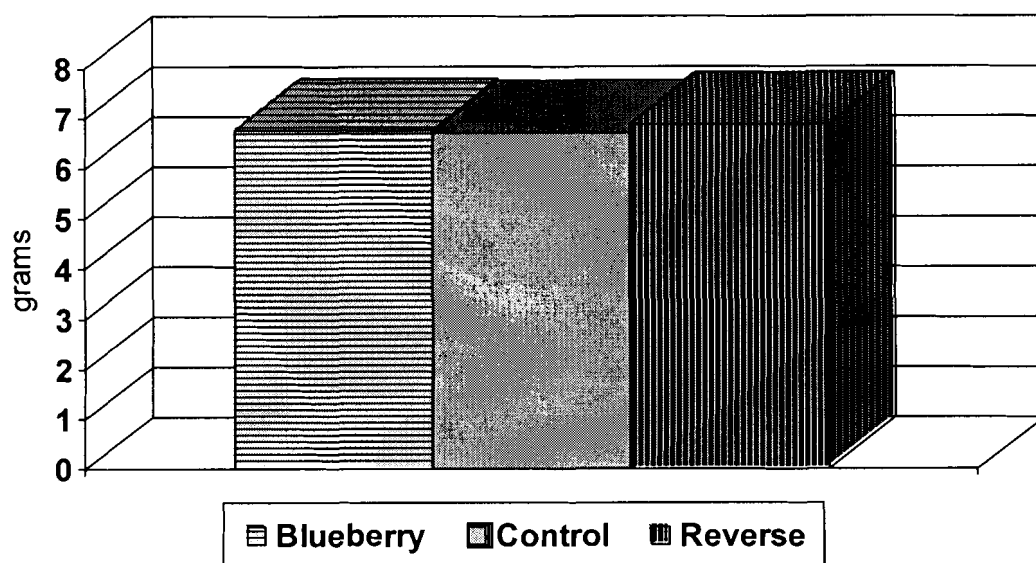
Table 4.5 and Figure 4.6 display the mean pD_2 values observed among the intact arterial rings. The mean pD_2 values for the intact arterial rings of the blueberry (6.751 ± 0.0791), control (6.715 ± 0.0791) and reverse (6.864 ± 0.0791) diet groups did not differ significantly from each other ($p < 0.05$).

Table 4.5: Effect of Whole Wild Blueberries on the Mean pD_2 of the Intact Arterial Rings

Diet Group	Vessel Sensitivity: pD_2 Value
Control Group	6.715 ± 0.0791
Blueberry Group	6.751 ± 0.0791
Reverse Group	6.864 ± 0.0791

Means are not statistically significant at $p < 0.05$.

Figure 4.5: Effect of Whole Wild Blueberries on the Mean pD_2 of Intact Arterial Rings



Not statistically significant at $p < 0.05$.

4.4.3 Denuded Arterial Rings Vessel Sensitivity

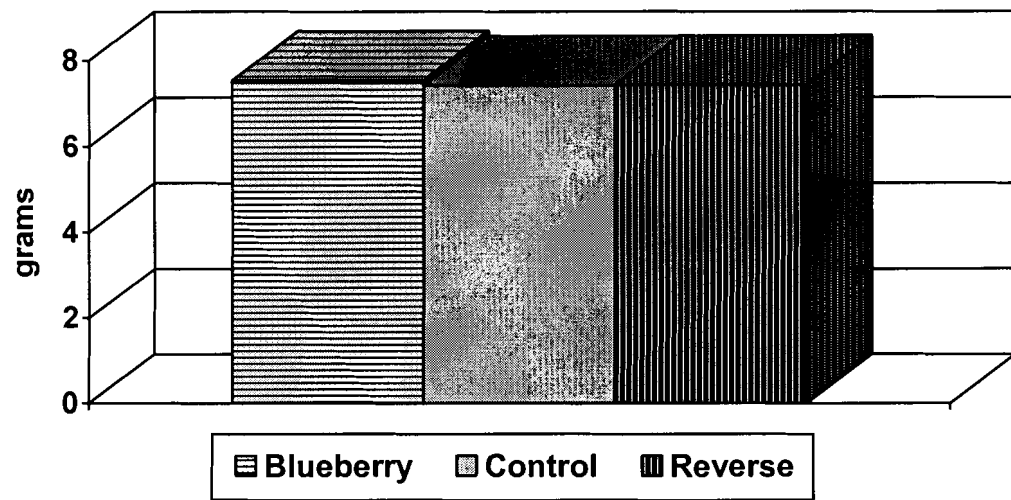
Table 4.5 and Figure 4.6 display the mean pD_2 values observed among the denuded arterial rings. The mean pD_2 values for the denuded arterial rings of the blueberry (7.548 ± 0.0509), control (7.426 ± 0.0509) and reverse (7.440 ± 0.0509) groups did not differ significantly from each other ($p < 0.05$).

