Comparing the Effects of Black and Navy Beans on Vascular Properties in Spontaneously Hypertensive Rats

by

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Abstract

Pulses such as dried beans may be useful in the treatment of hypertension. Vascular protection by beans may relate to their phenolic compounds, which are associated with darker-coloured seeds. Thus, black beans were compared to navy beans for their effects on hypertension and vascular remodelling in spontaneously hypertensive rats (SHR). Assessments of blood pressure, pulse wave velocity (PWV), and vessel morphology were conducted to ascertain the vascular effects of the beans. Results showed that neither black beans nor navy beans influenced blood pressure or PWV in SHR. However, vascular remodelling was mitigated by black beans but not by navy beans. This was indicated by the absence of a significant difference in the ratio of aortic elastin content to vessel wall area in black bean-fed SHR compared to normotensive Wistar Kyoto (WKY) rats. The consumption of black beans could therefore be a therapeutic dietary strategy for combating vascular remodelling in hypertension.

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Writing this thesis and completing this graduate program has been quite a journey. My decision to take on the role of a Master's student was not an easy one as my life was quite comfortable and stress free prior to this time. I didn't fully know what I would be getting into, but I knew it would be a challenge and that for me, it was a "now or never" type of event. I thank my parents, Carol and Greg Loader, as well as my partner, Max Porozny for being supportive of my decision to return to school and accomplish this personal mission.

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"*If your dreams do not scare you, they are not big enough*." – Ellen Johnson Sirleaf

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List of Abbreviations

ACE = angiotensin-converting enzyme
ADF = acid detergent fibre
ADHD = attention deficit hyperactivity disorder
AIN-93G = American Institute of Nutrition 93 Growth
ALT = alanine transaminase
AngII = angiotensin II
ARB = angiotensin receptor blocker
AST = aspartate transaminase
BH4 = tetrahydrobiopterin
BMI = body mass index
CDC = Crop Development Centre
CHMS = Canadian Health Measures Survey
CO = cardiac output
CSA = cross-sectional area
CVD = cardiovascular disease
DASH = Dietary Approaches to Stop Hypertension
DBP = diastolic blood pressure
***dd*H_2O** = double-distilled water
DOCA = deoxycorticosterone acetate
DPPH = 2,2-diphenyl-1-picrylhydrazyl
ECM = extracellular matrix
eNOS = endothelial nitric oxide synthase
ERK1/2 = extracellular signal-regulated kinase 1/2
ET-1 = endothelin-1
FMD = flow-mediated dilation
GAE = gallic acid equivalent
iNOS = inducible nitric oxide synthase
LDL = low-density lipoprotein
L-NAME = N^G-nitro-L-arginine methyl ester
L-NMMA = N^G-monomethyl L-arginine
MAP = mean arterial pressure
MAPK = mitogen-activated protein kinase
NAD(P)H = nicotinamide adenine dinucleotide (phosphate)
NDF = neutral detergent fibre
NO = nitric oxide
PI3K = phosphatidylinositol 3-kinase
PP = pulse pressure
PWV = pulse wave velocity
RAS = renin-angiotensin system
ROS = reactive oxygen species
SBP = systolic blood pressure
SD = Sprague Dawley
SHR = spontaneously hypertensive rat
SMAD3 = mothers against decapentaplegic homolog 3
TGF-β = transforming growth factor-β
TPR = total peripheral resistance
VSMC = vascular smooth muscle cells
WKY = Wistar Kyoto

Literature Review

Introduction

Hypertension, or high blood pressure, occurs in more than 1 in 5 Canadian adults (Padwal et al., 2016). Compared to normotensive individuals, the mortality rate of Canadians with hypertension is over 60% higher because they are more likely to develop dementia, kidney disorders, and cardiovascular disease (CVD) (Padwal et al., 2016). Furthermore, the etiology of hypertension is unknown in 95% of cases (referred to as essential or primary hypertension), making treatment a difficult task (Carretero et al., 2000). The goal of prescription drug therapy is to control high blood pressure, which is a treatment of the symptom rather than the cause of hypertension. Moreover, 30% of individuals with hypertension have uncontrolled high blood pressure, indicating that for some persons medications are unable to provide symptomatic relief (Padwal et al., 2016). Thus, it is apparent that new methods to reduce hypertension among Canadians are needed, and these methods should aim to treat the underlying causes of hypertension. At the same time, more research is required to establish the origins of primary hypertension, which will be needed to design therapies that target the underlying cause.

Dietary strategies to reduce hypertension have been previously explored. Increasing the consumption of certain macro- and micro-nutrients has been associated with blood pressure reductions among hypertensive individuals. Such nutrients include potassium, polyunsaturated fatty acids, protein (typically from plant sources), and fibre (Savica et al., 2010). In addition, non-nutritive dietary

components, namely phenolic compounds, have been shown to benefit the vasculature and exert hypotensive outcomes (Clark et al., 2015). Thus, foods that provide these aforementioned compounds should be examined for their therapeutic benefit in hypertensive individuals.

Pulses (beans, lentils, chickpeas, and peas) are crops grown in abundance throughout the Canadian prairies and comprise all of the aforementioned dietary characteristics. Previous literature has shown that pulses exert a blood pressure regulating effect and can mitigate the structural and functional changes to hypertensive blood vessels (Hanson et al., 2014; Jayalath et al., 2014). While various pulses have similar macro- and micro-nutrient contents, their effects on the vasculature are not the same. On the other hand, the composition and content of phenolic compounds differ among the pulse types. Therefore, it is likely that the vascular benefit of pulses is related to their phenolic compounds and thus pulses with greater concentrations of phenolics should be examined for their therapeutic potential.

As indicated by their dark seed coat colour, black beans typically have a greater total phenolic content and antioxidant activity than other bean types (Xu et al., 2012), and have yet to be examined for their vascular effects. Therefore, the purpose of the research described in this thesis was to compare black beans (a high phenolic content bean) to navy beans (a low phenolic content bean) on blood pressure, vascular function, and vascular remodelling in a rodent model of essential hypertension, while also comparing the effectiveness of the beans to green lentils, a

pulse that has previously been shown to exert vascular benefit (Hanson et al., 2016b; Hanson et al., 2014). The following sections will provide background from the literature on the aforementioned topics relevant to this thesis.

Hypertension

Hypertension is diagnosed when an individual has a systolic blood pressure (SBP) ≥140 mmHg and/or a diastolic blood pressure (DBP) ≥90 mmHg (Leung et al., 2016). Blood pressure is typically measured from the brachial artery using a sphygmomanometer operated by a medical doctor or other professional after the patient has been sitting in an upright and comfortable position for a minimum of five minutes (Leung et al., 2016). Three readings should be taken, with the average of the last two recorded as the patient's blood pressure (Leung et al., 2016). A blood pressure <120/80 mmHg is considered optimal as it indicates a low risk for future cardiovascular impairment, whereas 121-139/80-89 mmHg is high-normal and indicates a moderate risk for future cardiovascular impairment (Hypertension Canada, 2016a).

There are two main forms of hypertension. The majority of individuals with hypertension have essential, or primary, hypertension. Essential hypertension represents 95% of cases and is a form of hypertension that stems from an unknown cause (Carretero et al., 2000). The remaining 5% of hypertension diagnoses are due to secondary causes and thus are referred to as secondary hypertension (Viera et al., 2010). Secondary hypertension can stem from a variety of health ailments, with some common causes being renal disorders, thyroid dysfunction, aldosteronism,

and obstructive sleep apnea (Viera et al., 2010). Since essential hypertension has an unknown etiology, is a challenge to treat medically, and affects the majority of people with hypertension, essential hypertension will be the focus of this thesis. Going forward, essential hypertension will be referred to as hypertension.

Elevated blood pressure is a continuous and independent risk factor for the future development of CVD, which is the second leading cause of death in Canada after cancer (Kannel et al., 1971; Statistics Canada, 2015; Wade et al., 2015). Hypertension is often referred to as a "silent killer" because of its asymptomatic nature but severe negative consequences on health. According to data from the Canadian Health Measures Survey (CHMS), in 2012-2013, the prevalence of hypertension among Canadian adults was 22.6%, corresponding to over six million people (Padwal et al., 2016). Since 1998, the age-adjusted prevalence of hypertension among Canadian adults has increased by 60% (Padwal et al., 2016). In addition, data from the CHMS showed that the hypertensive population in Canada had mortality rates that were over 60% higher than the normotensive population (Padwal et al., 2016). Evidently, hypertension is a serious health concern.

While essential hypertension has no known etiology, Hypertension Canada has identified several modifiable lifestyle factors that increase the risk for developing hypertension. These risk factors include an unhealthy diet, smoking, excessive alcohol consumption, a sedentary lifestyle, persistent psychological stress, and the presence of sleep apnea and diabetes (Hypertension Canada, 2016b). In addition, the non-modifiable risk factors that have been identified are age and

family history of hypertension (Hypertension Canada, 2016b). On an annual basis, Hypertension Canada reviews their guidelines for the measurement, diagnosis, risk assessment, prevention, and treatment of hypertension to ensure medical professionals are assessing and treating patients with the most recent evidence-based approaches. The 2016 guidelines recommended seven health behaviour changes that patients could implement to manage their high blood pressure. These recommendations are to 1) accumulate 30-60 minutes of moderate-intensity exercise 4-7 days/week; 2) maintain or reduce body weight to achieve a body mass index (BMI) of 18.5-24.9 with a waist circumference <102 cm for men or <88 cm for women; 3) limit alcohol consumption to ≤2 drinks/day without exceeding 14 drinks/week for men or 9 drinks/week for women; 4) consume foods emphasized by the DASH diet; 5) reduce sodium intake to below 2000 mg/day; 6) increase potassium intake (as long as the patient is not at risk of hyperkalemia); and 7) incorporate relaxation techniques, or other cognitive-behaviour interventions, to reduce and manage psychological stress (Leung et al., 2016). The recommendation to increase dietary potassium intake is new to the guidelines as of 2016.

Also included in Hypertension Canada's guidelines are recommendations for the treatment of hypertension using prescription drugs. Initial therapy for hypertension should attempt to lower the high blood pressure using one of the following medications thiazine/thiazine-like diuretics, β-blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), or long-acting calcium channel blockers (Leung et al., 2016). If a single drug does not lower blood pressure in hypertensive patients, it is acceptable to use ≥2 different

medications (Leung et al., 2016). In addition, statins are recommended if the patient has three or more cardiovascular risk factors including, but not limited to male sex, age ≥55 years, peripheral artery disease, diabetes, smoking, and a total cholesterol/ high-density lipoprotein cholesterol ratio ≥6 (Leung et al., 2016). The goal of antihypertensive therapy is to reduce and maintain a blood pressure <140/90 mmHg (Leung et al., 2016). In a meta-analysis of 147 randomized, controlled trials including 464,164 participants, researchers found that a reduction in SBP by 10 mmHg or in DBP by 5 mmHg reduced the occurrence of stroke by ~33%, coronary heart disease by 25%, and heart failure by ~25% (Law et al., 2009). Thus, any intervention that reduces blood pressure by such amounts should be considered an effective therapeutic strategy.

Determinants of Blood Pressure

Cardiovascular Physiology

Blood is described as a type of dynamic connective tissue that has four main roles in the body 1) transporting nutrients, hormones, and electrolytes; 2) participating in the immune response; 3) clotting for healing and protection against blood loss during injury; and 4) maintenance of homeostasis within the body by regulating body temperature, blood glucose, blood pH, and blood pressure (Rhoades et al., 2009). The importance of continuous blood flow through the body to ensure these imperative tasks are accomplished cannot be overstated. An adequately functioning cardiovascular system, composed of the heart and blood vessels, is therefore essential for life.

Although blood circulation is a constant process in a continuous system, we can say that blood flow begins in the heart. The heart is a muscle that acts as a pump, propelling blood from the veins into pulmonary circulation for oxygenation before returning to the heart to be ejected into the arterial system for distribution to central and peripheral tissues (Hall, 2011). The pressure in the arteries during systole, when the heart contracts, is referred to as SBP. On the other hand, DBP is the pressure in the arteries during diastole, when the heart relaxes. Measures of SBP and DBP, in millimeters of mercury, are used to define a patient's blood pressure. Other measures of blood pressure include mean arterial pressure (MAP) and pulse pressure (PP). MAP is the weighted average of SBP and DBP, calculated as [(SBP + 2 × DBP)/3] (Moser et al., 2008). The difference between SBP and DBP is known as PP, and has been used as an indicator of arterial stiffness (Safar et al., 1987).

Blood pressure is a function of cardiac output (CO) factored by total peripheral resistance (TPR). CO is the volume of blood ejected from the heart each minute, and is determined by stroke volume (the volume of blood ejected per heart beat) and heart rate (the number of heart beats per minute) (Hall, 2011; Rhoades et al., 2009). Once the blood is ejected from the heart into the circulation it meets a certain level of resistance contributed by the tone of the blood vessels, referred to as the TPR (Hall, 2011). When there are increases in either CO or TPR, blood pressure will rise. However, TPR is the primary determinant of the increased blood pressure that occurs in hypertension as CO is usually unchanged (Brod et al., 1959). The main determinants of TPR are blood vessel diameter (determined by vascular tone and compliance), blood vessel length, and blood viscosity (Hall, 2011). It has been

suggested that contributors of peripheral resistance, namely endothelial dysfunction, augmented vascular reactivity, and vascular remodelling, may be causes of hypertension rather than consequences as they were previously regarded (Oparil et al., 2003). Thus, therapies for hypertension that address these aforementioned vascular impairments should be examined in hypertension research.

Within the cardiovascular system, there are five types of blood vessels that can be distinguished by their structures and functions. The five blood vessel types are arteries, arterioles, capillaries, venules, and veins. The walls of these blood vessels can contain up to three layers (or tunicas), each with distinct compositions and functions.

Common to all five blood vessel types, a single layer of endothelial cells constitutes the tunica intima, the innermost layer, of the blood vessel wall (Martini et al., 2009). The endothelium acts as both a barrier and communication medium between the vessel wall and its luminal contents. One of the major functions of endothelial cells is to produce nitric oxide (NO) to induce vasodilation. NO is produced from a reaction catalyzed by endothelial nitric oxide synthase (eNOS), which converts L-arginine and oxygen into L-citrulline and NO using tetrahydrobiopterin (BH4) as a cofactor (Klinger et al., 2013). In unstimulated conditions, eNOS remains affixed to caveolin-1, which renders eNOS catalytically inactive. Thus, eNOS must dissociate from caveolin-1 to enable NO production and subsequent vasodilation. This is accomplished by phosphorylating (thus activating)

eNOS. Blood flow-induced shear stress on endothelial cells can stimulate the activation of eNOS through a cell-signalling event involving Akt (also known as protein kinase B) and phosphatidylinositol 3-kinase (PI3K) (Dimmeler et al., 1999; Fulton et al., 1999).

A second layer of the blood vessel wall known as the tunica media can be found in arteries, arterioles, and veins, but not in venules or capillaries (Martini et al., 2009). This layer is composed of vascular smooth muscle cells (VSMC) and extracellular matrix (ECM) that are especially important for regulating vascular tone and compliance (Martini et al., 2009).

VSMC respond to endothelium-derived NO to produce the physical action of vasodilation. The counter to vasodilation is vasoconstriction, and this mechanical event opposes dilation to ensure the tone remains constant. Vasoconstriction is also mediated by other factors released from the endothelium, such as endothelin-1 (ET-1).

The ECM is composed of proteins that govern vascular compliance, namely collagen and elastin (Jacob et al., 2001; Wagenseil et al., 2009). While elastin offers extensibility, collagen provides strength and integrity to the vessel. Therefore, a delicate balance between these two ECM proteins is crucial for blood pressure homeostasis.

Finally, the tunica externa, or outermost layer, of all blood vessels (except capillaries and arterioles) contains connective tissue that is referred to as adventitia (Martini et al., 2009).

Endothelial Dysfunction

Endothelial dysfunction occurs when the ability of endothelial cells to produce NO becomes impaired (Kurowska, 2002). Endothelial dysfunction occurs in vascular diseases including hypertension. The loss of NO bioavailability due to endothelial dysfunction results in impaired blood vessel dilation thereby imposing a state of exaggerated vasoconstriction. Endothelial dysfunction contributes to a sustained increase in blood pressure, and may precede the initial rise in blood pressure in some cases.

Endothelial function of peripheral arteries can be measured in humans by flow-mediated dilation (FMD), finger plethysmography, or forearm plethysmography, and by measuring circulating NO species (Blanch et al., 2015; Flammer et al., 2012). FMD and finger plethysmography are measured in the brachial and finger arteries, respectively, after blood flow is impeded by inflation of a cuff around the limb containing the blood vessel being measured. When the cuff is deflated, blood flow is re-established, and in response, the vessel's endothelial cells produce NO, which causes vasodilation and thus enhances blood flow. This response of the endothelium can be measured using ultrasound or magnetic resonance imaging (Blanch et al., 2015). Similarly, flow can be measured after an NO agonist (such as acetylcholine) and an NO antagonist (such as N^G-monomethyl L-arginine [L-NMMA]) have been infused into the bloodstream, which is the principle behind forearm plethysmography (Blanch et al., 2015). In addition, circulating levels of NO metabolites, nitrate and nitrite, can indicate whether the endothelium is producing

NO or if this function is impaired. In animal or cell culture models, vascular proteins can be assessed by techniques such as Western blotting to determine if endothelial function is impaired.

One cause of endothelial dysfunction has been attributed to oxidative stress, or an imbalance of reactive oxygen species (ROS) to antioxidant compounds (Annor et al., 2015). ROS are detrimental to human health when their levels within the body become excessively elevated. In a healthy cell, ROS are produced during nutrient metabolism, as second messengers for certain intracellular signalling pathways, and as a part of the immune system response. ROS are also produced in the body after exposure to environmental pollutants, cigarette smoke, and ultra-violet radiation. Given that ROS have unpaired electrons they actively seek to fill their valence shell by acquiring electrons from other molecules, and thus are considered highly reactive species. Oxidative stress can contribute to endothelial dysfunction through several mechanisms. As previously mentioned, BH4 is a cofactor required for the production of NO from L-arginine and oxygen by eNOS (Klinger et al., 2013). During this reaction, the role of BH4 is to become oxidized by the electron transfer from molecular oxygen (Klinger et al., 2013). Normally, BH4 is quickly recycled back to its reduced state to participate in the reaction again (Klinger et al., 2013). However, if the endothelial cell is burdened with high intracellular ROS, BH4 can be "held up" in an oxidized state (Alp et al., 2004). Without BH4 to accept the electron transfer, eNOS generates superoxide anion and peroxynitrite instead of NO (Klinger et al., 2013). In this situation, eNOS is said to be "uncoupled" (Klinger et al., 2013).

Nullifying intracellular ROS may therefore serve as an approach to prevent eNOS uncoupling and reduce high blood pressure (Harrison et al., 2009).