Table 4.6: Effect of Whole Wild Blueberries on the Mean pD_2 of Denuded Arterial Rings

Diet Group	Vessel Sensitivity: pD_2 Value
Control Group	7.426 ± 0.0509
Blueberry Group	7.548 ± 0.0509
Reverse Group	7.440 ± 0.0509

Means are not statistically significant at $p < 0.05$.

Figure 4.6: Effect of Whole Wild Blueberries on the Mean pD_2 of Denuded Arterial Rings



Not statistically significant at $p < 0.05$.

CHAPTER 5

DISCUSSION

5.1 Overall Results

The objective of the present study was to evaluate the *in vivo* dietary effect of whole wild blueberries on the biomechanical properties of the Sprague-Dawley rat aorta as related to cardiovascular disease. Our results demonstrate for the first time that whole wild blueberries affect the vascular contractile machinery causing a decrease in the mean maximum contraction force (F_{max}) in response to the contractile effect of the alpha-1-adrenoreceptor agonist L-phenylephrine (PE). These results indicate that blueberries require a functional endothelium to exert their effect on the vascular contractile machinery. However, based on the results from vessel reactivity index, pD₂, blueberries most likely do not function through cell membrane receptor-agonist interactions. The effect of blueberries to improve arterial integrity, decrease vasoconstriction and reduce vascular resistance has implications for blood pressure regulation and overall cardiovascular health. Cardiovascular disease (CVD) continues to claim more lives each year in developed countries than any other disease. An alarming fact is that, only 30% of the morbidity associated with CVD can be attributed to the traditional risk factors. Consequently, the observed cardiovascular benefit of whole wild blueberries could provide a therapeutic mechanism for the maintenance of arterial health and the prevention of a dysfunctional endothelium.

The endothelium plays a central role in the pathogenesis of cardiovascular disease (5, 8). By modulating the arterial damage when individuals are exposed to stress or risk

factors for atherosclerosis. Endothelial dysfunction, one of the earliest stages in atherosclerosis, impairs the release and action of vasoactive substances and precedes the morphological changes of the arterial wall (8, 9). The dysfunctional endothelium, created by mechanically removing the intima, mimics the endothelium damage that occurs with atherosclerosis. The alpha-adrenergic receptors of the endothelial cells produce vasoconstrictor and vasorelaxing factors in response to stimuli (117). Vasoactive substances produced by a functional endothelium exert their action on the VSMC or the endothelium to elicit a contraction or relaxation. Consequently, Kawabe et al. proposes that the alpha-adrenergic receptors may allow for endothelial cell regulation of the VSMC contraction (117). VSMC regulation via the alpha-adrenergic receptors could prevent complete closure of the vasculature (117). The endothelium is also critical for the vessel relaxation induced by other vasoactive substances including bradykinin, substance P, histamine, serotonin and arachidonic acid (12).