ROS-producing enzymes in the endothelium that have been implicated in hypertension are nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase and xanthine oxidase (Paravicini et al., 2008). Inhibition of these enzymes has been demonstrated to reduce ROS and benefit endothelial function (in cell culture and animal models) and blood pressure (in animal models) (Gomez-Guzman et al., 2012; Kou et al., 2008; Suzuki et al., 1998). Interestingly, a reduction in NAD(P)H oxidase activity may be achieved by flavonoid treatment as this was shown with epicatechin treatment in hypertensive rats (Gomez-Guzman et al., 2012). The beneficial effect of epicatechins was attributed to a reduction in endothelin-1 (ET-1), which is known to stimulate NAD(P)H oxidase activity (Gomez-Guzman et al., 2012). Interestingly, the researchers observed reductions in NAD(P)H oxidase activity with the epicatechin treatment and this translated to significant attenuations in both ROS production and a rise in the SBP of hypertensive rats (Gomez-Guzman et al., 2012). A similar result was observed in spontaneously hypertensive rats (SHR) treated with resveratrol, which also produced a significant attenuation in blood pressure rise (Bhatt et al., 2011). This finding was accompanied with reductions in ROS and eNOS uncoupling, along with increased eNOS expression and NO production (Bhatt et al., 2011). Overall, flavonoids may act to improve endothelial function and blood pressure via ROS reduction in animal models of hypertension.

Systematic reviews and meta-analyses of clinical trials have revealed improvements in endothelial function after the consumption of foods containing high amounts of polyphenols, such as chocolate and cocoa (Hooper et al., 2012), grape seed (Feringa et al., 2011), and berries (Amiot et al., 2016). Other dietary components also improve endothelial function, including L-arginine (Bai et al., 2009) and potassium (Blanch et al., 2015). In addition, an overall healthful diet can benefit endothelial function as reported in a systematic review and meta-analysis of clinical trials employing components of the Mediterranean diet (Schwingshackl et al., 2014). The sum of this evidence suggests that diet and dietary compounds (polyphenols and nutrients) can benefit endothelial cells to positively influence vascular function, and may be especially helpful to hypertensive individuals with endothelial dysfunction.

Vascular Remodelling and Arterial Stiffness

Vascular remodelling is a term used to describe the physical changes that occur to blood vessels that can be observed during hypertension. These changes include the growth and proliferation of VSMC and changes in the ECM protein composition, resulting in a growth of the medial layer of the blood vessel wall. The media can grow inwards causing a reduction in lumen diameter, or outwards without a change to the lumen diameter (Feihl et al., 2008). In humans, vascular structure can be estimated using forearm plethysmography, or directly by myography of biopsied small arteries (Feihl et al., 2008). In experimental animals, large arteries can be excised for morphological examination. Additionally, vessel

cross-sections can be stained for VSMC nuclei (to estimate cell proliferation) or ECM proteins, such as collagen and elastin. These approaches provide specific information regarding the structural changes to the vessel wall.

Vascular remodelling has been heavily debated for being both a cause and a consequence of hypertension. Cell-signalling events that contribute to the onset of vascular remodelling processes have been previously explored. Hormone peptides such as angiotensin II (AngII) and ET-1 have been implicated in the initiation of vascular remodelling (Lee et al., 1995). On the other hand, growth inhibitors such as NO are suggested to prevent vascular remodelling (Lee et al., 1995). Altered levels and activities of AngII and ET-1, as well as reductions in NO bioavailability, are commonplace in hypertension and thus may represent important targets for the treatment of vascular remodelling.

AngII and ET-1 are both vasoconstricting peptide hormones that contribute to vascular remodelling through similar pathways. AngII is produced from angiotensin I (a product of angiotensinogen conversion by renin) by ACE, and acts both peripherally and centrally to increase blood pressure (Sparks et al., 2014). ET-1 also has a role in vasoconstriction, but its production occurs in vascular endothelial cells (Bohm et al., 2007). Aberrant production of AngII and ET-1 set the stage for pathological conditions within the cardiovascular system. Previous work has exhaustively demonstrated that stimulation of VSMC with either AngII or ET-1 induces cell proliferation and growth, as well as collagen production (Belmadani et al., 2008; Bouallegue et al., 2013; Chen et al., 2009; Kubo et al., 1998; Kwon et al.,

2003; Kyaw et al., 2001; Lambers et al., 2013; Lee et al., 2007; Luo et al., 2006; Paravicini et al., 2012; Romero et al., 2010; Touyz et al., 2001; Ushio-Fukai et al., 1998; Yogi et al., 2007). The above listed studies all showed that the mechanism through which AngII and ET-1 induce vascular remodelling involves the mitogen-activated protein kinases (MAPKs), namely p38 MAPK, c-Jun N-terminal kinase (JNK), and/or extracellular signal-regulated kinase 1/2 (ERK1/2).

Endothelial dysfunction can also contribute to vascular remodelling in hypertension. When NO signalling to VSMCs is absent, the function and structure of these cells and the ECM becomes altered. In Wistar rats administered with the eNOS inhibitor, N^{G}-nitro-L-arginine methyl ester (L-NAME), blood pressure rose in parallel with an increase in thickness of the media in the aorta (Neto-Ferreira et al., 2011). This suggests that the absence of NO contributes to vascular remodelling. Likewise, Sprague Dawley (SD) rats administered L-NAME experienced hypertension and vascular remodelling as observed by an increased media thickness and media: lumen ratio in resistance arteries (Maranon et al., 2014). Interestingly, treatment of these animals with the superoxide dismutase mimetic, Tempol, increased NO levels and prevented vascular remodelling but did not lower the high blood pressure (Maranon et al., 2014). This study indicates that vascular remodelling and blood pressure rises occur through similar but also independent pathways, and also clarifies the causal role of NO deficiency in vascular remodelling. Furthermore, this study suggests that treatment with antioxidants may reverse vascular remodelling. Indeed, a six-week treatment with curcumin in hypertensive

rats resulted in attenuated vascular remodelling as observed by lower media thickness, vessel CSA, and media: lumen ratio in the aorta (Boonla et al., 2014).

When vessels undergo structural remodelling, their compliance (ability to stretch) can become impaired. This is referred to as arterial stiffness. Arterial stiffness is described as a reduction in arterial compliance whereby the arteries can no longer stretch in response to blood flow, and this condition contributes to the blood pressure rise (Kelly et al., 1992). Arterial stiffness can be measured by pulse wave velocity (PWV), which is the speed at which a wave of pressure, generated by the ejection of blood from the heart into the aorta, travels through the blood vessels. PWV is measured between two points in the arterial system, such as between the carotid and femoral arteries or between the brachial and ankle arteries (Oparil et al., 2003). Stiffer arteries exhibit greater PWV compared to compliant arteries. Vascular remodelling can cause arterial stiffness when there are changes to the ECM protein composition such that the elastin/collagen ratio is decreased (Clark et al., 2005). As previously mentioned, collagen is a stiff protein. Thus, a disproportional increase in collagen relative to elastin in the vessel wall results in vascular stiffness. Currently, there are no medications or treatments aimed at reducing arterial stiffness for blood pressure management. Interestingly, the most recent update from the Canadian Hypertension Education Program recommended that future research should be focused on developing approaches to improve arterial compliance (Padwal et al., 2016).

Dietary Influences

The DASH Diet

The DASH diet was developed through the National Heart, Lung, and Blood Institute as an alternative intervention to prescription medications for the management of high blood pressure (Sacks et al., 1999). It was understood that a combination of healthful nutrients was likely to be more effective for reducing high blood pressure than individual nutrients alone, and thus the DASH diet was developed (Sacks et al., 1995). The diet was designed so that it was rich in protein, minerals, and fibre while being low in saturated fat, total fat, and cholesterol. Thus, it mainly contains fruits, vegetables, nuts, whole-grain cereals, low-fat dairy, fish, chicken, and lean meats (Sacks et al., 1995).

It was hypothesized that an individual with hypertension following this diet would see reductions in their blood pressure. Indeed, research on the DASH diet has showed such beneficial outcomes. In the first studies, the DASH diet was compared to a typical American diet and also to an American diet that was enriched with fruits and vegetables (Sacks et al., 1999; Sacks et al., 1995). The study examined 459 healthy adults who followed one of the three diets for eight weeks (Sacks et al., 1999). At baseline, approximately 29% of the study population had mild hypertension, and the remaining subjects had high-normal or normal blood pressure (Sacks et al., 1999). After two weeks on the diets, those that followed the DASH diet or modified American diet experienced significant reductions in blood pressure compared to the control. The blood pressure lowering effects were

independent of weight loss and were equally observed in both men and women (Sacks et al., 1999).

Interestingly, the fruit and vegetable enhanced American diet reduced blood pressure by only half as much as the DASH diet and so the authors speculated that the fruits and vegetables contributed to 50% of the blood pressure reduction seen with the DASH diet while the other components of the DASH diet contributed to the additional 50% decrease (Sacks et al., 1999). In a recent systematic review examining the effect of fruit and vegetable consumption on vascular function, as measured by arterial stiffness or endothelial function, it was found that fruit and vegetable interventions were beneficial 50% of the time (Blanch et al., 2015). This indicates that more than just a high consumption of fruit and vegetables is required to improve vascular function.

Based on the estimated phytochemical composition, the DASH diet has high levels of flavonols, flavanones, flavanols, anthocyanidins, isoflavones, carotenoids, and phytosterols but not flavones compared to a typical American diet (Most, 2004). It was suggested that the beneficial effects of the DASH diet might be attributed to the antioxidant properties of the foods supplied by the diet thereby improving endothelial function and promoting vascular relaxation (Most, 2004).

Beyond the initial studies on the DASH diet, many others have examined the therapeutic benefits of this diet. In a systematic review and meta-analysis of 17 randomized controlled trials totaling 2561 participants, the DASH diet was found to be effective for lowering SBP and DBP in both hypertensive and normotensive adult

subjects (Saneei et al., 2014). Thus, the DASH diet has been generally accepted as a healthful diet for blood pressure management, and is promoted by organizations in Canada such as the Heart and Stroke Foundation of Canada and the Canadian Diabetes Association.

The Mediterranean Diet

The Mediterranean diet is another therapeutic diet well-accepted for its cardiovascular health benefits, being comparable to interventions such as physical activity, aspirin, statins, and anti-hypertensive drugs (Widmer et al., 2015). The composition of the Mediterranean diet is similar to the DASH diet, but is based on the dietary habits of Crete, Greece, and Southern Italy in the early 1960s when life expectancy in these regions was among the highest in the world accompanied with the lowest rates of coronary heart disease, some cancers, and other diet-related chronic diseases(Willett et al., 1995). The foundation of the Mediterranean diet consists of plant-based foods, including fruits, vegetables, breads, cereals, potatoes, beans, nuts, and seeds (Willett et al., 1995). Olive oil is the main source of dietary fat, with its health benefits related to the high content of monounsaturated fatty acids and vitamin E and low content of saturated fatty acids (Willett et al., 1995). Dairy (mainly as yogurt and cheese), fish, poultry, and eggs are consumed in low to moderate amounts while red meat consumption is restricted in the Mediterranean diet (Willett et al., 1995). In addition, a low to moderate red wine consumption during meals is encouraged (Willett et al., 1995).

Like the DASH diet, the mechanism by which the Mediterranean diet reduces blood pressure has not been elucidated, however, relationships between this diet and vascular outcomes have been observed. A cross-sectional study of Mediterranean-residing elderly adults found that better adherence to the Mediterranean diet was significantly and inversely associated with MAP and incident hypertension (Tyrovolas et al., 2014). Other vascular health benefits of the Mediterranean diet are improved endothelial function and reduced inflammation. In a systematic review of 17 randomized control trials with 2300 subjects, it was reported that dietary interventions with components of the Mediterranean diet resulted in significant decreases in the inflammatory markers C-reactive protein, interleukin-6, and intracellular adhesion molecule-1, as well as increases in FMD compared to control groups (Schwingshackl et al., 2014). In addition, the antioxidant effects exerted by olive oil polyphenols, fruits and vegetables, whole grains, nuts and legumes, and alcohol in the Mediterranean diet may act to reduce ROS thereby improving vascular outcomes (Widmer et al., 2015). As previously mentioned, reducing vascular ROS may alleviate endothelial dysfunction via improving NO bioavailability.

Beans and Lentils

Definition of Pulses

Pulses are the dried, edible seeds of leguminous plants (Pulse Canada, 2017b). They differ from the other legumes including soybeans and peanuts that are harvested for their oils, and string beans and peas that are harvested as fresh

vegetables (Pulse Canada, 2017b). Similar to other legumes, pulses grow in pods producing ~1-10 seeds per pod depending on the pulse. Each pulse seed has a fibrous outer coating (referred to as a hull or seed coat) wrapped around a starchy seed body (also referred to as a cotyledon). The four pulse crop types grown in Canada are beans, peas, chickpeas, and lentils. This literature review, however, will primarily focus on beans and lentils, as they were the two pulse types to be examined in my study, and they will be justified in a later section.

Consumption in Canada

Despite the numerous environmental, economical, and human health benefits (to be discussed), the consumption of pulses remains very low among Canadians, with only 13.1% of the population ingesting pulses on any given day (Mudryj et al., 2012). The low consumption is not a problem of supply, as Canada is among the top producers of pulses worldwide. Thus other factors for the low consumption patterns appear to be responsible. Some individuals experience issues with the digestibility of pulses, as these crops are known to stimulate intestinal gas production and bloating. However, properly cooking pulses or taking a digestive enzyme supplement can aid the digestion of pulses. On the other hand, some consumers lack familiarity with pulses and are unsure how to prepare and incorporate them into their usual meals.

To encourage consumption, pulses have been a recent focus of food product development research in Canada. The production of pulse flours and fractions (concentrated in fibre or protein) has stimulated innovative uses for pulses in

common foods such as breads, tortillas, muffins, brownies, crackers, chips, pasta, noodles, and snack bars to name a few. Many of these aforementioned products have been introduced to the consumer market. Pulses, due to their health and environmental benefits, have the potential to fit into a variety of current food trends such as healthy snacks, free-from (e.g. allergen-, gluten-, wheat-, lactose-, and dairy-free), meat alternatives, natural and environmentally friendly, a whole food, and overall good for health and weight management. With the end of the 2016 International Year of Pulses, which promoted pulse-based recipes to the public, it is hoped that the average intake among Canadians will have risen, although data on this is not yet available.

Production in Canada

Beans and lentils are grown primarily in the Canadian Prairies (Alberta, Saskatchewan, and Manitoba) with some bean production also occurring in Ontario. The production of lentils is far greater than beans with the 2015-2016 Canadian crop year yielding 2541 thousand metric tonnes of lentils and only 249 thousand metric tonnes of beans (Agriculture and Agri-Food Canada, 2017). Given the vast agricultural landscape, it is no surprise that Canada is the number one producer of lentils worldwide (Food and Agriculture Organization of the United Nations, 2013b). In addition, Canada is also the number one exporter of lentils worldwide (Food and Agriculture Organization of the United Nations, 2013a). While the production of beans is far less than lentils, Canada nonetheless ranks among the top twenty bean producers and exporters worldwide (Food and Agriculture Organization of the

United Nations, 2013a, 2013b). Some of the top importers of beans and lentils include Turkey, Sri Lanka, India, Pakistan, Egypt, Brazil, the United States, the United Kingdom, Italy, and Mexico (Food and Agriculture Organization of the United Nations, 2013a).

Different types and varieties of beans and lentils are grown in Canada. Bean and lentil types are differentiated based on their size and seed coat colour. The primary bean types grown in Canada are navy beans (white pea beans), black beans, great northern beans, red kidney beans, small red beans, pink beans, pinto beans, and cranberry (Romano) beans (Agriculture and Agri-Food Canada, 2013a). Lentils primarily have either green or red seed coats, and in Canada, green lentils are more commonly grown than red. Some of the main green lentil varieties include the Crop Development Center (CDC) Greenland, CDC Plato, CDC Improve, CDC Impower, and CDC Greenstar (Agriculture and Agri-Food Canada, 2013b; Saskatchewan Pulse Growers, 2015, 2016)

Compared to other agricultural commodities produced in Canada, pulses are an environmentally sustainable food resource. This is attributed to their effects on reducing greenhouse gases and water demand.

A remarkable feature of pulses is their ability to utilize atmospheric nitrogen to generate their own nourishment (Pulse Canada, 2017a). By fixing atmospheric nitrogen, pulses reduce the demand for inorganic fertilizer during their production. It is well known that inorganic fertilizer contributes to greenhouse gases when it leeches into the atmosphere after conversion into nitrous oxide in the soil (Pulse

Canada, 2017a). Growing pulses in place of fertilizer-requiring crops therefore reduces greenhouse gas emissions from nitrous oxide. In addition, pulses aid in lowering atmospheric carbon, another greenhouse gas, by sequestering this element in the soil. The recently released Pan-Canadian Framework on Clean Growth and Climate Change from the Government of Canada lists "increasing stored carbon" in soils as a top priority for Canada's climate change goals (Government of Canada, 2016). Thus, the practice of cultivating pulses fits nicely with current political policies in Canada.

When pulses are grown in rotation with cereal crops, they benefit the production of the latter. In general, pulses have shorter roots than cereal crops, and will typically utilize water from only the first 30 cm of soil leaving behind water in the deeper soil for plants to access in subsequent years (Wang et al., 2012). Thus, when pulses are grown in a particular field in one year, the soil will retain more water and nutrients for the following year's crop. As a result, cultivating pulses not only benefits the environment, but also the production of other important Canadian crops.

Nutritional Content

Beans and lentils are exceptionally healthful foods containing many essential nutrients. The nutrient compositions for navy beans, black beans, and lentils can be found in Table 1, which is based on data compiled in the Canadian Nutrient File (Health Canada, 2017). The nutrient content of beans and lentils varies slightly with beans contributing marginally more fibre and magnesium, and lentils having

modestly more protein, iron, and B vitamins. Nonetheless, it is evident that the consumption of beans or lentils would make significant contributions towards meeting dietary reference intakes for many nutrients. According to the recommendations set out by Health Canada, 175 mL of cooked beans or lentils would meet ≥10% of the recommended intake for protein, carbohydrate, fibre, iron, magnesium, phosphorous, potassium, zinc, folate, and thiamine (Health Canada, 2010). The 175 mL portion represents Health Canada's suggested serving size (as a meat alternative) for pulses.

Compared to nutrient analyses in the Canadian Nutrient File of other grains (such as quinoa, oat, barley, rice, spelt, buckwheat), pulses provide more protein per serving at ~11-13 g/175 mL cooked seeds making this crop the best choice for sourcing dietary plant protein. On the other hand, like all plant protein sources, pulses are not considered complete sources of protein because they lack the sulphur-containing amino acids (methionine and cysteine). Nonetheless, pulses can be complemented with cereal crops, such as wheat, to provide a complete protein. Indeed, Health Canada recognizes pulses as a suitable alternative to meat under Canada's Food Guide (Health Canada, 2011; Pulse Canada, No Date). In addition to this, pulses have been adopted into the DASH diet, which is recommended by The Heart and Stroke Foundation of Canada for blood pressure management. The DASH diet encourages 4–5 servings/week of nuts, seeds, and pulses (Heart and Stroke Foundation of Canada, 2017).

Table 1. Select Nutrient Contents from the Canadian Nutrient File for Cooked Lentils, Black Beans, and Navy Beans per 175 mL

	Lentils	Black Beans	Navy Beans
Energy (kcal)	170	168	189
Protein (g)	13.2	11.3	11.1
Fat (g)	0.56	0.69	0.83
Carbohydrate (g)	29.5	30.2	35.1
Fibre (g)	6.20	8.90	9.20
Calcium (mg)	28.0	34.0	93.0
Iron (mg)	4.88	2.67	3.18
Magnesium (mg)	53.0	89.0	71.0
Phosphorous (mg)	264	178	194
Potassium (mg)	540	452	524
Sodium (mg)	3.00	1.00	0.00
Zinc (mg)	1.86	1.42	1.39
Copper (mg)	0.37	0.27	0.28
Selenium (mg)	4.10	1.50	3.90
Folate (μg)	265	190	189
Niacin (niacin equivalents)	1.55	2.87	3.12
Pantothenic acid (mg)	0.93	0.31	0.36
Riboflavin (mg)	0.11	0.08	0.09
Thiamine (mg)	0.25	0.31	0.32
Vitamin B_6 (mg)	0.26	0.09	0.19

Phenolic Compounds

In addition to the many essential nutrients found in beans and lentils, there are also non-nutritive components, such as phenolic compounds. Unlike the micro- and macronutrients, there is far greater variability in the concentrations and types of phenolic compounds present in different pulse types. These differences are due to the location of the phenolic compounds, which are primarily contained within the hull (vs. the cotyledon) and thus differ between pulses based on their seed coat colour (Dueñas et al., 2002; Madhujith et al., 2004).

Differences among pulses in terms of their phenolic content was demonstrated by Xu & Chang (2012) who determined and compared the total phenolic content of thirteen different pulse types. Analysis by Folin-Ciocalteau assay revealed that, indeed, the phenolic content (expressed as gallic acid equivalents [GAE]) of pulses varies according to type and seed coat colour. Lentils ranked at the top, having significantly higher total phenolic content (14.3 mg GAE/g) than any other examined pulse (Xu & Chang, 2012). This was followed by the dark beans, namely adzuki beans, black beans, black soybeans, and small red beans (8.53–12.5 mg GAE/g), which had significantly greater phenolic contents than the lighter coloured pulses, black eyed peas and yellow soybeans (2.14 – 2.54 mg GAE/g) (Xu & Chang, 2012). At the bottom of the list were green and yellow peas and chickpeas with 1.13 – 1.44 mg GAE/g total phenolic content (Xu & Chang, 2012).

When comparing bean types, the variability in phenolic concentration based on seed coat colour is evident in the literature. For example, black bean hull extracts have a significantly greater total phenolic content compared to red, brown, and white bean hull extracts (Madhujith et al., 2004). The presence of phenolic compounds in the hull (and thus the basis for characterization by seed coat colour) was demonstrated by researchers who examined flours from bean hulls and compared them to flours from dehulled beans (i.e. beans with the hull removed). In this study, it was found that bean hull millstreams were over thirteen times more concentrated in total phenolics than the dehulled bean millstreams (Oomah et al., 2005). The types of phenolic compounds in these millstreams were also examined. Researchers found that flavonols were significantly and positively associated with

total phenolics for all beans (r=0.857), and additionally, flavonols were associated with antioxidant activity as determined by the β-carotene-linoleate model system (r=0.681) and the antioxidant activity coefficient method (r=0.881) (Oomah et al., 2005). Thus, the majority of phenolic compounds found in bean hulls are flavonols, which exert antioxidant capabilities.

However, when individual bean types were examined, black beans had significantly greater concentrations of anthocyanins compared to cranberry, dark red, light red, navy, and pinto beans (Oomah et al., 2005). Interestingly, anthocyanins were significantly and positively correlated with anti-radical activity (r=0.807) and trolox equivalent antioxidant capacity (r=0.826), which was unlike flavonols (Oomah et al., 2005). The dissimilarities in antioxidant and anti-radical activities of flavonols and anthocyanins suggests that these phenolic compounds may act differently in the body and thus black beans may bring about unique health benefits over other beans. Antioxidants inhibit the oxidation of a molecule by interfering with the reaction, while anti-radicals are capable of interacting specifically with ROS to neutralize their free radical (Tirzitis et al., 2010).

In lentils, similar to beans, the hull contains far greater concentrations of phenolic compounds compared to the cotyledon (Duenas et al., 2006; Dueñas et al., 2002). The phenolic compounds in highest concentration in lentil hulls are flavanols, specifically, catechins and procyanidins (Dueñas et al., 2002). Procyanidins fall under the category of proanthocyanidins, which are also known as condensed tannins (Amarowicz et al., 2010). In agreement, Zhang et al. (2015)

found that condensed tannins comprised the majority of the total phenolics in twenty different varieties of Canadian grown lentils. While flavonols and flavones are present in lentil hulls, their concentration is much lower than that of flavanols (Dueñas et al., 2002). Thus, the phenolic content of lentils is much different from beans, which primarily contain flavonols. Condensed tannins from lentils, while being anti-nutritional compounds, have also been studied for their antioxidant activity. Zhang *et al.* (2015) found that the condensed tannins within lentils were strongly and positively correlated (r=0.9652) with antioxidant activity as determined by the ferric reducing antioxidant power assay.

In a study comparing the activities of phenolic compounds among different pulse types, including lentils, black kidney beans, and navy beans, it was shown that lentils exhibited the greatest total antioxidant capacity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and total reducing power while having the highest phenolic content (Zhao et al., 2014). In contrast, navy beans had the lowest phenolic content in parallel with the lowest activities of the aforementioned tests (Zhao et al., 2014). The black kidney beans, on the other hand, ranked among the second highest for total phenolic content, total antioxidant capacity, DPPH radical scavenging activity, and total reducing power (Zhao et al., 2014). Interestingly, the black kidney beans exhibited the highest hydroxyl radical scavenging activity, while the lentils ranked among the lowest activity (Zhao et al., 2014). Although this study examined black kidney beans and not black common beans (which were used in this thesis), the indistinguishable seed coat colour (black) suggests that these two beans contain the same (or similar) phenolic

compounds and thus similar results for black common beans would be expected. Nonetheless, the results from Zhao *et al.* (2014) indicate that pulses may exert diverse effects in the body due to their unique compositions of phenolic compounds that act through different means. Moreover, these results suggest that lighter coloured beans may exert less bioactivity since their phenolic compounds are in lower concentration and have less antioxidant potential compared to darker coloured beans. On the other hand, the actual bioactivity and modes of action of phenolic compounds from pulses within the human body warrants further assessment. Since beans and lentils contain different types and concentrations of phenolic compounds, they may have distinct actions in the body.

Vascular Benefits

Pulses have been examined in a number of studies for their therapeutic potential against chronic diseases and metabolic conditions, including cancer, CVD, diabetes, metabolic syndrome, and obesity (Mudryj et al., 2014). While clinical studies examining pulses for their cholesterol lowering, blood sugar regulating, and anti-obesity effects have also reported blood pressure outcomes, the power to detect blood pressure reductions was not considered nor were hypertensive subjects always examined. The current available literature that has reported the vascular effects of pulses in humans and animal models is summarized in Tables 2 and 3, respectively.

Clinical Studies

Fourteen clinical studies published between 2009 and 2015 have reported blood pressure outcomes after a pulse-based dietary intervention. Six of these studies were conducted in Canada. Baseline blood pressures showed a range of blood pressure control, with authors reporting normal, prehypertensive, and hypertensive baseline values. Only four studies had subjects with blood pressure in the prehypertensive or hypertensive category and, of these studies, two reported that some of their subjects were using antihypertensive medications. In addition, three of the studies reporting normal baseline blood pressures reported that some of their subjects were using antihypertensive medications.

The sample sizes ranged from 7 to 132 subjects who were overweight or obese (in twelve studies) adult males and females (age ≥ eighteen years). Study durations ranged from eight weeks to eighteen months with five of the studies having a crossover design and the remainder being a parallel design. The form of the pulse intervention varied among studies and included reconstituted pulse protein isolates/hydrolysates (in water or juice), whole cooked pulses (either alone, or incorporated into a meal), powdered pulses incorporated into wheat products (such as breads or pasta), and reconstituted pulse powder (as a soup). Six of the studies had unspecified doses for the pulse intervention, as these studies encouraged a recommended intake of pulses rather than providing specific amounts to consume. In five of the studies, other changes were made to the background diet, which included calorie restriction, a low glycemic index diet, or nutritional

guidance/encouragement (e.g. to follow dietary recommendations laid out by a national heart association).

Among the studies that examined subjects with higher than normal blood pressure, two reported significant reductions in blood pressure vs. baseline (Bahr et al., 2013) or vs. control (Li et al., 2011), whereas one found non-significant reductions vs. control (Venn et al., 2010), and one did not witness blood pressure reductions vs. baseline (Zahradka et al., 2013). Other vascular assessments in these studies are described below.

Interventions with lupin protein isolate (Bahr et al., 2013) and pea protein hydrolysate (Li et al., 2011) resulted in significant SBP reductions of 8.4 mmHg and 10.0 mmHg, respectively, compared to baseline. These SBP reductions would be similar to what would be produced from prescribed antihypertensive medications. Li *et al.* (2011) also found significant blood pressure reductions compared to the control group (mean SBP change of approximately −9.0 mmHg in 3 g/day pea protein hydrolysate group vs. −4.0 mmHg in control arm). However, this study had only seven participants and reported no other anthropometric or metabolic parameters.

Venn *et al.* (2014) found SBP reductions of 10.0 mmHg, but these reductions were not significant compared to baseline or the control. This study encouraged participants to consume two servings/day of cooked pulses while also following dietary recommendations laid out by the National Heart Foundation of New Zealand, which advocates other additional healthy eating choices. However, only

10% of the participants in the pulse intervention group had hypertension, and from the three-day food records, it was apparent that the two servings/day of pulses were not consumed by all participants although their pulse consumption did increase from baseline and was higher than the control group (Venn et al., 2010). Had the pulse intervention been more precisely followed, the blood pressure reductions in this study may have reached significance. The only significant outcomes for the pulse intervention in this study were reductions in total and low-density lipoprotein (LDL) cholesterol and waist circumference.

In agreement, Zahradka *et al.* (2013) also observed significant reductions in LDL cholesterol and BMI after participants consumed ½ cup servings of pulses for eight weeks. Although Zahradka *et al.* (2013) did not observe blood pressure reductions from the pulse intervention, 23 of their 26 participants were already on antihypertensive therapies suggesting that the pulses were not able to provide additional blood pressure lowering effects beyond the mechanisms of these drugs. However, vascular outcomes other than blood pressure were found to have significantly changed after the pulse intervention. These changes included an increase in ankle-brachial index and right ankle blood pressure, which indicates increased blood flow to the peripheral arteries – an important finding for this study considering the subjects had peripheral artery disease. While LDL cholesterol was significantly lowered by the pulse intervention, this outcome was not significantly associated with ankle-brachial index or SBP suggesting that circulating lipids do not impact vascular function. On the other hand, body weight and BMI were significantly associated with ankle-brachial index, suggesting that body weight management may

also be related to other vascular parameters. Although BMI was significantly reduced by the pulse intervention, the reduction was not clinically relevant. Thus, another explanation aside from lipid lowering and BMI reduction is warranted to explain the benefits of pulses on the vasculature.

Three studies found significant reductions in blood pressure from baseline and compared to the control, although these studies examined subjects with blood pressure in the normal range. One of these studies examined subjects with type 2 diabetes put on a low glycemic index diet, with the intervention group also consuming pulses (Jenkins et al., 2012). Subjects consuming 1 cup/day mixed pulses combined with a low glycemic index diet experienced significant reductions in SBP (−4.0 mmHg) and DBP (−3.0 mmHg) vs. baseline (Jenkins et al., 2012). In addition, the change in blood pressure in the pulse group was significantly greater than the change experienced by the control group (Jenkins et al., 2012). Interestingly, both the pulse and control groups experienced reductions in body weight, HbA1c, and fasting blood glucose as would be expected with a low glycemic index diet, however, only the pulse group experienced a reduction in blood pressure (Jenkins et al., 2012). This suggests that the pulses had specific effects on vascular function that were due to a mechanism beyond blood glucose and body weight regulation. While there were no differences between groups in terms of their baseline blood pressure, 61 and 72% of subjects were taking antihypertensive medications in the control and pulse groups, respectively (Jenkins et al., 2012). The slightly higher medication use in the pulse group may explain some of the additional blood pressure lowering outcomes observed in this group.

On the other hand, in a calorie-restriction dietary study, pulses had an added benefit on blood pressure regulation in subjects who were normotensive and not on antihypertensive medication. In this study, subjects consuming four servings/week of mixed pulses experienced significant SBP reductions of 9.0 mmHg after eight weeks (Hermsdorff et al., 2011). Again, the blood pressure lowering was an added benefit of the pulses considering that both the intervention and control groups lost body weight as observed by a reduction in BMI after consuming the calorie-restricted diet (Hermsdorff et al., 2011). Furthermore, the inflammatory markers, C-reactive protein and Complement C3, were significantly reduced by the pulse intervention, which was not observed in the control group (Hermsdorff et al., 2011). This evidence suggests that pulses may act through anti-inflammatory or antioxidant means to influence vascular function.

Similarly, a hypocaloric diet incorporating four servings/week of pulses had significant blood pressure lowering effects in normotensive individuals not on antihypertensive medications (Abete et al., 2009). These results were significant vs. baseline and the control group (Abete et al., 2009). Furthermore, there were no differences between the pulse and control diets in terms of calories or macronutrient compositions (Abete et al., 2009). However, the incorporation of pulses significantly lowered the glycemic index of the diet even though fibre contents were not significantly different (Abete et al., 2009). Interestingly, these researchers had also examined a hypocaloric pulse-free diet that was high in protein. However, this diet had no effect on blood pressure compared to baseline or the control even though these participants lost significantly more weight and

experienced an increase in mitochondrial oxidation, a finding that was similar to the pulse group (Abete et al., 2009). These data suggest that the reductions in blood pressure in the pulse group were unlikely due to the reduction in body weight, and that some other component of the pulses contributed to the blood pressure lowering effect.

Finally, significant reductions in SBP, DBP, high-density (HDL) cholesterol, circulating insulin, and insulin resistance were observed after lupin-enriched wheat-based products were consumed in place of 100% wheat products for twelve months (Belski et al., 2011). Lupins are a pulse crop primarily grown in Australia. A similar study using lupin-enriched wheat products also found that SBP was significantly reduced after sixteen weeks on the intervention compared to the control (Lee et al., 2009). Although the actual lupin intake was not assessed in either of the two aforementioned studies, protein and fibre intake were increased compared to the control suggesting the altered macronutrient profiles of the diet could have contributed to the beneficial outcomes of the pulse intervention (Belski et al., 2011; Lee et al., 2009).

The final four studies showed no effect of pulses on blood pressure (Abeysekara et al., 2012; Gravel et al., 2010; Saraf-Bank et al., 2016; Veenstra et al., 2010). All of these four studies examined normotensive subjects, which may partially explain why the pulse interventions had no effect on blood pressure. Furthermore, the power to detect blood pressure changes in these studies was not assessed and could also explain the lack of observed benefits.

Overall, the clinical studies reporting blood pressure outcomes after dietary interventions with pulses indicate that pulses can reduce blood pressure (observed in seven of thirteen studies). In a systematic review and meta-analysis of eight of these aforementioned randomized controlled feeding trials, it was found that the weighted mean reduction in SBP was 2.25 mmHg following a mean consumption of ⅝ cup/day of pulses for a mean duration of ten weeks (Jayalath et al., 2014). The blood pressure regulating mechanisms of pulses are likely independent of alterations in body weight/composition and circulating lipids, glucose, and insulin. On the other hand, the increased fibre and protein, and/or reduced inflammation provided by pulses may explain some of the hypotensive benefits. In a systematic review and meta-analysis of eight randomized trials representing a total of 464 participants, there was a trend ($p = 0.068$) towards reductions of plasma C-reactive protein with pulse consumption (Salehi-Abargouei et al., 2015). Furthermore, the OmniHeart randomized trial demonstrated that adjustment of the DASH diet to replace some of the carbohydrates with protein resulted in a reduction in blood pressure in pre-hypertensive and hypertensive individuals (Appel et al., 2005). Interestingly, 50% of the protein incorporated into the higher protein diet was from plant sources that were primarily described as non-soy legumes (Appel et al., 2005).

Animal Studies

To better define the mechanisms by which pulses exert their vascular effects, animal studies can provide ample amounts of information. Of the thirteen animal studies reporting blood pressure as an outcome of pulse consumption, all showed

blood pressure reductions. Seven of the ten studies examined a pulse protein isolate/hydrolysate, three examined phenolic–rich pulse extracts, one examined ground pulse hulls, and two examined whole ground pulses. The SHR was used for ten studies, while the remaining three studies used rat models of type 2 diabetes made hypertensive by a high-salt diet, or polycystic kidney disease, or healthy rats made hypertensive by AngII perfusion. Study durations ranged from acute (six hours) examinations to eight-week interventions.

In the acute studies, pulse protein hydrolysates or isolates were examined for their ability to impact blood pressure over a six to twenty-four hour period. Protein isolates are a concentrated source of protein where most of the carbohydrates and fats have been eliminated, whereas protein hydrolysates are isolates that have been enzymatically or heat treated to make the amino acids and peptides more bioavailable.

Pea proteins were examined in two of the studies, where it was found that hydrolysates were more effective than isolates at reducing SBP short-term (Girgih et al., 2016; Li et al., 2011). In the study by Li *et al.* (2011), 100 and 200 mg/kg body weight doses of pea protein hydrolysate were equally as effective as captopril (an ACE inhibitor) at reducing SBP at two, four, six, and eight hours post-treatment in ten-week old SHR. On the other hand, the SHR were not fully hypertensive at ten weeks of age and therefore the blood pressure reductions do not necessarily reflect what might occur in hypertensive blood vessels (mean SBP=135–150 mmHg; mean DBP=80–90 mmHg;). Alternatively, in twenty-week old SHR, 100 mg/kg body

weight of a 5 kDa sized pea protein hydrolysate resulted in greater SBP reductions at two and four hours post-administration compared to captopril (Girgih et al., 2016).

To examine the chronic vascular effects of the pea protein hydrolysates and isolates, the two aforementioned studies investigated five and eight week interventions with pea protein isolates in thirty-week old SHR as well as weanling rats with polycystic kidney disease. In the older SHR, a diet containing 1% weight/weight (w/w) of 5 kDa pea protein hydrolysate was more effective for reducing SBP than either an equal dose of heat treated pea protein isolate or a lower dose (0.5% w/w) of 5 kDa pea protein hydrolysate (Girgih et al., 2016). Furthermore, these treatments were ineffective in Wistar Kyoto (WKY) rats, suggesting that they correct an abnormality that is observed only within SHR. *In vitro*, the 5 kDa pea protein hydrolysate showed 50% ACE inhibition at a concentration of 0.1 mg/mL while being less effective at inhibiting renin, thus suggesting that these peptides exert their antihypertensive benefits by inhibiting ACE rather than renin (Girgih et al., 2016). In the weanling rats with polycystic kidney disease, 0.5% and 1.0% (w/w) pea protein hydrolysates were equally effective at reducing SBP and DBP beginning at six weeks of treatment. In addition, the pea protein hydrolysate was able to reduce plasma AngII, plasma ACE activity, renin mRNA levels in the kidney, and ACE mRNA levels in the kidney (Li et al., 2011).