The role of denudation is to remove the functional endothelial layer responsible for the production of vasoactive substances. A dysfunctional endothelium is unable to modulate the balance of endothelium-dependent vasorelaxation and contraction that is required to maintain vascular integrity (5, 9). Thus the effect of denudation allowed us to elucidate where blueberries act to affect the VSMC or the endothelium of the artery.

Impairment of endothelium-dependent NO arterial relaxation occurs with a dysfunctional endothelium in the early stages of atherosclerosis (118). Nitric oxide, a vasodilator, functions to inhibit VSMC contraction. Thus, a possible physiological influence of NO includes regulating blood pressure by affecting vasoconstriction of the vessel (22). The endothelial-dependent vasorelaxation, stimulated by increases in the

cAMP-NO system is impaired with a dysfunctional endothelium. Thus, the bioavailability of NO may be responsible for the impaired VSMC function that occurs with a dysfunctional endothelium (16, 117). Numerous studies have documented the beneficial effects of NO, including: vasodilation, inhibition of vasoconstriction, inhibition of platelet adhesion, inhibition of leukocyte adhesion, reduction of smooth muscle cell proliferation, and the scavenging of superoxide anions (9, 12, 43). Therefore, reduced NO production, occurring with a dysfunctional endothelium, is instrumental in the progression of cardiovascular disease.

5.2 Effect of Denudation

The effectiveness of denudation was evaluated with the effect of acetylcholine on each arterial ring. Furchgott and Zawadzki determined that acetylcholine requires the endothelium to cause arterial vasorelaxation (116). Thus, endothelial denudation was confirmed with the application of an acetylcholine dose and subsequent lack of vessel relaxation. In our study, the denuded arterial rings had an increased maximum contraction force (F_{max}) compared to the intact rings. The observed contractile ability of the denuded rings' VSMCs indicates that the integrity of the contractile machinery was not compromised during the mechanical denudation procedure. In addition, the denuded rings displayed less than 5 % relaxation upon exposure to the acetylcholine dose. Our results are in agreement with Adeagbo et al. who observed acetylcholine-induced relaxation of intact rings and no relaxation among the denuded rings (27). Acetylcholine exerts its effect through an indirect mechanism involving endothelium derived relaxing factor (NO) that diffuses from the endothelium to the VSMC (16). In addition,

acetylcholine mediated vasodilation can occur through NO, PGI₂, and EDHF (117). Our results validate that our denudation procedure effectively removed the endothelial layer of the denuded arterial rings without damaging the VSMC layer.

In our animals, the maximum contraction force (F_{max}) developed in response to L-phenylephrine agonist was increased among the animals with denuded arterial rings compared to animals with intact arterial rings. The effect of denudation increases the concentration of receptor sites available for α -1- agonists binding (27). Thus the absence of the endothelium enhances the contraction, via binding of L-phenylephrine to VSMC α -1-adrenoceptors (119). The structural integrity of the denuded rings was confirmed with the expected absence of relaxation upon application of an acetylcholine dose. It has been known for some time that endothelium-derived nitric oxide (NO) mediates vascular relaxation (27). Endothelial dysfunction impairs the production and release of endothelium-dependent vasodilating substances (i.e NO) to cause vessel relaxation. Thus the endothelium-denuded vessels prevented the endothelium from functioning as a modulator of vasoconstriction and vasodilation (118). As expected, we observed a reduced F_{max} to PHE among the endothelium-intact arterial rings. The presence of a functional endothelium among the intact rings, reduces the concentration of receptor sites for PHE to bind, because the endothelial layer lines the vessel and prevents direct exposure of the VSMC to the agonists.

Vessel reactivity, pD_2 , is an index of membrane receptor agonist interactions which in this study indicates whether blueberries act to affect the pathway of the agonist. Vessel reactivity is derived from the EC_{50} . The EC_{50} is the concentration of the agonist fraction required to inhibit 50% of the vessel response. The EC_{50} was obtained by

transforming the dose response curves to semi-log curves (120). The pD_2 value was calculated as the negative log (base 10) of the EC_{50} value and serves as an indicator of receptor agonists interactions and vessel sensitivity to each fraction of agonist added to each tissue bath. As expected, differences were observed in the pD_2 value between intact and denuded arterial rings (117). The reduction that we observed in the pD_2 of intact arterial rings suggests a depressed membrane-related effect of receptor agonist interactions. The observed increased pD_2 value of denuded arterial rings occurs because of the increased receptor-agonist interactions occurring in the absence of the endothelial cell layer (16, 117). With the removal of the endothelium, agonist receptors on the VSMC are exposed and present in a higher concentration which allows an increased binding and action of agonists.

5.3 Effect of Diet

This is the first study to document the *in vivo* effect of whole wild blueberries on the biomechanical properties of the artery of Sprague-Dawley rats. Among the intact arterial rings, blueberries decreased the F_{max} to PHE when compared to their effect on the control animals. The differences in F_{max} between the blueberry and control group's intact arterial rings indicate that blueberries function on the vascular contractile machinery of the artery. Our results also demonstrated for the first time a reduced maximum contraction force to PHE between the intact rings of the reverse and the control diet groups. This suggests that the addition of blueberries to older animals may be beneficial in maintaining endothelial function and regulating vascular tone. The reverse diet group consisted of animals consuming the control diet for thirteen weeks and then

the blueberry diet for eight weeks. Additionally, there were no differences in the maximum contraction force to PHE between the blueberry and reverse diet groups. Consequently, these results indicate that blueberries can function to reduce the maximum contraction force to PHE among animals that had previously consumed a diet without blueberries. Our results suggest that blueberries require a functional endothelium in order to affect the biomechanical properties of the aorta. In addition, the present study indicates that blueberries could function to not only maintain but also to improve arterial integrity of older animals.

Our results are supported by the *in vitro* observation that blueberry extract, enriched with anthocyanins, functioned to cause endothelial-NO dependent relaxation of the rat aorta (121, 105). Even though our results are the first to evaluate dietary consumption and the corresponding effect of whole blueberries on arterial function, other anthocyanin containing foods have been evaluated. The effects of red wine polyphenolic compounds (RWPC) on endothelial function have received considerable attention by researchers. Andriambeloson et al. observed the *in vitro* effect of red wine polyphenolic compounds (RWPC) on the endothelium to induce endothelium-dependent vasorelaxation (106). Andriambeloson et al. concluded that the effect of RWPC to induce vasorelaxation is associated with an enhancement of the NO-cGMP system rather than the ability of RWPC to protect NO from breakdown (105, 107). In addition, red-wine improved arterial health by preventing homocysteine from decreasing eNOS levels and resulting in endothelial dysfunction (121).

We did not observe differences in the vessel reactivity (pD₂) of the intact arterial rings between the control, reverse or blueberry groups. This suggests that the ability of

the blueberry diet to affect the vascular tone of the artery does not involve interactions between agonist membrane receptors and blueberry components. In addition, there were no differences among the denuded arterial rings from the different diets that affected the membrane-receptor agonist interactions. These results further suggest that the effect of blueberries does not involve membrane-receptor agonists on the VSMC.

Most likely, blueberries' effect of the vascular contractile machinery requires the presence of a functional endothelium, as indicated by differences in F_{max} , but does not involve membrane-receptor agonist interactions on the endothelium or VSMC. Our results suggest for the first time that whole wild blueberries, as a component of the diet, may be capable of improving the arterial integrity by affecting the vascular contractile machinery of the artery when challenged with PHE.

An effect of diet was not observed among the denuded arterial rings from the three dietary groups. Hence, the observed maximum contraction force and vessel reactivity of the endothelial-denuded arterial rings from the blueberry, control, or reverse diet groups were not significantly different. As previously stated, differences were observed in vessel reactivity between the intact and denuded rings within each diet group. Thus, the mechanism by which blueberries elicit an effect on the aortic VSMC contractile machinery to improve arterial integrity requires the presence of a functional endothelium. These results indicate that although blueberries do not have membrane-related effects of receptor-agonist interactions, they function through a pathway in the endothelium to ultimately affect the vascular contractile machinery of the artery. Thus the mechanism by

which blueberries may act to reduce arterial contraction in response to alpha-1-adrenergic agonist could occur through a pathway such as NO or cAMP that requires an intact endothelial layer.