Like pea protein, isolated proteins from lupin, mung bean, and common bean have been demonstrated to have antihypertensive capabilities. Salt-induced hypertensive rats with type 2 diabetes fed a diet containing 20% (w/w) lupin protein isolate for two weeks had significantly lower SBP than control fed rats (Pilvi et al., 2006). While potential ACE inhibitory mechanisms of action were not investigated as was done with the pea protein studies, it was found that the lupin protein isolate improved vascular function. Specifically, it was observed that endothelium-dependent vasodilation was improved by the lupin protein isolate diet, since vessel relaxation was significantly greater than in the control hypertensive animals and was not different from the normotensive Wistar Kyoto (WKY) rats (Pilvi et al., 2006). Interestingly, soy protein isolate did not have this effect on the vasculature although it did lower SBP, suggesting that proteins from these sources may act through different mechanisms to regulate blood pressure (Pilvi et al., 2006). The authors speculated that ACE inhibition by the lupin proteins might have contributed to the blood pressure regulation in their study (Pilvi et al., 2006).

A single dose of mung bean protein hydrolysates (600 mg/kg body weight) orally administered to twelve-week old SHR acutely lowered SBP up to eight hours after administration (Li et al., 2006). In these animals, blood pressure returned to baseline by twelve hours after administration (Li et al., 2006). The blood pressure lowering effect of the mung bean hydrolysate was thought to be due to an ACE inhibitory action of the peptides, which was supported by results from an *in vitro* ACE inhibition assay (Li et al., 2006).

A single dose of a 3–10 kDa protein fraction from Azufrado Higuera beans given orally to SHR resulted in significant reductions in SBP up to four hours after administration, with the blood pressure returning to baseline at the six hour time point (Ariza-Ortega et al., 2014). Strangely, this study did not present information on the age of the SHR or the number of animals per group and thus it is difficult to know if these results could be reproduced. Although in agreement with the previously discussed studies, the Azufrado Higuera beans exerted antioxidant and ACE inhibitory potential as determined through *in vitro* antioxidant activity and ACE inhibitory assays (Ariza-Ortega et al., 2014).

Collectively, these aforementioned studies have determined that pulse proteins exert bioactivity within the vasculature of various animal models and they regulate blood pressure and blood vessel function likely through an ACE inhibitory mechanism.

Conversely, four animal studies attributed the blood pressure regulating effects of pulses to their polyphenol content. In AngII-induced hypertensive rats, lentil polyphenol extract added to drinking water caused significant reductions in MAP, media/lumen ratio (of heart and kidney arteries), perivascular fibrosis (of heart and kidney arteries), and aortic ROS levels, while also increasing serum total phenolics and serum oxygen radical absorbance capacity in comparison to hypertensive SD rats without lentil treatment (Yao et al., 2012). Furthermore, the lentil-treated hypertensive rats were not different from normotensive SD rats in terms of MAP, media/lumen ratios, and aortic ROS levels (Yao et al., 2012). This

research suggests that the lentil treatment was able to mitigate the negative effects of AngII on the vasculature.

Similarly, azuki bean extracts significantly reduced SBP in nineteen-week old SHR and five-week old SHR compared to untreated SHR (Mukai et al., 2009; Sato et al., 2008). The alterations in blood pressure were attributed to the ability of the extract to increase NO production as observed by an increase in urinary nitrate/nitrate excretion, and reduced levels of eNOS and inducible nitric oxide synthase (iNOS) in the aorta and kidneys (Mukai et al., 2009). It was speculated that the lower levels of eNOS and iNOS in the normotensive and azuki bean extract-treated SHR were due to the reduced ROS levels in these animals, as an increase in ROS is known to cause uncoupling of NOS enzymes and thus upregulate eNOS and iNOS expression as a compensatory mechanism (Mukai et al., 2009). As previously discussed, the seed coats of pulses are known to contain more polyphenols than the cotyledon. Therefore, these researchers also examined a diet enriched with azuki bean seed coat to examine if this portion of the pulse would have enhanced beneficial effects compared to the whole bean extract. Indeed, after eight weeks on the diet, SBP was significantly reduced in SHR treated with the azuki bean seed coat extract compared to untreated SHR (Mukai et al., 2011). This benefit was attributed to the ability of the extract to regulate aortic ROS levels, as observed by reductions in aortic NAD(P)H-stimulated superoxide production and p47 phox mRNA levels (Mukai et al., 2011). In addition, cyclooxygenase-2, an aortic inflammatory marker, was significantly reduced by the extract compared to untreated SHR, suggesting that

azuki bean seed coats may attenuate vascular remodelling induced by inflammation in hypertensive vessels (Mukai et al., 2011).

These four aforementioned studies with lentil and azuki bean extracts demonstrate that pulse polyphenols, likely from the seed coat, can reduce blood pressure in the SHR. The blood pressure lowering mechanism may be mediated through antioxidant and anti-inflammatory actions, which can improve endothelial function and thus blood pressure.

The final two animal studies examined diets containing whole ground pulses at 30% (w/w) inclusions. The first study, conducted in fifteen-week old SHR, found that a mixed lentil diet was able to attenuate the rise in blood pressure that occurs as SHR age (Hanson et al., 2014). Surprisingly, no other pulse type was effective at lowering the rise in blood pressure in these animals. In addition to regulating blood pressure, the lentil diet also attenuated vascular remodelling as observed by no difference in aortic media/lumen ratio compared to the WKY rats (Hanson et al., 2014). On the other hand, this alteration in vascular structure did not coincide with improvements to vascular compliance, as PWV was not different from the control SHR and was significantly faster than the WKY rats (Hanson et al., 2014). Given that these diets were not isolated compounds of pulses (i.e. isolated proteins or polyphenols), it is interesting to see that the whole food was still able to influence the vasculature, as this would more likely reflect human consumption patterns.

Given that the lentils, but not other pulses, exerted vascular benefits, it seems evident that lentils may influence vascular structure and function in a way that

other pulses cannot. Lentils have been previously shown to contain higher concentrations of phenolic compounds, namely tannins, compared to other pulse types (Xu et al., 2012). Interestingly, when urinary metabolites of SHR fed the control, lentil, bean, pea, or chickpea diets were compared by a non-target metabolomics approach, it was found that the urine of only the lentil-fed SHR contained arginine-related and citrulline compounds (Hanson et al., 2016a). As previously mentioned, citrulline and arginine are involved in the production of NO by eNOS for vasodilation. The presence of these compounds suggests that the lentil diets may have preserved endothelial function thereby attenuating the blood pressure rise of SHR.

On the other hand, explanations for the lack of effect by the mixed bean diet in the Hanson *et al.* (2014) study can be provided. As previously discussed, different bean types have unique compositions and concentrations of phenolic compounds. Therefore, certain bean types may be more beneficial to the vasculature than others. Thus, a mixed bean diet may have hindered the benefits of a bean highly concentrated in phenolics if beans with low concentrations were present. Therefore, future work is required to differentiate the effects of presumed high and low polyphenol beans, such as black beans vs. navy beans.

A second study by the same group further examined the vascular effects of the lentils by separately testing green and red lentils. Here it was found that green lentils were more effective than red lentils for attenuating vascular remodelling (Hanson et al., 2016b). It was observed that the mesenteric arteries from green

lentil-fed SHR were structurally similar to WKY rats as observed by no differences between the groups in terms of lumen diameter, media width, and external diameter (Hanson et al., 2016b). Furthermore, the mesenteric arteries from green lentil–fed SHR had significantly larger lumen and external diameters along with smaller media/lumen ratios than control SHR (Hanson et al., 2016b). Moreover, results from pressure myography indicated that the mesenteric arteries from green lentil-fed SHR were also more compliant than control SHR, and were not different from WKY rats (Hanson et al., 2016b). The combination of these results suggests that the green lentils attenuated the effects of high blood pressure on blood vessel function and vascular remodelling in SHR. On the other hand, tail-cuff blood pressure was not improved by the green lentil diet, although it was improved by the mixed lentil diet, which is in agreement with the first study conducted by this group (Hanson et al., 2016b; Hanson et al., 2014).

Mechanisms of action for the lentils to improve vascular structure and function were examined by Western blotting of extracted aorta proteins. The animals consuming lentils (mixed, red, or green) had phosphorylated-p38 MAPK levels similar to WKY rats and lower than control SHR (Hanson et al., 2016b). In the vasculature, p38 MAPK is activated by ROS but it also increases intracellular ROS, the combination of which leads to endothelial dysfunction and vascular remodelling in hypertension. Therefore, this result suggests that the lentils may have acted through a p38 MAPK-dependent pathway to attenuate vascular remodelling and dysfunction in the SHR, although this mechanism has yet to be confirmed.

Table 2. Clinical Studies Reporting Blood Pressure[1] Change as a Result of Pulses

Reference	Study Design	Population	Age (years)	Pulse Intervention	Control	Study Duration	SBP Effects (mmHg)[2]	DBP Effects (mmHg)[3]
Saraf-Bank *et al.,* 2016	R SB CO	Adults with diabetes, M and F, living in Iran; n=26	43–57	65 g/day dry pulses (lentils and pinto beans) cooked and consumed 4 days/week on a background healthy diet	Habitual diet	14 weeks	–3.4±2.2	–3.4±1.9
Bähr *et al.,* 2013	R C DB CO	Adults with high cholesterol, M and F, living in Germany; n=33	18–80	25 g/day lupin protein isolate dissolved in 500 mL water	25 g/day milk protein isolate, dissolved in 500 mL water	20 weeks	–8.4±13.6###	–2.7±7.5#
Zahradka *et al.,* 2013	SB	Adults with peripheral artery disease, M and F, living in Canada; n=26	>40	½ cup/day cooked pulses (pinto, kidney, black, and navy beans, green and yellow peas, green and red lentils, and chickpeas)	NA	8 weeks	+1.6	+2.3

Reference	Study Design	Population	Age (years)	Pulse Intervention	Control	Study Duration	SBP Effects (mmHg)[2]	DBP Effects (mmHg)[3]
Abeysekara *et al.,* 2012	R SB CO	Older, inactive adults, M and F, living in Canada; n=87	≥50	150 g/day dry pulses (chickpeas, peas, and lentils), cooked and divided into 2 servings/day	Participants' regular diet	8 weeks	–0.0	–0.0
Jenkins *et al.,* 2012	R C P	Adults with type 2 diabetes, M and F, living in Canada; n=60–61/ group	>50	Encouraged to increase pulse (chickpeas, lentils, and beans) intake by 1 cup (190 g)/ day as part of a low GI diet	Encouraged to increase intake of whole wheat products (insoluble fibre) as part of a low GI diet	3 months	–4.0#***	–3.0#***
Belski *et al.,* 2011	R C DB P	Overweight or obese, healthy adults, M and F, living in Australia; n=46–63/ group	20–71	Lupin flour added to bread, biscuits, and pasta at 20–40% (w/w) inclusions replacing wheat flour; consumed in place of 100% wheat products	100% wheat products	12 months	–2.4*	–1.7*

Reference	Study Design	Population	Age (years)	Pulse Intervention	Control	Study Duration	SBP Effects (mmHg)[2]	DBP Effects (mmHg)[3]
Hermsdorff *et al.,* 2011	R C P	Obese adults, M and F, living in Spain; n=15/group	≥36	160 –235 g/day cooked pulses (chickpeas, peas, lentils, and beans) consumed 4 times/week on a background calorie restricted diet	Calorie restricted diet	8 weeks	–9.0##*	–6.0##
Li *et al.,* 2011	R C DB CO	PreHTN or HTN adults (SBP ranging from 125-170 mmHg), M and F, living in Canada; n=7	30–55	PPH divided into 3 doses dissolved in 50 mL orange juice consumed at breakfast, lunch, and dinner a) 1.5 g/day PPH b) 3 g/day PPH	50 mL orange juice	8 weeks	a) 1.5 g/day PPH: approx. –5.0 b) 3 g/day PPH: approx. –9.0*	a) 1.5 g/day PPH: NR b) 3 g/day PPH: NR
Gravel *et al.,* 2010	R C P	Adults with metabolic risk factors, F, living in Canada; n=65–68/ group	30–65	150 mL/day cooked pulses (chickpeas, peas, lentils, and beans) prepared into a meal consumed 5 days/week	Isocaloric pulse-free meals consumed 5 times/week	16 weeks	–0.6	–0.6

Reference	Study Design	Population	Age (years)	Pulse Intervention	Control	Study Duration	SBP Effects (mmHg)[2]	DBP Effects (mmHg)[3]
Veenstra *et al.,* 2010	R C DB CO	Healthy adults, M, living in Canada; n=21	19–40	100 g/day spray-dried pulse powder, rehydrated as a soup a) Chickpeas b) Green lentils c) Green peas	50 g/day dehydrated potato flakes, rehydrated as a soup	7 months	a) Chickpeas: –5.8 b) Green lentils: +2.6 c) Green peas: –0.8	a) Chickpeas: –0.4 b) Green lentils: –1.3 c) Green peas: +0.3
Venn *et al.,* 2010	R C P	Obese healthy adults, M and F, living in New Zealand; n=53–55/ group	>30	Encouraged to consume 180 g/day cooked pulses (chickpeas, lentils, red kidney beans, butter beans, baked beans, or mixed beans) divided into 2 servings/day while following rec'ds from the National Heart Foundation of New Zealand	Encouraged to follow rec'ds from the National Heart Foundation of New Zealand	18 months	–10.0	–4.0

Reference	Study Design	Population	Age (years)	Pulse Intervention	Control	Study Duration	SBP Effects (mmHg)[2]	DBP Effects (mmHg)[3]
Abete *et al.,* 2009	R C P	Obese adults, M, living in Spain; n=8–10/ group	31–45	4 servings/week cooked pulses (unspecified dose and pulse type) on a background hypocaloric diet	Hypocaloric diet without pulses	8 weeks	–9.6±5.1#*	–10.1±7.1#
Lee *et al.,* 2009	R C P	Overweight or obese adults, M and F, living in Australia; n=37/group	20–70	Lupin kernel flour added to bread at 40% (w/w) inclusion, replacing 15–20% of usual energy intake	100% wheat flour bread, replacing 15–20% of usual energy intake	16 weeks	–0.1*	+0.4

Abbreviations: C, controlled; CO, cross-over; DB, double-blind; DBP, diastolic blood pressure; F, female; GI, glycemic index; HTN, hypertensive; M, male; NC, no change; P, parallel; PPH, pea protein hydrolysate; R, randomized; rec'ds, recommendations; SB, single-blind; SBP, systolic blood pressure; w/w, weight/weight.

[1]Blood pressure measured at the brachial artery.

[2,3]SBP and DBP reductions are shown relative to baseline values.

Significantly different from baseline ($p \leq 0.05$).

Significantly different from baseline ($p \leq 0.01$).

Significantly different from baseline ($p \leq 0.001$).

* Significantly different from control ($p \leq 0.05$).

** Significantly different from control ($p \leq 0.01$).

*** Significantly different from control ($p \leq 0.001$).

No letter or * or # indicates no significant difference from baseline or control, respectively.

Table 3. Animal Studies Reporting Blood Pressure Change as a Result of Pulses

Reference	Animal Model (Age at Intervention)	Treatment Groups[1]	N/ Group	Study Duration	Select Vascular Effects[2,3]
Girgih *et al.,* 2016 (Part 1)	SHR (20 weeks)	Single TRT by oral gavage: a) Saline (HTN Ctl) b) 10 mg Captopril/kg BW (Positive HTN Ctl) c) 100 mg UTPPI/kg BW d) 100 mg HTPPI/kg BW e) 100 mg PPH-5/kg BW	6	24 hours	**SBP reductions:** (e)>(b) at 2 and 4 hours; (d)>(c) at all time points; (c) ineffective at all time points.
Girgih *et al.,* 2016 (Part 2)	SHR and WKY (30 weeks)	TRT in diet: a) Untreated (HTN Ctl)-SHR b) 0.5% PPH-5-SHR c) 1.0% PPH-5-SHR d) 1.0% HTPPI-SHR e) Untreated (NTN Ctl)-WKY f) 1.0% PPH-5-WKY g) 1.0% HTPPI-WKY	6–7	5 weeks	**SBP:** (a)>(b)=(d)>(c); (e)=(f)=(g)
Hanson *et al.,* 2016	SHR and WKY (17 weeks)	TRT in diet: a) Untreated (HTN Ctl)-SHR b) 30% mixed lentil-SHR c) 30% red lentil-SHR d) 30% green lentil-SHR e) Untreated (NTN Ctl)-WKY f) 30% mixed lentil-WKY	10	8 weeks	**SBP:** no diet effect; (a-d)>(e-f) **DBP:** (a)=(c)=(d)>(b)>(e)=(f) **MAP:** (a)=(c)=(d)>(b)>(e)=(f) **PWV:** no diet effect; (a-d)>(e-f) **Media/lumen ratio (mesenteric artery):** (a)>(b)=(d)=(f)>(e); (a)=(c)>(e) **Lumen diameter (mesenteric artery):** (d)=(c)=(e)=(f)>(a); (e)>(a)=(b) **Pressure myography, circumferential stress**

Reference	Animal Model (Age at Intervention)	Treatment Groups[1]	N/ Group	Study Duration	Select Vascular Effects[2,3]
					(mesenteric artery): (a)=(b)>(c)>(e)>(d)=(f) **Pressure myography, elastic modulus (mesenteric artery):** (a)>(d)=(e)=(f); (a)>(c)=(d)=(e) **Aortic phospho-p38MAPK levels:** (a)>(b-f)
de Jesús Ariza-Ortega *et al.,* 2014	SHR (NR)	Single TRT by intraperitoneal administration: a) 2.5 mL distilled water/kg BW (HTN Ctl) b) 10 mg Captopril/kg BW (Positive HTN Ctl) c) 4 mg AH F3–10/kg BW	NR	6 hours	**SBP:** (b) and (c)<baseline at 2 and 4 hours, but not at 1 or 6 hours; (a) not different from baseline at any measured time point
Hanson *et al.,* 2014	SHR and WKY (16 weeks)	TRT in diet: a) Untreated (HTN Ctl)-SHR b) 30% mixed bean-SHR c) 30% mixed pea-SHR d) 30% mixed lentil-SHR e) 30% chickpea-SHR f) 30% mixed pulse-SHR g) Untreated (NTN Ctl)-WKY	8	4 weeks	**SBP:** (a) and (f)>(d); (a–f)>(g) **DBP:** (a) and (f)>(d); (a–f)>(g) **MAP:** (a) and (f)>(d); (a–f)>(g) **PWV:** no diet effect, (a-f)>(g) **Media/lumen ratio (aorta):** (c)=(g)

Reference	Animal Model (Age at Intervention)	Treatment Groups[1]	N/ Group	Study Duration	Select Vascular Effects[2,3]
Yao *et al.,* 2012	AngII-induced HTN SD rats (NR)	TRT in drinking water: a) Untreated (NTN Ctl) b) Untreated (HTN Ctl) c) HTN +1% LPE d) NTN +1% LPE	5	8 weeks	**MAP:** (b)>(c)=(a)=(d) **Media/lumen ratio (cardiac artery):** (b)>(c)=(a)=(d) **Media/lumen ratio (renal artery):** (b)>(c)=(a)=(d) **Aortic ROS levels (DHE fluorescence):** (b)>(c)=(a)=(d) **Aortic ROS levels (DCFH fluorescence):** (b)>(c)=(a)=(d) **Serum total phenolics:** (c)=(d)>(a)=(b) **Serum ORAC:** (c)=(d)>(a)=(b)
Li *et al.,* 2011 (Part 1)	SHR (10 weeks)	Single TRT by oral gavage: a) Saline (HTN Ctl) b) 3 mg Captopril/kg BW (Positive HTN Ctl) c) 100 mg PPH/kg BW d) 200 mg PPH/kg BW e) 200 mg PPI/kg BW	6	24 hours	**SBP reductions:** (b)=(c)=(d)>(a)=(e) at 2, 4, 6, and 8 hours, but not at 24 hours
Li *et al.,* 2011 (Part 2)	Han:SPRD-cy rats (Weanling)	TRT in diet: a) Untreated (HTN Ctl) b) 0.5% PPH c) 1.0% PPH	13	8 weeks	**SBP:** (a)>(b) and (c) at 5, 6, 7, and 8 weeks, but not at 4 weeks **DBP:** (a)>(b) and (c) at 6, 7, and 8 weeks, but not at 3 or 5 weeks **Plasma AngII:** (a)>(b) and (c) **Renal renin mRNA expression:** (a)>(b) and (c)

Reference	Animal Model (Age at Intervention)	Treatment Groups[1]	N/ Group	Study Duration	Select Vascular Effects[2,3]
Mukai & Sato, 2011	SHR and WKY (18 weeks)	TRT in diet: a) Untreated (Ctl)-SHR b) 1% ABSC-SHR c) Untreated (Ctl)-WKY d) 1% ABSC-WKY	8	8 weeks	**SBP:** (a)>(b)>(c)=(d) **Aortic NAD(P)H-stimulated superoxide production:** (a)>(b)=(c)=(d) **Aortic p47phox mRNA expression:** (a)>(b)>(c)=(d) **Aortic iNOS expression:** (a)>(b)=(c)=(d)
Mukai & Sato, 2009	SHR and WKY (19 weeks)	TRT in diet: a) Untreated (HTN Ctl)-SHR b) 0.9% ABE-SHR c) Untreated (NTN Ctl)-WKY d) 0.9% ABE-WKY	7–8	8 weeks	**SBP:** (a)>(b)>(c)=(d) **Urinary nitrate/nitrite excretion:** (b)>(a)=(c)=(d) **Aortic eNOS expression:** (a)>(b)=(c)=(d) **Aortic iNOS expression:** (a)>(b); (a)=(c)=(d); (b)=(c)=(d) **Renal eNOS expression:** (a)>(b)=(c)=(d) **Renal iNOS expression:** (a)>(b)=(d); (a)=(c); (b)=(c)
Sato *et al.,* 2008	SHR and WKY (5 weeks)	TRT in diet: a) Untreated (HTN Ctl)-SHR b) 0.8% ABE-SHR c) Untreated (NTN Ctl)-WKY d) 0.8% ABE-WKY	8	8 weeks	**SBP:** (a) and (b)>(c) and (d) at 2, 4, 6, and 8 weeks; (b)<(a) at 6 and 8 weeks but not at 0, 2, or 4 weeks
Li *et al.,* 2006	SHR (12 weeks)	Single TRT by oral gavage: a) 1.5 mL distilled water (HTN Ctl) b) 600 mg MBPH/kg BW	12	12 hours	**SBP reductions:** (a)>(b) at 2, 4, 6, and 8 hours, but not at 12 hours

Reference	Animal Model (Age at Intervention)	Treatment Groups[1]	N/ Group	Study Duration	Select Vascular Effects[2,3]
Pilvi *et al.*, 2006	Salt-induced HTN GK rats and WKY (NR)	TRT in diet: a) Untreated (HTN Ctl)-GK b) 20% LPI-GK c) 20% SPI-GK d) Untreated (NTN Ctl)-WKY	8–10	2 weeks	**SBP:** (a)>(b)=(c)=(d) **Contractile force of mesenteric artery rings:** (b)=(c)=(d)>(a) **Endothelium-dependent vasorelaxation:** (b)=(d)>(a); (c)=(a), (b), and (c)

Abbreviations: ABE, azuki bean extract; ABSC, azuki bean seed coat; ACE, angiotensin-converting enzyme; AH F3–10, Azufrado Higuera Fraction 3–10 kDa; AngII, angiotensin II; BW, body weight; COX-2, cyclooxygenase-2; Ctl, control; DBP, diastolic blood pressure; DCFH, dichlorofluorescin diacetate; DHE, dihydro-ethidium; eNOS, endothelial nitric oxide synthase; GK, Goto-Kakizaki; HTN, hypertensive; HTPPI, heat treated pea protein isolate; iNOS, inducible nitric oxide synthase; LPE, lentil polyphenol extract; LPI, lupin protein isolate; MAP, mean arterial pressure; MBPH, mung bean protein hydrolysate; NAD(P)H, nicotinamide dinucleotide phosphate; NTN, normotensive; NR, not reported; ORAC, oxygen radical absorbance capacity; PPH, pea protein hydrolysate; PPH-5, pea protein hydrolysate-5 kDa; PPI, pea protein isolate; PWV, pulse wave velocity; ROS, reactive oxygen species; SBP, systolic blood pressure; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat; SPI, soy protein isolate; TRT, treatment; UTPPI, untreated pea protein isolate; WKY, Wistar Kyoto.

[1] % pulse indicates % weight of intervention/weight of diet (i.e. weight/weight), unless otherwise indicated.

[2] Differences between treatment groups were considered significant when ">" was used as a comparator.

[3] Differences between treatment groups were considered not significant when "=" was used as a comparator.

The Spontaneously Hypertensive Rat (SHR)

Animals are often employed in scientific research to examine the effects of experimental variables on biological processes and diseases in greater detail than can be accomplished through clinical research alone. While the results from an animal experiment cannot necessarily be extrapolated to humans, animal studies have some benefits over clinical research. For example, animal studies are far less expensive and time consuming than human studies and also allow for invasive measurements that could not be performed in humans such as vessel morphology and protein analysis. Several animal models of hypertension are available; however, the SHR is the most commonly utilized in scientific research.

The SHR is currently the only animal model of essential hypertension. Okamoto and Aoki first bred the SHR in 1963 by mating a male WKY rat showing spontaneous hypertension with a female WKY rat that had slightly above average blood pressure (Okamoto et al., 1963). The offspring were successively interbred until 100% of the rats spontaneously developed hypertension (Okamoto et al., 1963). These researchers defined the rats as hypertensive if they developed SBP above 150 mmHg as measured by the tail-water-plethysmographic method (Okamoto et al., 1963). After weekly measurements of blood pressure throughout the lifespan of the SHR, it was determined that these animals become fully hypertensive by fifteen weeks of age (Okamoto et al., 1963). After fifteen weeks, the SHR continue to experience a steady rise in blood pressure over time reaching an SBP >190 mmHg by sixty weeks of age (Okamoto et al., 1963). In WKY rats, SBP

reaches 130-136 mmHg after ten weeks of age and does not deviate throughout the rest of their lifespan (Okamoto et al., 1963). In addition to having hypertension, other vascular effects are also observed in SHR. In particular, endothelial dysfunction is evident in the SHR (Pinto et al., 1998). To lower the high blood pressure in SHR, the use of inhibitors of the renin-angiotensin system (RAS), calcium antagonists, and vasodilators have been shown to be effective, while diuretics and endothelin antagonists have been less effective (Pinto et al., 1998). Interestingly, hypertension in the SHR does not progress to the development of strokes, atherosclerosis, or vascular thrombosis (Pinto et al., 1998). This is unlike humans, where hypertension is a significant and independent risk factor for CVD, as previously mentioned. However, cardiac hypertrophy occurs in the SHR with many, but not all, SHR experiencing heart failure after twenty-four months of age (Pinto et al., 1998).

Some debate has existed on the suitability of WKY rats to act as a normotensive control for the SHR. Although SHR from different suppliers in the United States have been found to maintain their original characteristics thus indicating that the genetics of this strain have been well-preserved, WKY rats sourced from different American suppliers have been found to vary in their body weights and blood pressure. These observations suggest that potential genetic variations may exist among differently sourced WKY rats (Kurtz et al., 1987). The biological differences among differently sourced WKY rats have been attributed to the claim that the WKY rats were distributed before becoming fully inbred (Kurtz et al., 1987). Furthermore, a claim was made that some SHR were also distributed

before becoming fully inbred (Yamori, 1987). Thus, genetic disparity in differently sourced SHR may also be observed. Regardless of these aforementioned statements, the SHR is the most frequently used animal model of essential hypertension in research and the WKY rat remains the best choice for a normotensive control when employing the SHR in research studies (Kurtz et al., 1987; Louis et al., 1990; Pinto et al., 1998).

Other animal models of hypertension develop high blood pressure by surgical or dietary means. The two-kidney one clip rat experiences hypertension after surgical constriction of one renal artery (Pinto et al., 1998). This type of hypertension is known as Goldblatt hypertension (Pinto et al., 1998). The Dahl salt-sensitive and deoxycorticosterone acetate (DOCA)-salt rats are induced into a hypertensive state after being fed a high sodium diet (Pinto et al., 1998). The DOCA-salt rats also require the administration of DOCA, which is a hormone that stimulates renal sodium reabsorption. Thus, in the Dahl salt-sensitive and DOCA-salt rats, the hypertension is caused by increased blood volume due to high concentrations of sodium in the blood. The Dahl salt-sensitive rats do not require any additional surgical or endocrine manipulations and thus the impaired renal sodium handling observed in this model is thought to be a genetic disorder (Pinto et al., 1998). Other studies have employed rats with renal disorders, diabetes, or without health ailments and administered vasoconstrictors (such as AngII) or a high-salt diet to induce hypertension. Many of these aforementioned animal models are utilized in research; however, none of them are models of essential hypertension. Since essential hypertension is the form of high blood pressure that

affects 95% of people with hypertension, it is only appropriate to use an animal model of essential hypertension when examining treatment or prevention strategies. The SHR is the only animal model of essential hypertension currently available and thus is the best choice for research on essential hypertension.

Knowledge Gaps

In both experimental animal models and humans, it is evident that hypertension is associated with endothelial dysfunction and vascular remodelling. These processes can be initiated in situations where there are chronic elevations of vascular ROS, ET-1 production, and circulating AngII. Currently, there is no cure for essential hypertension, and patients with hypertension usually require two or more different medications to help manage their high blood pressure. These medications can have side effects on the human body, and moreover were not specifically designed to treat endothelial dysfunction or vascular remodelling.

It is evident from the literature that pulses may have a useful role in hypertension management. Indeed, the consumption of pulses by human subjects has resulted in blood pressure reductions in controlled feeding trials (Table 2). The antihypertensive mechanism for pulses may relate to their bioactive peptides (for ACE inhibition) or phenolic compounds (potential antioxidants) as demonstrated in animal studies (Table 3). However, mechanistic research involving whole pulses (to mimic human consumption) in animal models is lacking. Moreover, clinical studies rarely examine individual pulse types. For example, black beans have never been studied for their effects on the vasculature in either animal models or human subjects.

A previous study in SHR found that green and red lentils exerted different effects on the vasculature with only green lentils improving vascular compliance (Hanson et al., 2016b). Given that there are no differences in nutrient contents of

green and red lentils, the vascular effects may be attributed to the antioxidant/phenolic compounds because these constituents can vary between green and red lentils (Zhang et al., 2015). Moreover, the lentils were found to inhibit p38 MAPK phosphorylation, which is a ROS-sensitive protein that contributes to vascular remodelling. Although Hanson *et al.* (2014) found that a mixed bean diet was ineffective, it is possible that differently coloured beans may act uniquely in the vasculature (as was found with lentils).

Thus, the following questions remain to be answered:

1. Can beans exert blood pressure regulating effects and mitigate vascular remodelling in hypertension?
2. Are there differences between different coloured beans in terms of their effect(s) on hypertension and vascular remodelling?
3. If beans are successful, what cell-signalling mechanisms could explain their mode of action?

Study Rationale

This project was designed to explore, and compare, the use of black beans and navy beans in alleviating the progression of hypertension, a disease that affects 30% of Canadian adults. Specifically, we sought to determine if either bean type could mitigate the development of hypertension by attenuating both the rise in blood pressure and vascular remodelling in SHR, and if such changes would be associated with altered cell-signalling events at the vascular level (i.e. modifications in pathways leading to endothelial dysfunction and vascular remodelling).

Recent work in our laboratory showed that the consumption of lentils by SHR attenuated rises in MAP and SBP while concurrently altering blood vessel structure and function, resulting in greater arterial compliance (Hanson et al., 2016b; Hanson et al., 2014). These findings were unique to lentils and not observed with diets of mixed beans, mixed peas, or chickpeas. The ability of the lentils to alleviate symptoms of hypertension was attributed to an inhibition of aortic p38 MAPK activity (Hanson et al., 2016b). As previously mentioned, aberrant p38 MAPK activation in the vasculature contributes to excessive ROS accumulation, which causes endothelial dysfunction and vascular remodelling. Interestingly, when green lentils and red lentils were directly compared, it was found that green lentils produced greater vascular benefit than did red lentils. Thus, it became evident that different varieties of lentils exhibit unique effects on the vasculature, and so it was postulated that similar findings might be observed if individual bean varieties were examined in separate diets rather than as a mixture in one diet. The literature

suggests that pulse seed coat colour is indicative of the phenolic content and antioxidant potential of pulse, where darker coloured seeds have greater total phenolic concentrations and activities than lighter coloured seeds (Garcia-Lafuente et al., 2014; Xu et al., 2012).

Thus, this study was developed to examine whether the effects of black beans and navy (white) beans on blood vessel structure and function were different. This was achieved by examining a black bean-based diet vs. a navy bean-based diet on vascular structure and function in SHR in comparison to SHR fed a green lentil diet, SHR fed a pulse-free control diet, and WKY rats fed a pulse-free control diet.

Hypothesis

Black beans, but not navy beans, will improve blood vessel function and structure in SHR. More specifically, SHR fed a diet containing black beans will exhibit attenuated progression of hypertension and vascular remodelling during an eight-week dietary intervention. This will not be observed with SHR fed a navy bean diet. Compared to SHR control, the black bean-fed SHR will have:

1. Lower blood pressure as measured by the tail-cuff method, and greater arterial compliance as measured by PWV.

2. Improved blood vessel structure as observed by a smaller media and/or larger lumen, accompanied with an altered protein composition in the vessel wall of the aorta.

3. Altered levels of proteins involved in endothelial function (such as eNOS = increased by black beans) and vascular remodelling (such as MAPKs = decreased by black beans) in aortic tissue.

Objectives

To determine and compare the effects of an eight-week dietary intervention with a black bean or a navy bean diet, relative to a green lentil diet and a pulse-free diet, in SHR by examining:

1. Blood pressure (SBP, DBP, and MAP), determined by the non-invasive tail-cuff method.

2. Arterial compliance, determined by PWV.

3. Vascular remodelling, determined by sectioning and staining aorta cross-sections with an Elastin Stain Kit to assess ECM protein composition and the lumen and media sizes.

4. Cell-signalling pathways involved in endothelial dysfunction and vascular remodelling, as determined by Western immunoblotting of proteins extracted from aortic tissue.

Materials and Methods

Experimental Animals and Diets

Forty SHR and ten WKY rats were ordered from Charles River Laboratories (St. Constant, QC, Canada). The rats were housed at the R.O. Burrell Laboratory animal facility at the St. Boniface Hospital Albrechtsen Research Centre (Winnipeg, MB, Canada). The University of Manitoba's Bannatyne Campus Animal Care Committee approved the protocol for this experiment prior to its commencement. Proper animal care and experimentation as outlined by the Canadian Council on Animal Care was followed for the duration of the experiment. Upon arrival, the rats were individually caged and housed under controlled laboratory conditions with twelve hours of day (light)/twelve hours of night (darkness), 40-60% humidity, and controlled temperature. The rats were acclimated for a minimum of two weeks prior to experiment commencement, during which time they were allowed free access to water and fed a standard chow diet (for the first week) followed by the semi-purified control diet (for the second week). Following acclimatization and at seventeen weeks of age, the rats were randomly assigned to one of the following groups (n = 10 rats/group):

1. SHR fed a diet containing black beans (30% w/w) for eight weeks.
2. SHR fed a diet containing navy beans (30% w/w) for eight weeks.
3. SHR fed a diet containing green lentils (30% w/w) for eight weeks. This group served as a positive control.

4. SHR fed a pulse-free control diet for eight weeks. This group served as a hypertensive reference group and a negative control.

5. WKY fed a pulse-free control diet for eight weeks. This group served as a normotensive reference group.

The diets were fed *ad libitum*, where 50 g of powdered diet was allocated per rat per day and offered in a lidded jar. Weighing of the previous day's unconsumed feed before provision of fresh feed was done to monitor daily feed intake. The amount of pulses in the experimental diets (30% w/w) and duration of the intervention (eight weeks) was based on the study by Hanson *et al.* (2014) where feeding a 30% (w/w) lentil diet to SHR for eight weeks improved blood pressure and blood vessel structure and function in SHR. As well, the sample size of n=10 per group was considered sufficient based on the same previously mentioned study (Hanson et al. 2014). In addition, the sample size was justified by a power calculation based on SBP data from a study where SHR were fed a mixed bean diet (SBP = 174 mmHg) and compared to SHR fed a pulse-free diet (SBP = 200 mmHg) (Hanson et al. 2014). Using the aforementioned data, a sigma of 16.5, alpha of 0.05, and 90% power, a sample of n=9/group was calculated to be sufficient for this experiment.

Preparation of the experimental and control diets was as follows. The black beans (~90% Eclipse and ~10% unknown variety) and navy beans (~75% T9905, ~20% Envoy, and ~5% unknown variety) were obtained from Legumex Walker

(Plum Coulee, MB, Canada), while the green lentils (CDC Greenland) were obtained from Simpson Seeds (Moosejaw, SK, Canada).

To mimic typical human consumption, the beans and lentils were cooked according to Pulse Canada's *Guide to Cooking Beans, Chickpeas, Lentils and Peas* (2008). Unlike lentils, beans require overnight soaking prior to cooking. This was accomplished by weighing 1000 g dry beans on an electronic scale, and soaking them in 3000 g tap water overnight at 4°C. The next day, 1000 g soaked beans (after draining) or 1000 g dry lentils were weighed on an electronic scale, rinsed under cold running water (to remove saponins), and brought to boil in 3000 g water. Once boiling, the beans were cooked for one hour and the lentils were cooked for twenty-five minutes, after which time the cooking water was drained and the beans and lentils were rinsed under cold running water to prevent over-cooking. The cooked pulses were then manually mashed and pressed into nine-inch round foil pans at one inch depth, covered with foil, and freeze-dried in a VirTis freeze-dryer (SP Scientific; Warminister, PA, USA). After freeze-drying, the beans and lentils were powdered in an Ultra Centrifugal Mill (Retsch ZM 200; Retsch, Haan, Germany) set at 12 000 rpm until passing through a 0.5 mm screen. The pulse powders were then ready for addition to the experimental diets, which, along with the control diet, were based on the American Institute of Nutrition-93 Growth (AIN-93G) formulation (Reeves et al., 1993a; Reeves et al., 1993b).

The AIN-93G formulation contains carbohydrates from cornstarch, maltodextrin and sucrose; protein from casein (with additional L-cysteine due to the

limiting amount in casein); fat from soybean oil (to meet the requirements for linoleic and linolenic acids); tertiary-butylhydroquinone, an antioxidant, to prevent oxidation of the polyunsaturated fatty acids; fibre from cellulose; an essential vitamin mix (which is void of vitamin C as rats are able to synthesize this nutrient *de novo*); and finally, an essential mineral mix (Reeves, 1997).