5.4 Blueberry Components

This is the first time an *in vivo* effect of diet with whole wild blueberries on the endothelium has been observed. Previous studies have evaluated the *in vitro* effect of both blueberry and other polyphenolic components on endothelial function. Potentially, the polyphenolic components eliciting the vessel relaxation may also function to reduce the vessel contraction by preserving NO. Of the 17 known anthocyanins, only 6 are found in higher plant forms and 5 of these anthocyanins are found in the blueberry. The five anthocyanins identified in whole wild blueberries, delphinidin, malvidin, petunidin, cyanidin and peonidin, are shown in figure 2.3 and table 2.2 (54, 122). The results of our dietary study indicate the *in vivo* effects of the whole wild blueberry on the endothelium. On the other hand, previous studies evaluated the *in vitro* beneficial cardiovascular effect of individual anthocyanin extracts from blueberries and other polyphenolic containing foods. RWPC extracts, malvidin and cyanidin did not induce endothelium-dependent relaxation, while delphinidin induced endothelial vasorelaxation was completely mediated by NO (105). The location of the hydroxyl group on the anthocyanin may influence its antioxidant and antiproliferative activity (71). Consequently, the hydroxyl group position may also influence its ability to induce endothelial vasorelaxation (123). The increased synthesis of NO observed by Andriambeloson et al. may partially explain how polyphenolic compounds of whole wild blueberries in our study reduced vasocontraction.

However, the results we observed reflect the bioavailability of the whole wild blueberry in addition to the synergistic effects of its components on the endothelium.

The *in vitro* cardioprotective mechanism of polyphenolic compounds may be associated with the elicited endothelial NO dependent relaxation. Peroxynitrite is a toxic oxidant, formed from the reaction of NO and superoxide anion. Cyanidin-3-*O*-glucoside (C3G) extract from blackberries, displayed an *in vitro* protective effect against endothelial dysfunction by scavenging peroxynitrite in human umbilical vein endothelial cells (124). Therefore, cyanidin-3-*O*-glucoside may be capable of improving the bioavailability of NO. Enhanced endothelial NO-synthase-mediated relaxation was observed in internal mammary aortic rings following pre-incubation with procyanidins from grape seed extract. The procyanidins protected the endothelial cells from peroxynitrite damage and increased endothelium-dependent relaxation. Thus, this study supports the possibility that structurally related anthocyanin compounds affect the NO and influence the endothelium (125).

Intact aortic rings exposed to RWPC displayed an increase in cGMP formation (106). Delphinidin produced the same profile as the RWPC and is also one of the five anthocyanins in blueberries (54, 105). The possible role of the involvement of the NO-cGMP system is reinforced by the fact that blueberry extracts enriched with anthocyanins caused an NO-endothelial dependent relaxation of rat aorta (106). The effect of anthocyanins on the NO is further evidenced by the ability of anthocyanins to increase the activity of NO synthase and cause relaxation of the aortic rings (107). Thus, blueberries act on the endothelium to affect a pathway in the VSMC possibly by affecting the NO-cGMP system.