For the experimental diets, the pulse powders were incorporated at 30% (w/w); this level of pulses does not alter growth. The final formulations of the four diets can be found in Table 4. To make the experimental diets isonitrogenous and isocaloric with the control diet, the amounts of casein and maize starch were adjusted. These adjustments were determined by calculation based on proximate analysis of the pulse powders (as conducted by Central Testing Labs Ltd.; Winnipeg, MB, Canada). More detail on the calculations and proximate analysis results of the pulse powders can be found in Appendix A. In addition, proximate analysis of the four diets was conducted by Central Testing Labs Ltd. (Winnipeg, MB, Canada) to verify that the diets had similar protein and energy contents (Table 5). Cellulose was not added to the pulse diets as the pulse powders provided sufficient fibre to meet the minimum daily requirements for rats. However, it should be noted that proximate analysis results for fibre revealed differences in content among the diets (Table 5). Neutral detergent fibre (NDF) is approximately the total content of insoluble fibre (TestDiet, 2017), which was higher in the pulse containing diets compared to the control diet. Acid detergent fibre (ADF) and crude fibre represent specific types of insoluble fibre, and their contents would be reflected in NDF (TestDiet, 2017).

The diets were prepared by combining all dry ingredients using a Hobart (Toronto, ON, Canada) countertop mixer on low speed for five minutes, followed by the addition of the soybean oil and mixing on low speed for ten minutes. All ingredients were weighed on an electronic scale during preparation of the diets. For long-term storage, the diets were kept at -20°C, vs. 4°C for short-term storage in the animal facility.

Table 4. Diet Formulations

	AIN-93G Control	Black Bean	Navy Bean	Green Lentil
Ingredients[1,2] **(g/kg)**				
Casein	200	121.5	118	106
Maize Starch	397	226	229.5	241.5
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Cellulose	50	0	0	0
L-Cysteine	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5
Mineral Mix[3]	35	35	35	35
Vitamin Mix[4]	10	10	10	10
Soybean Oil[5]	70	70	70	70
Pulse powder[6] **(g/kg)**				
Black Bean	-	300	-	-
Navy Bean	-	-	300	-
Green Lentil	-	-	-	300

Abbreviations: AIN-93G, American Institute of Nutrition-93 Growth diet.

[1]Ingredients (except the pulse powders) ordered from Dyets, Inc. (Bethlehem, PA, USA).

[2]Adjustments to casein, maize starch, and cellulose were done as described in the Materials and Methods section with additional information in Appendix A.

[3]AIN-93G MX.

[4]AIN-93 VX.

[5]With 0.02% tert-butylhydroquinone.

[6]Pulse powders were prepared as described in the Materials and Methods section.

Table 5. Proximate Analysis of Diets[1,2]

	AIN-93G Control	Black Bean	Navy Bean	Green Lentil
Moisture[3]	7.15	5.48	5.35	5.25
Dry Matter	92.9	94.5	94.7	94.8
Crude Protein[4]	16.8	16.5	17.8	17.1
Crude Fibre[5]	1.04	3.04	2.32	3.51
ADF[6]	0.59	3.35	3.60	3.35
NDF[7]	1.85	5.73	6.59	4.34
Carbohydrate[8]	65.7	64.0	64.3	64.4
Fat[9]	6.89	7.76	7.04	6.78
Ash[10]	2.39	3.25	3.19	2.99
Energy (kcals/g)[11]	3.92	3.92	3.92	3.87

Abbreviations: AIN-93G, American Institute of Nutrition-93 Growth diet; ADF, acid detergent fibre; NDF, neutral detergent fibre.

[1]Proximate analysis conducted by Central Testing Labs Ltd., Winnipeg, MB, Canada.
[2]Results are expressed as % of weight, as is.
[3]Moisture was determined by the AOAC 930.15 method.
[4]Crude protein was determined by a modified AOAC 990.03 method.
[5]Crude fibre was determined by the AOCS Ba6a-05 method.
[6]ADF determined by the ANKOM Technology (Macedon, NY, USA) method 08-16-06.
[7]NDF determined by the ANKOM Technology (Macedon, NY, USA) method 08-16-06.
[8]Non-fibre carbohydrate was determined by calculation.
[9]Fat was determined by the AOCS Am 5-04 method.
[10]Ash was determined by the AOAC 923.03 method.
[11]Energy was determined by calculation assuming 4 kcal/g for protein and carbohydrate, and 9 kcal/g for fat.

Assessment of Disease Risk

Body Weight

Body weights were measured at baseline and weekly by placing each animal in a tared bowl rested on an electronic scale. Weight was recorded after the scale reading was steady for at least three seconds.

Body Composition

Determination of fat mass, lean mass, free water mass, and total water mass was done by using an EchoMRI-700TM whole body Quantitative Magnetic Resonance (QMR) instrument (EchoMRI; Houston, TX, USA). Each animal was measured three times, and the average of the three values was used in calculations for group averages. Negative free water values were excluded. Body composition was assessed at baseline and eight weeks.

Serum Biochemistry

Blood was collected from the rats at baseline and week eight. The rats were fasted (with free access to water) in metabolic cages for six hours, after which time 1–2 mL of blood was collected from the saphenous vein into microvette blood collection tubes. The blood was put on ice and allowed to clot before being spun for fifteen minutes at 1000 *g* in a centrifuge maintained at 4°C. The serum was then obtained and preserved at -80°C until analyzed. Levels of total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, alanine transaminase (ALT), aspartate transaminase (AST), urea, and creatinine in the serum were measured with a Cobas C111 auto-analyzer (Roche; Penzberg, BY, Germany).

Urine Biochemistry

Urine was collected from the rats at baseline and week eight. The rats were fasted (with free access to water) in metabolic cages where the urine would drain to the bottom of the cage and accumulate in a pre-weighed vial. After the rats spent six

hours in the metabolic cages, the vial containing the urine was retrieved and the weight of the urine was determined. The urine was stored at -80°C until analyzed. Levels of urea and creatinine in the urine were measured with a Cobas C111 auto-analyzer (Roche; Penzberg, Germany).

Assessments of Arterial Stiffness and Vascular Function

Blood Pressure

SBP, DBP, and MAP were assessed non-invasively by the tail cuff method using a CODA system (Kent Scientific; Torrington, CT, USA) at baseline, four, and eight weeks. Briefly, the rats were placed in clear plastic tubes and rested on a warming platform to encourage blood flow to the tail. Occlusion and volume-pressure recording cuffs were then placed on the tails of the rats. The occlusion cuff would inflate to obstruct blood flow and then deflate to establish pulsatile blood flow to the tail allowing the volume-pressure recording cuff to record a pressure reading. The machine would perform five acclimation cycles before recording the blood pressure readings that were used for analysis. Each animal's blood pressure was measured ten times, and the average of the accepted readings for individual rats was used in calculations for group averages.

Pulse Wave Velocity (PWV)

PWV is the speed at which blood travels between two defined points in the arterial system, and was assessed at baseline and eight weeks. Briefly, the rats were lightly anesthetized using isoflurane and placed in a supine position while a trained

technician from the R.O. Burrell Laboratory animal facility used an ECG-triggered 10-MHz Doppler probe (Indus Instruments; Weber, TX, USA) to measure the passage of sequential pulse waves between the aortic arch and the proximal abdominal aortic bifurcation. Three PWV measurements were recorded per animal, and the average of the three measurements was used in calculating group averages. Readings of peak flow velocity (cm/second), minimum flow velocity (cm/second), and mean flow velocity (cm/second) were reported.

Aorta Collection and Morphology

At the end of the eight-week dietary intervention, the rats were euthanized by an overdose of pentobarbital. The aorta was retrieved from the carcass. The aorta is the largest artery in the body and thus a sufficient amount of tissue is provided for morphological and protein analysis. Upon dissection of the aorta, the aortic arch was removed and a ~1 cm portion from the center of the descending vessel was embedded vertically in Optimal Cutting Temperature compound (Sakura Finetek USA, Inc.; Torrance, CA, USA), then frozen in an ethanol and dry ice bath, and stored at -80°C until sectioned. The remainder of the aortic tissue was wrapped in foil, flash frozen in liquid nitrogen, and stored at -80°C until the protein was extracted for Western Blotting as described under Western Immunoblotting.

Using a Thermo Shandon Cryotome (Thermo Fisher Scientific; Waltham, MA, USA), a minimum of two non-consecutive 5 μm thick slices of aorta cross-sections were obtained from the embedded tissue and adhered to Superfrost™ Plus microscope slides (Thermo Fisher Scientific; Waltham, MA, USA) and subsequently

stored at -20°C. The slides were then fixed in 1% paraformaldehyde for ten minutes prior to staining.

The aorta cross-sections were treated with an Elastin Stain Kit (Sigma-Aldrich; St. Louis, MO, USA) so that elastin was black, collagen was red, and cells were yellow. Briefly, the fixed slides were washed in 1× phosphate buffer solution (Appendix B) for ten minutes, hydrated in double-distilled water for fifteen minutes, then stained according to the manufacturer's protocol. Differentiation in the working ferric chloride solution was done for three minutes, and staining in the Van Gieson solution was done for ninety seconds. After dehydrating the slides in xylene and allowing them to air-dry for one hour, the slides were mounted with VectaMount Permanent Mounting Medium (Vector Laboratories; Burlingame, CA, USA).

Two images per slide were captured under an EVOS® microscope (AMG Solutions; Seattle, WA, USA) with 4× and 20× objectives. These images were evaluated using ImageJ software (http://imagej.net) to determine vessel morphology (with images captured using 4× objective) and elastin content (20× objective). Additional details on these procedures are outlined in Appendix C. Briefly, the inner (lumen) and outer circumferences of the vessel cross-sections were manually traced using a pen tablet (Wacom Technology Corporation; Portland, OR, USA). Based on those traces, ImageJ quantified the lumen circumference, lumen cross-sectional area (CSA), outer media diameter, and whole-vessel CSA. In Microsoft Excel, the lumen diameter, media thickness, media CSA, and media/lumen

ratio were calculated (according to the formulas outlined in Appendix C). Two sets of morphological measurements were obtained per animal, the average of which was used in calculations for group averages.

Western Immunoblotting

Proteins were extracted from the rat aortae for the assessment of relative levels of proteins involved in endothelial function and vascular remodelling. Protein extraction from the excised rat aortic tissue was performed as follows. Approximately forty milligrams of frozen tissue was weighed on an electronic scale, placed in a mortar, immersed in liquid nitrogen, and ground to a powder. Subsequently, 1200 µL (or 30 µL/mg tissue) of 3× sample buffer (Appendix B) was added to the powdered tissue and allowed to digest the sample for fifteen minutes. After this, the protein extract was pipetted into 1.5 mL microcentrifuge tubes and spun for twenty minutes at 13 000 rpm in a centrifuge (Model 5804 R, Eppendorf; Hamburg, Germany) maintained at 4°C. The supernatant was then collected, and the samples were sonicated prior to storage at -80°C.

To determine the concentration of proteins within each sample, a bicinchoninic acid (BCA) colourimetric protein assay (Pierce; Rockford, IL, USA) was performed. The standards used in this assay contained increasing concentrations of bovine serum albumin (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mg/mL) diluted with double-distilled water. In triplicate, the protein extracts and standards were pipetted at 10 µL/well into NUNC 96-well plates and 200 µL of reaction mixture (Pierce; Rockford, IL, USA) was added to each well. The plates were then incubated

at 30°C for thirty minutes, after which time absorbance at 562 nm was quantified in a FLUOstar OMEGA microplate reader (BMG Labtech; Ortenberg, BW, Germany). Results were accepted if the regression analysis of the standard curve produced a coefficient of determination (r^2) of ≥0.99, and if the coefficient of variation (CV) for each sample was <10%.

Proteins from the extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane for specific protein detection by Western Immunoblotting. Transfer of the proteins onto the membrane was confirmed by incubating the membrane with Ponceau S Stain (Amresco, LLC; Solon, OH, USA) for five minutes. Before incubation with antibodies, the membranes were blocked in 3% bovine serum albumin-Tris-buffered saline with 0.1% Tween 20 (BSA-TBST) for one hour. Following the blocking step, membranes were incubated with primary antibodies diluted in 3% BSA-TBST for a specified period of time, washed four times with 1× TBST, incubated with secondary antibodies diluted in 1% BSA-TBST for one hour, and washed four times with 1× TBST. β-Tubulin antibody was used as the loading control for each membrane. Specific details on the antibodies used are provided in Table 6. After incubation with the antibodies, the membranes were dipped in EMD Millipore Luminata™ Western HRP Chemiluminescence Substrate (EMD Milipore; Darmstadt, HE, Germany) and visualized with a FluorChem Q Western Blot imager (Protein Simple; Santa Clara, CA, USA). AlphaView SA software (Protein Simple; Santa Clara, CA, USA) was used for quantification of the relative protein levels, and calculations were done in Microsoft Excel.

Table 6. Antibodies Used in Western Blotting of Rat Aorta Protein Extracts

Antibody	Supplier	Catalog/ Lot #	Acrylamide (%)	1°AB Dilution	Incubation Time	2°AB	2°AB Dilution
eNOS	Cell Signalling[1]	CS952S/ 3	6	1:500	o/n @ 4°C	Rabbit	1:10000
p-ERK1/2 (Thr202/Tyr204)	Cell Signalling	CS9101L/ 26	10	1:1000	o/n @ 4°C	Rabbit	1:10000
ERK1/2	Cell Signalling	CS9102L/ 23	10	1:1000	o/n @ 4°C	Rabbit	1:10000
p-HSP27 (Ser82)	Cell Signalling	CS2401S/ 11	10	1:1000	o/n @ 4°C	Rabbit	1:10000
HSP27	Santa Cruz[2]	SC1048/ J316	10	1:1000	o/n @ 4°C	Goat	1:10000
p- p38 MAPK (Thr180/Tyr182)	Cell Signalling	CS9211S/ 23	10	1:1000	o/n @ 4°C	Rabbit	1:10000
p38 MAPK	Cell Signalling	CS9212/ 23	10	1:1000	o/n @ 4°C	Rabbit	1:10000
Scleraxis	Custom antibody, preparation described by Espira *et al.* 2009		10	1:1000	o/n @ 4°C	Rabbit	1:10000

Abbreviations: AB, antibody; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; o/n, overnight; p, phosphorylated; Ser, serine; Thr, threonine; Tyr, tyrosine.
[1]Cell Signalling Technology, Danvers, MA, USA.
[2]Santa Cruz Biotechnology, Dallas, TX, USA.

Statistical Analysis

Time course data were analyzed by analysis of variance (ANOVA) and endpoint data were analyzed by ANOVA using SAS statistical software (version 9.4). Duncan's multiple range post hoc test was utilized for means testing. Outliers were removed if values were outside the result of (mean ± [3 × standard deviation]). To ensure datasets met the ANOVA requirements for normality and homogeneity, the Shapiro-Wilk normality test and Levene's test for homogeneity of variances were conducted. If a dataset was not normal or homogeneous (as indicated by $p < 0.05$ for the Shapiro-Wilk or Levene's test, respectively), then results from the log-transformed data were reported. Results were considered statistically significant at $p < 0.05$. In addition, a trend towards significance was considered for results with p-values >0.05 and <0.1.

Results

Feed Intake, Body Weight, and Body Composition

Feed intake was measured to ensure dietary compliance and to observe any diet related effects on apparent appetite. Average daily feed intake is presented in Figure 1a and weekly averages as well as total intake can be found in Table D-1 (Appendix D). WKY rats consumed less feed throughout the trial compared to SHR, with the exception of the navy bean-fed SHR that had feed intake not different from WKY rats. However, the average daily intake was similar among the SHR groups, with no significant influence of diet type on feed intake.

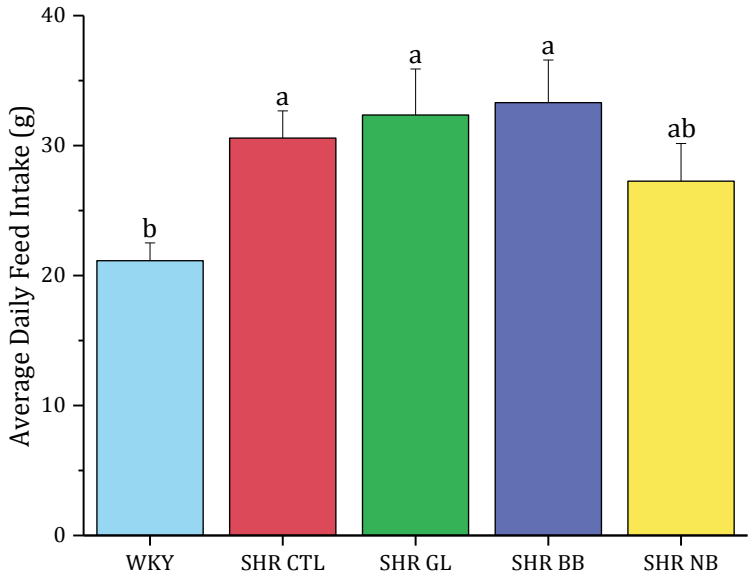
Body weight and composition were measured to confirm adequate growth of the rats throughout the study duration and to determine any diet-related effects on body weight and composition. Results for final (week eight) body weight are presented in Figure 1b and weekly body weights are found in Table D-2 (Appendix D). Results for body composition can be found in Table 7 (fat and lean mass) and Table D-3 (water) (Appendix D).

The final body weights of all SHR were significantly lower than the WKY rats, and there were no differences in body weight among SHR groups. Total lean mass was not different among all animals at baseline or week eight. However, total fat mass was significantly higher in the WKY rats compared to all SHR groups at baseline and week eight. As a ratio to body weight, lean mass was significantly lower and fat mass was significantly higher in WKY rats compared to all SHR groups. Thus,

the larger body weight of the WKY rats was attributed to the greater proportion of fat mass per 100 g body weight compared to SHR. Furthermore, the change in fat mass from baseline to week eight was significantly greater in WKY rats than SHR, while the change in lean mass was not significantly different among the groups. This confirms that the body weight gain in the WKY rats was due to body fat accumulation.

Among SHR groups, there were no significant diet-related influences on body weight or composition. The absence of significant differences in body weight and feed intake among the SHR implies there were no differences in the dose of pulses ingested throughout the study.

a)



b)

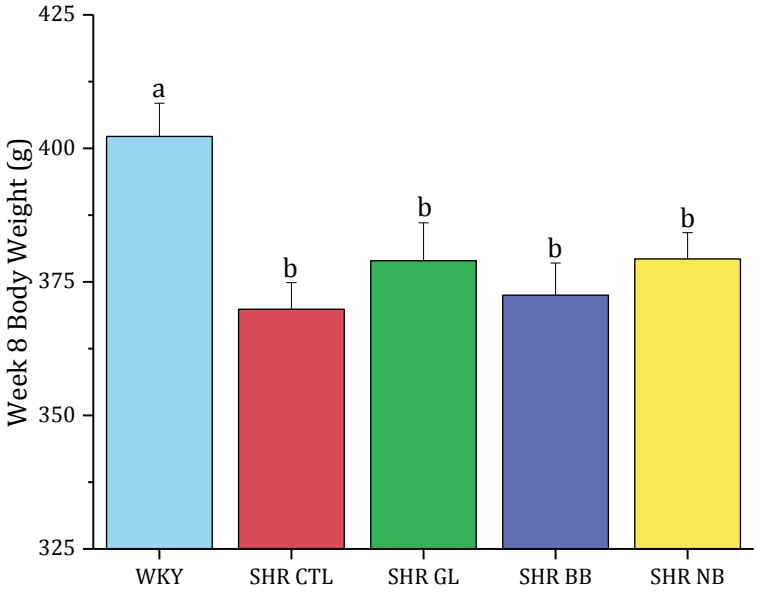


Figure 1. Daily Feed Intake (a) and Week 8 Body Weight (b)

Results are expressed as mean ± SE. Different letters represent significant differences ($p<0.05$). *Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Table 7. Body Composition of WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Lean Mass (g)					
Baseline	246 ± 4	252 ± 8	249 ± 6	243 ± 6	257 ± 5
Week 8	257 ± 3	251 ± 3	255 ± 5	254 ± 4	262 ± 3
Baseline vs. Week 8	10.9 ± 3.2	-0.8 ± 7.7	6.5 ± 7.7	11.0 ± 1.9	5.0 ± 4.4
Lean Mass (g/100 g Body Weight)					
Baseline	74.4 ± 0.5	77.0 ± 1.6	74.8 ± 1.9	72.6 ± 0.6	75.8 ± 1.8
Week 8	63.9 ± 0.9 b	68.3 ± 1.3 a	67.8 ± 0.7 a	68.0 ± 0.8 a	68.9 ± 0.6 a
Fat Mass (g)					
Baseline	23.2 ± 1.1 a	16.9 ± 1.3 b	16.2 ± 1.0 b	17.3 ± 0.9 b	17.1 ± 1.65b
Week 8	37.8 ± 1.9 a	21.3 ± 1.8 b	21.2 ± 2.6 b	21.5 ± 1.5 b	19.0 ± 1.2 b
Baseline vs. Week 8	15.1 ± 2.0 a	4.4 ± 1.3 b	5.0 ± 2.6 b	4.1 ± 1.6 b	1.8 ± 1.6 b
Fat Mass (g/100 g Body Weight)					
Baseline	7.02 ± 0.25 a	5.18 ± 0.37 b	4.87 ± 0.31 b	5.19 ± 0.27 b	5.08 ± 0.45 b
Week 8	9.38 ± 0.46 a	5.75 ± 0.42 b	5.61 ± 0.64 b	5.81 ± 0.43 b	5.00 ± 0.31 b

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained *in vivo* with a QMR instrument. Results are expressed as mean ± SE (n = 9–10/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Serum and Urine Biochemistry

Circulating lipids are commonly used markers of CVD risk. Results for fasting serum lipids can be found in Figure 2 and Table E-1 (Appendix E). At beginning and end of study, all SHR groups had significantly lower levels of serum triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol compared to WKY rats. The only exception was baseline LDL cholesterol, which was not different in control and green lentil-fed SHR compared to WKY rats. On the other hand, ratios of total cholesterol, LDL cholesterol, and triglycerides to HDL cholesterol were not significantly different between WKY rats and SHR at week eight (Table E-2, Appendix E). The only observed effect of diet on serum lipids among the SHR groups was a significantly lower total cholesterol level in the black bean-fed SHR compared to control-fed SHR at week eight. Thus, there were no differences in triglycerides or HDL or LDL cholesterol among the SHR groups at baseline or week eight.

Hypertension can cause the kidneys and liver to become dysfunctional. Thus, serum markers of kidney and liver function are indicative of end-organ damage in hypertension. Urinary markers of kidney function can also be measured as indicators of kidney damage. The results for fasting serum kidney and liver function can be found in Table 8, while the results for urine markers of kidney function can be found in Table 9.

With regard to kidney function, some significant differences at baseline for serum urea were present where SHR typically had higher levels than WKY rats

(with the exception of the green lentil-fed SHR that was not different from the WKY rats). However, no significant differences in serum urea were present at week eight. In addition, serum creatinine was not different among all groups at baseline or week eight, and furthermore, no significant differences were found for urine concentrations of urea or creatinine among all groups at baseline or week eight.

WKY rats had significantly lower serum ALT and AST levels compared to SHR at baseline and week eight. The pulse-fed SHR had significantly higher levels of serum ALT and AST compared to control SHR at week eight. The only exception was for the green lentil-fed SHR, whose week eight serum AST was not significantly different from control SHR.

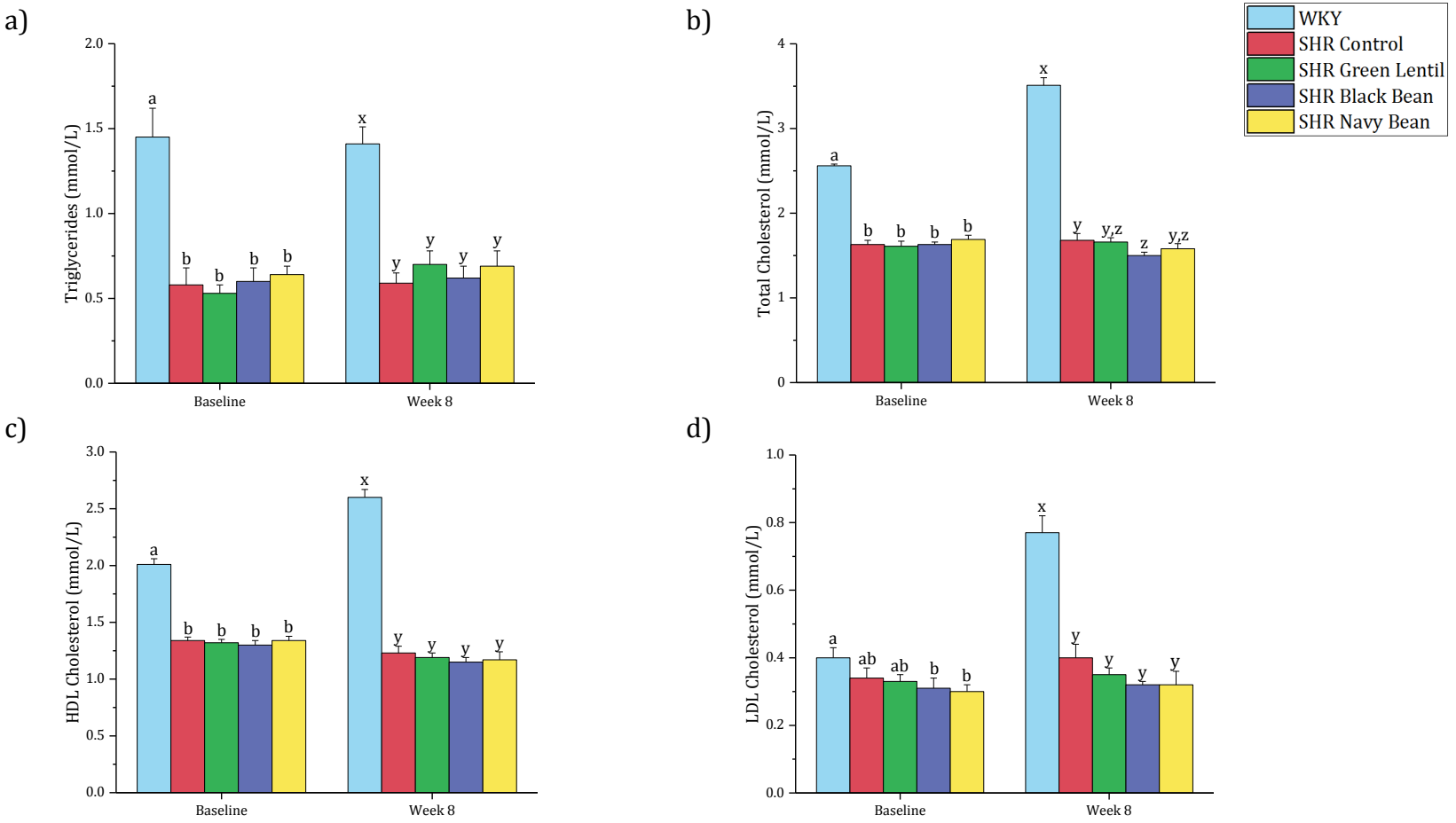


Figure 2. Fasting Serum Triglycerides (a), Total Cholesterol (b), HDL Cholesterol (c), and LDL Cholesterol (d) at Baseline and 8 Weeks

For means within the same time point, different letters represent significant differences ($p<0.05$). *Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Table 8. Fasting Serum Markers of Kidney and Liver Function in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Kidney Function					
Creatinine (µmol/L)					
Baseline	23.0 ± 1.1	25.6 ± 5.2	25.5 ± 1.3	26.3 ± 0.9	26.9 ± 0.9
Week 8	30.5 ± 0.9	34.7 ± 1.8	30.2 ± 1.4	31.5 ± 0.8	29.8 ± 1.9
Baseline vs. Week 8	7.49 ± 1.25	8.33 ± 3.15	4.68 ± 2.20	5.19 ± 1.24	2.84 ± 2.50
Urea (mmol/L)					
Baseline	4.86 ± 0.15 c	5.77 ± 0.20 a	5.21 ± 0.19 bc	5.58 ± 0.15 ab	5.63 ± 0.14 ab
Week 8	4.07 ± 0.09	4.77 ± 0.08	4.98 ± 0.29	4.69 ± 0.19	4.46 ± 0.38
Baseline vs. Week 8	-0.79 ± 0.14	-0.59 ± 0.53	-0.39 ± 0.37	-0.78 ± 0.23	-1.14 ± 0.41
Liver Function					
ALT (U/L)					
Baseline	37.8 ± 2.6 b	62.0 ± 2.8 a	63.2 ± 2.2 a	60.8 ± 1.9 a	62.8 ± 2.0 a
Week 8	27.9 ± 1.6 c	47.9 ± 3.4 b	64.0 ± 4.8 a	76.1 ± 5.9 a	74.8 ± 7.2 a
Baseline vs. Week 8	-8.2 ± 2.7 bc	-16.9 ± 3.6 c	1.7 ± 5.0 ab	15.5 ± 6.0 a	12.5 ± 7.7 a
AST (U/L)					
Baseline	77 ± 3 b	132 ± 6 a	143 ± 5 a	137 ± 7 a	140 ± 6 a
Week 8	89 ± 6 c	127 ± 6 b	142 ± 7 ab	150 ± 7 a	155 ± 8 a
Baseline vs. Week 8	17.1 ± 7.3	-5.7 ± 10.7	-0.3 ± 6.6	10.1 ± 8.7	18.5 ± 11.1

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained using a Cobas CIII auto-analyzer. Results are expressed as mean ± SE (n = 7–9/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Table 9. Urine Markers of Kidney Function in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Creatinine (mmol/L)					
Baseline	5.53 ± 1.61	6.87 ± 1.58	5.63 ± 1.18	6.03 ± 1.43	6.54 ± 1.28
Week 8	6.04 ± 1.49	7.37 ± 1.49	5.67 ± 1.07	6.98 ± 0.97	5.45 ± 0.92
Baseline vs. Week 8	1.07 ± 2.56	0.53 ± 1.82	0.15 ± 1.89	2.18 ± 3.80	-1.55 ± 1.49
Urea (mmol/L)					
Baseline	675 ± 193	702 ± 168	562 ± 139	616 ± 144	692 ± 149
Week 8	548 ± 107	620 ± 143	410 ± 76	490 ± 64	419 ± 85
Baseline vs. Week 8	-59 ± 247	-78 ± 197	-147 ± 176	6 ± 113	-302 ± 192

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained using a Cobas CIII auto-analyzer. Results are expressed as mean ± SE (n = 6–8/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Blood Pressure

Blood pressure was the primary outcome for this study and was the determinant for the sample size of this study based on the power calculation. The tail-cuff method is commonly performed in rodent models of hypertension to determine blood pressure and so it was employed for this study. Results for SBP, DBP, and MAP can be found in Figure 3 and Table F-1 (Appendix F).

At baseline, week four, and week eight, all SHR groups had significantly higher SBP, DBP, and MAP compared to WKY rats. Among the SHR groups, there were no significant diet-related influences on SBP, DBP, or MAP at any time point measured during the study. However, the control SHR in our study experienced a decline in blood pressure over time, which is unusual for this animal model, whereas pulse-fed SHR exhibited the normal increase in blood pressure over time. Due to this discrepancy, the black bean- and navy bean-fed SHR showed significantly greater changes in SBP from baseline to week eight (+12.5 ± 8.5 and +17.9 ± 7.4 mmHg, respectively) compared to the SBP change experienced by the control SHR (-12.9 ± 5.2 mmHg). However, the SBP changes in the black bean- and green lentil-fed SHR were not significantly different from the WKY rats. Furthermore, no significant differences were observed in the DBP or MAP change from baseline to week eight among all groups.

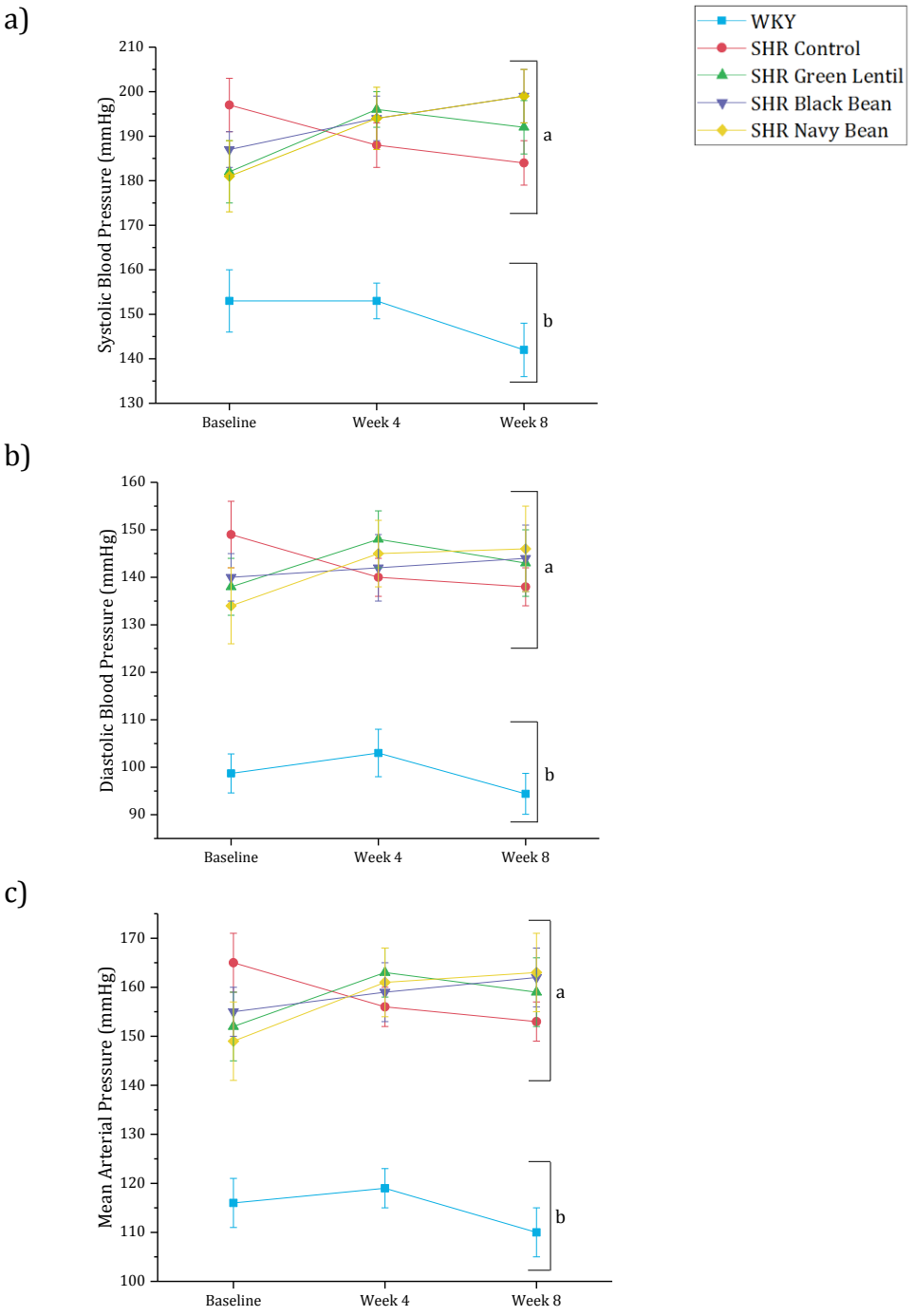


Figure 3. Baseline, Week 4, and Week 8 Systolic Blood Pressure (a), Diastolic Blood Pressure (b), and Mean Arterial Blood Pressure (c)

Blood pressure was determined by the tail-cuff method. Data are expressed as mean ± SE (n = 8–10/group). Different letters represent significant differences ($p<0.05$) for all time points. *Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

PWV

PWV is a measure of the speed at which a pulse wave travels through the arterial system and a higher velocity is indicative of arterial stiffness. In this study, PWV was measured at the femoral artery. Results for PWV are presented in Table 10.

There were no differences in baseline measurements between WKY rats and SHR except for two of the SHR groups having a higher mean flow velocity than the WKY rats. No significant differences between peak flow velocity, minimum flow velocity, or mean flow velocity were found at week eight among all groups. Furthermore, the changes from baseline to week eight measurements of PWV were not significantly different among all groups. Thus, no significant diet-related effects on PWV were observed.

Table 10. Pulse Wave Velocity Measurements in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Peak Flow Velocity (cm/second)					
Baseline	26.4 ± 1.8	28.3 ± 0.6	26.0 ± 1.6	27.6 ± 0.9	27.6 ± 1.1
Week 8	24.6 ± 1.3	27.9 ± 1.1	27.9 ± 1.0	27.7 ± 1.4	27.3 ± 1.9
Baseline vs. Week 8	-1.78 ± 2.69	-0.39 ± 1.20	1.90 ± 1.79	-0.70 ± 1.21	-0.28 ± 2.21
Minimum Flow Velocity (cm/second)					
Baseline	1.60 ± 0.78	1.90 ± 1.10	2.53 ± 0.33	2.98 ± 0.54	3.32 ± 0.35
Week 8	2.74 ± 0.32	3.25 ± 0.73	3.07 ± 1.00	3.85 ± 0.59	2.00 ± 0.76
Baseline vs. Week 8	1.15 ± 0.72	1.36 ± 1.23	0.53 ± 1.07	0.88 ± 0.74	-1.33 ± 0.93
Mean Flow Velocity (cm/second)					
Baseline	6.37 ± 0.59 b	7.44 ± 0.88 ab	7.81 ± 0.44 ab	8.67 ± 0.48 a	8.55 ± 0.44 a
Week 8	6.85 ± 0.45	8.20 ± 0.79	8.09 ± 0.74	8.69 ± 0.72	7.30 ± 0.56
Baseline vs. Week 8	0.49 ± 0.96	0.76 ± 1.31	0.29 ± 0.83	-0.22 ± 0.56	-1.25 ± 0.88

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained *in vivo* with an ECG-triggered 10 mHz Doppler probe (Indus Instruments). Results are expressed as mean ± SE (n = 7–9/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Vessel Morphology

Vascular remodelling occurs in hypertensive vessels, and thus measuring the structural features of the vessel wall can be used to establish whether a dietary intervention can modify vessel morphology. Measurements of remodelling were achieved in this study by morphometry of elastin-stained aortae cross-sections. Use of the Elastin Staining Kit (Sigma) allowed areas rich in elastin to be stained black and thus quantifying the area of blackness determined elastin content. Results are presented in Figures 4 and 5, and Table 11.