5.5 Hypothetical Mechanism

The results of our *in vivo* study are reinforced by the *in vitro* observations previously stated. Although the beneficial cardiovascular mechanism of whole wild blueberries is unknown, endothelium-dependent vasocontraction and vasorelaxation may be attributed to the enhanced synthesis of NO. We hypothesize that a mechanism involving enhanced NO production or increased NO bioavailability may be responsible for the reduced maximum contraction force observed among the blueberry groups intact rings in our study. The pathway for NO production, as depicted in Figure 2.2, involves the guanylate cyclase catalyzing the formation of cGMP. Increased formation of cGMP, causes smooth muscle relaxation via protein kinase phosphorylation. Continued stimulus of the endothelial cells may increase in cGMP and cause a relaxation response of the VSMC (107). Nitric oxide functions to activate VSMC guanylate cyclase, produce cAMP, cause vasodilation locally and may also be deactivated by superoxide and other O_2^- free radicals (12). Red wine increased endothelial NOS activity in human endothelial cells *in vitro* (126). Duarte et al investigated the effect of flavonoids on rat aortic smooth muscle and revealed that a possible mechanism of flavonoids involved the reduction of cGMP breakdown by inhibiting cyclic nucleotide phosphodiesterases (127). Activation of phospholipase C, phospholipase A, protein kinase C, and tyrosine kinase are not involved in the endothelial NO-dependent vasorelaxation induced by wine polyphenols in the rat thoracic aorta (128). Nitric oxide bioavailability is based upon intracellular availability of L-arginine, altered NOS activity (eNOS or iNOS), impaired receptor mediated release of NO and generation of reactive oxygen species (12). Thus, the preservation of NO or increased production of NO may occur through several

mechanisms and affect the contractile machinery of the VSMC. The increased bioavailability of NO could be responsible for the effect of whole wild blueberries on the endothelium.

Numerous studies have documented the absorption and metabolism of anthocyanins from the blueberry to increase the serum antioxidant status (58, 63, 113). The absorption of polyphenols by human subjects was detected by Paganga and Rice-Evans to increase polyphenolic plasma concentration to 0.5-1.6 μmol . The plasma concentration detected was comparable to the polyphenolic concentration required to induce 50% of the vessel maximal relaxation (62). Youdim et al. determined that anthocyanins extracted from the elderberry can be incorporated into the endothelial cell's cytosol and membrane to enhance resistance to reactive oxygen species *in vitro* (97). These studies confirm the absorption and incorporation of anthocyanins in humans and suggest the bioavailability of anthocyanins allows a concentration to provide an *in vivo* effect. Furthermore, based on these studies on absorption and incorporation of berry anthocyanins, we hypothesize that the effect of whole wild blueberries observed in the Sprague-Dawley rat aorta may also be observed in humans to improve arterial integrity and possibly cardiovascular function.

5.6 Clinical Relevance

Blueberries and other polyphenolic containing fruits have been recognized as having various health benefit's in protecting against chronic diseases including; preventing glaucoma, fibrocystic disease of the breast in humans, cancer, cerebrovascular conditions, diabetes, atherosclerosis, and cardiovascular disease (30). Blueberries have

the highest polyphenolic content of all fruits and vegetables (48, 49). Investigators have also observed a direct correlation between polyphenolic content and antioxidant properties of blueberries (48, 49). Our results confirm that whole wild blueberry components are bioavailable and can elicit an *in vivo* effect on endothelial function. The present study indicates that blueberries aid in arterial relaxation by acting on the vasodilator tone of the artery. In addition to improved vasorelaxation, studies have documented blueberries and blueberry component benefits on the cardiovascular include; reduced capillary permeability, decreased platelet aggregation and leukocyte adhesion, increased arterial flexibility, inhibition of oxidation and Maillard reactions and reduced inflammation (68). Thus far, blueberries have been implicated to be beneficial in preventing the progression of atherosclerosis and for improving cardiovascular health through various mechanisms. The vast majority of studies have been conducted *in vitro*.

Hypertension (high blood pressure) is a known risk factor for the development of atherosclerosis. The arteries of hypertensive individuals have increased vascular tone, increased vasoconstriction and a reduced a vascular response to sheer stress (129). Pomegranate juice was shown to have an inhibitory effect on serum angiotensin converting enzyme (ACE) levels and to slightly reduce the blood pressure of hypertensive patients after two weeks of consumption (130). Our results indicate that blueberries act through NO to affect the vascular tone of the artery. The vasorelaxant effect that blueberries appear to be capable of eliciting in arteries could have implications on blood pressure regulation. Furthermore, whole wild blueberries could have therapeutic implications for blood pressure regulation.

5.7 Conclusion

This is the first study to identify the role of whole wild blueberries and the location of their effect on the vascular endothelium. In addition, this is the first dietary study evaluating the possible implications whole wild blueberries could have on endothelial function as related to cardiovascular disease. Our results confirm that sufficient absorption of the whole blueberries occurred to create an *in vivo* effect. This study determined that whole wild blueberries affect the vascular contractile machinery of the rat aorta to reduce the maximum contraction force generated in response to the alpha-1-adrenergic agonist L-phenylephrin *in vivo*. Blueberries do not affect vessel reactivity as indicated by the pD₂ value. They seem to act on the VSMC through the endothelium and may involve the NO-cGMP system. Our results indicate that blueberries can improve arterial integrity and alter the responsiveness of the artery to factors such as high blood pressure or other factors increasing vessel contractility and predisposing the cardiovascular system to CVD. This suggests that blueberries improve the vascular tone of the artery. Consequently, whole wild blueberries may have implications in blood pressure regulation and could prove to have a therapeutic role in improving cardiovascular health.

5.8 Limitations and Future Recommendations

The majority of studies conducted thus far on the vasorelaxant benefits of polyphenolic compounds with reference to cardiovascular health have focused on RWPC. Although blueberries and red wine have a similar polyphenolic profile they do have significant differences. This study served the purpose of identifying the location of the

action of blueberries on the artery and allowed us to speculate the mechanism of blueberries action involving NO to be proposed. Further studies are needed to identify the pathways that whole wild blueberries function to affect the contractile machinery of the aorta. Studies are also needed to elucidate the dietary effectiveness of individual blueberry extracts in comparison to the dietary effect of whole wild blueberries on the aortic vascular contractile machinery. For instance, delphinidin, an anthocyanin common to red wine and blueberries, was documented as producing a vasorelaxant effect comparable to red wine. It is unknown if the benefit of the whole blueberry or what combination of polyphenolic compounds or individual anthocyanins are responsible for the vasorelaxant effect observed in this study. In addition, studies in other animal models are required to evaluate the dietary effect of whole wild blueberries on the endothelium and VSCM. An investigation should also be conducted to evaluate the possible role of whole wild blueberries on blood pressure regulation. The hypertensive rat would be an ideal model to evaluate the dietary effect of whole wild blueberries on blood pressure regulation. Another study would be to evaluate the effect of whole blueberry consumption within a population of hypertensive human subjects. It is apparent that based on our *in vivo* observations; there are numerous avenues a researcher could select to better understand the pathway blueberries utilize to affect the endothelium as related to cardiovascular health. Hopefully, the consumption of whole wild blueberries will prove to be an alternative therapeutic modality for improving endothelial function and overall cardiovascular health.

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Cynthia Norton was born in Alton, IL on October 21, 1976. Cindy was raised in Brighton, IL and graduated from Jerseyville Community High School in 1994. In May of 1998, she graduated from Westminster College with a Bachelor of Arts in Biology. She was employed by Genome Incyte Pharmaceuticals as a Hybridization Lab Technician from May 1998 until July 1999. Cindy then completed two years of national volunteer service with AmeriCorps. From 1999 she served as an Emergency Responder with the St. Louis Partners AmeriCorps Emergency Response Team and from 2000-2001 she served as a Patient Educator with the DownEast Community HealthCorps in Lubec, ME. In fall of 2001 she entered the graduate program in Food Science at the University of Maine.

While at the University of Maine she has become a member of Kappa Omicron Nu Honorary Society and Phi Tau Sigma Honorary Society. She presented her thesis research as a poster at the FASEB meeting in San Diego, CA in spring 2003. Cindy is a candidate for the Master of Science degree in Food Science and Human Nutrition from The University of Maine in December, 2003.