With respect to the morphological measurements, which were quantified using the software program ImageJ, no significant differences among groups were found for lumen diameter, lumen cross-sectional area (CSA), external diameter, adventitia thickness, or adventitia CSA. On the other hand, WKY rats had significantly smaller media thickness, media CSA, and media/lumen ratios than all SHR groups.

The total elastin content in the aorta was not significantly different among all groups, including WKY rats vs. SHR. On the other hand, the elastin content relative to media area was significantly lower in the control-, green lentil-, and navy bean-fed SHR compared to WKY rats. However, there was no difference in this outcome between the WKY rats and black bean-fed SHR.

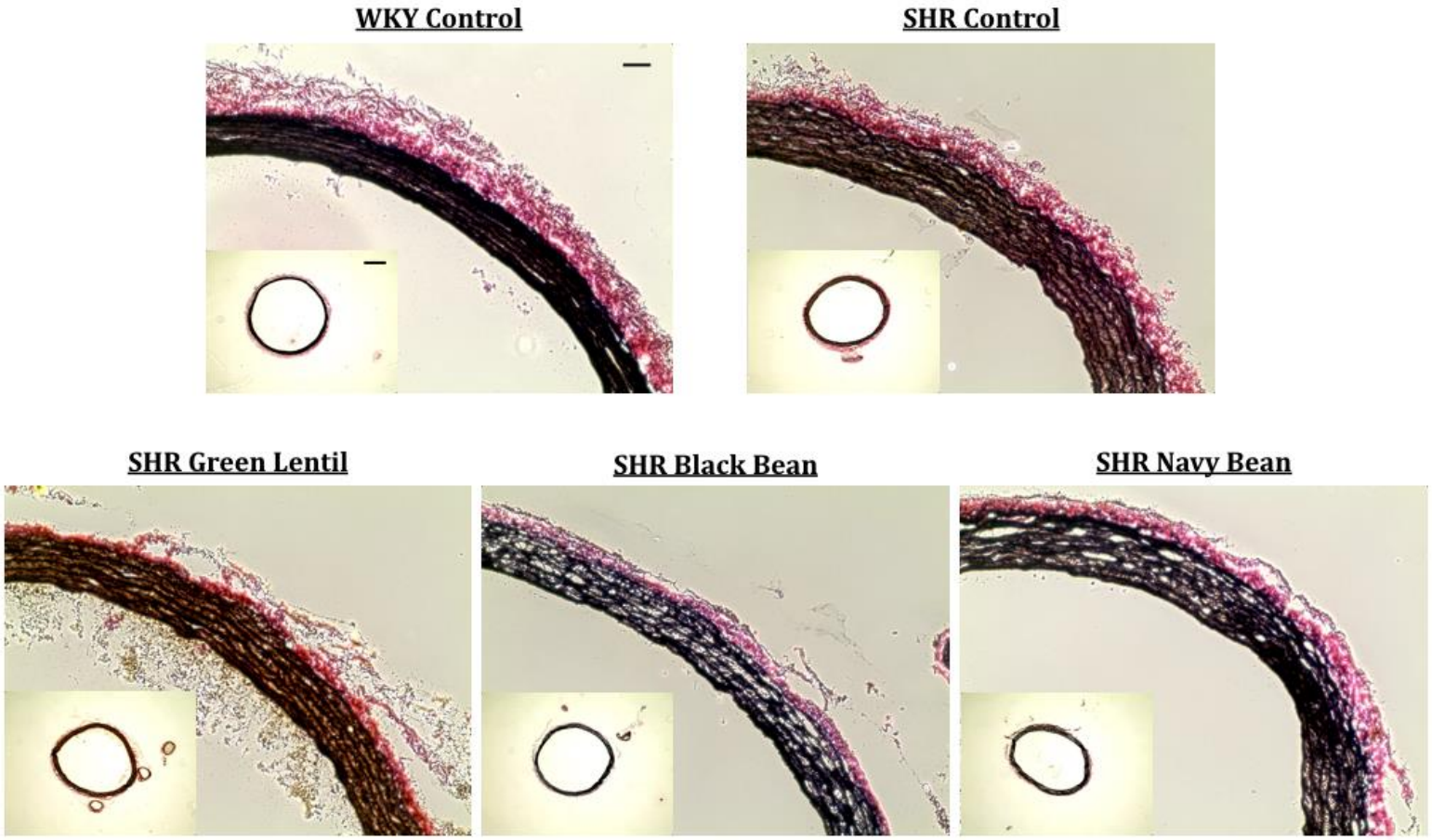


Figure 4. Representative Elastin Stained Aorta Cross-Sections
Bar = 0.1 mm; inset bar = 0.5 mm. *Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Table 11. Vessel Morphological Measurements in WKY Rats and SHR Aortae

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Lumen Diameter (μm)	1680 ± 33	1710 ± 20	1660 ± 44	1670 ± 22	1710 ± 20
Lumen CSA (μm^2)	1970 ± 90	2040 ± 72	2010 ± 110	1990 ± 54	1980 ± 86
Media Thickness (μm)	63.1 ± 1.9 b	93.4 ± 3.3 a	92.0 ± 2.9 a	84.7 ± 2.1 a	85.9 ± 5.0 a
Media CSA (μm^2)	326 ± 12 b	498 ± 24 a	504 ± 22 a	444 ± 19 a	478 ± 27 a
Media/Lumen Ratio (×100)	3.76 ± 0.14 b	5.48 ± 0.21 a	5.44 ± 0.21 a	5.08 ± 0.12 a	5.12 ± 0.32 a
External Diameter (μm)	1810 ± 34	1900 ± 19	1840 ± 22	1840 ± 23	1860 ± 38
Adventitia Thickness (μm)	81.4 ± 9.7	64.8 ± 9.3	52.4 ± 8.1	61.0 ± 4.9	62.5 ± 10.9
Adventitia CSA (μm^2)	358 ± 20	302 ± 49	317 ± 36	384 ± 28	337 ± 51

Abbreviations: CSA, cross-sectional area; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained from aorta sections stained using an Elastin Stain Kit (Sigma), and measured on ImageJ. Results are expressed as mean ± SE (n = 5–9/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

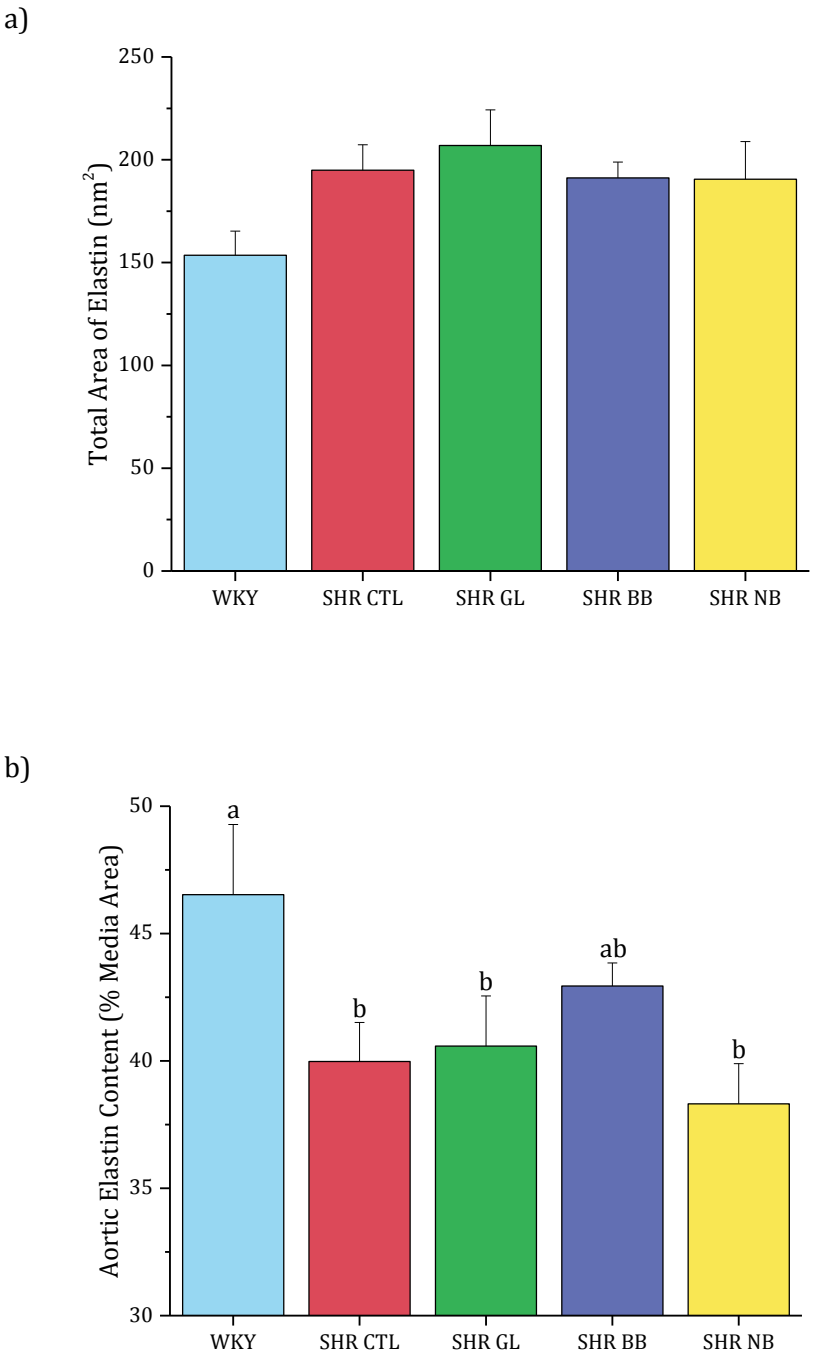


Figure 5. Elastin Content in Aorta as Total Area (a) and % of Media Area (b)
Elastin content was determined by quantifying the area of blackness on aorta cross-sections stained with an Elastin Stain Kit (Sigma). Data are expressed as mean ± SE (n = 6–9/group). Different letters represent significant differences ($p<0.05$). An absence of letters indicates no significant differences. *Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Protein Analysis

Of the five aortic proteins analyzed by Western blotting, significant differences in scleraxis and phosphorylated ERK1/2, as well as a trend for differences in phosphorylated p38 MAPK were observed among certain groups. No significant differences in levels of phosphorylated HSP27 were found, nor were there differences in total levels of ERK1/2, p38 MAPK, HSP27, or eNOS. Results for all tested antibodies are presented in Figures 6–10.

Scleraxis is associated with arterial stiffness and vascular ageing. Compared to all SHR groups, scleraxis was found in significantly greater abundance in WKY rats. Furthermore, black bean-fed SHR had significantly lower levels of scleraxis compared to control and navy bean-fed SHR.

ERK1/2 is involved in vascular remodelling through upregulation of collagen production and VSMC hypertrophy and hyperplasia. In our study, all SHR groups had significantly higher levels of phosphorylated ERK1/2 compared to WKY rats.

Similar to ERK1/2, p38 MAPK is implicated in the vascular remodelling process. There was a statistical trend ($p=0.0902$) for higher levels of phosphorylated p38 MAPK in control SHR compared to WKY rats.

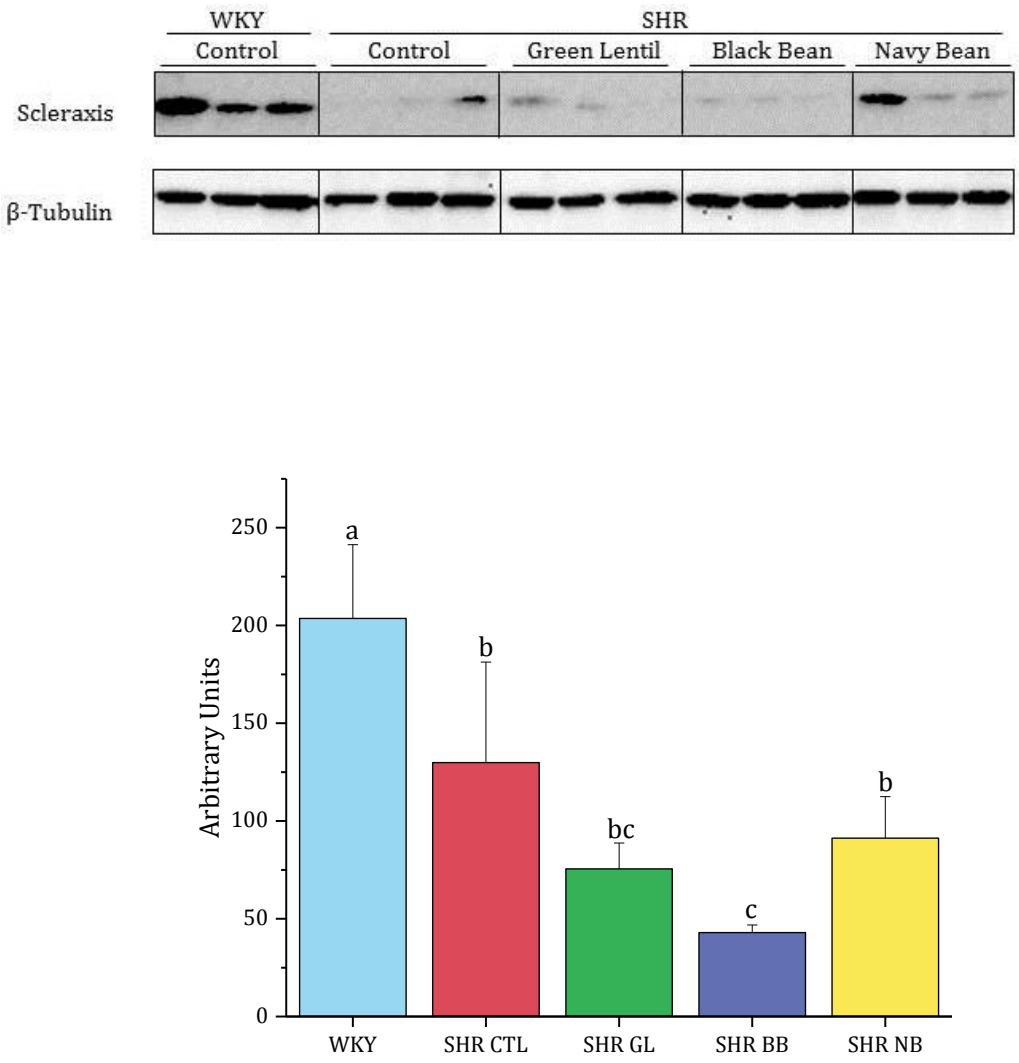


Figure 6. Levels of Scleraxis in Aorta

Aorta lysates were subjected to Western blotting analysis using specific antibodies against Scleraxis and β-tubulin (loading control). Scleraxis and β-tubulin antibodies were applied at a concentration of 1:1000 and incubated overnight (scleraxis) or for 1 hour (β-tubulin). Rabbit secondary was applied in a 1:10000 concentration and incubated for 1 hour. Relative protein levels for each sample were calculated as the ratio of raw signal strength for band intensity of scleraxis/β-tubulin as the loading control. Data are expressed as mean ± SE (n = 7–10/group). Bars with different letters are significantly different ($p<0.05$). *Abbreviations: BB, black bean; CTL, control; GL, green lentil; NB, navy bean; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

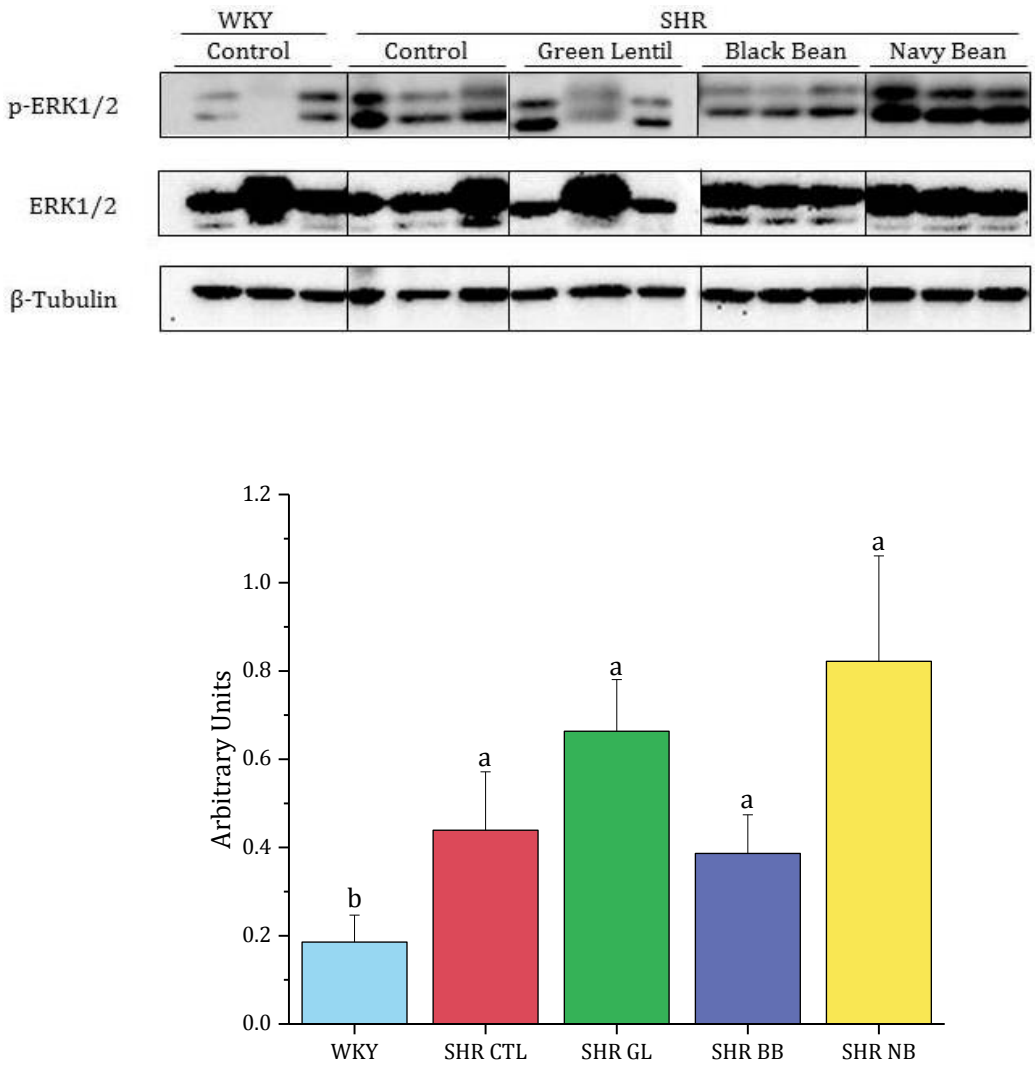


Figure 7. Levels of p-ERK1/2 in Aorta

Aorta lysates were subjected to Western blotting analysis using specific antibodies against p-ERK1/2 and ERK1/2. p-ERK1/2 and ERK1/2 antibodies were applied at a concentration of 1:1000 and incubated overnight. Rabbit secondary was applied in a 1:10000 concentration and incubated for 1 hour. Relative protein levels for each sample were calculated as the ratio of raw signal strength for band intensity of p-ERK1/2/ERK1/2. Data are expressed as mean ± SE (n = 8–10/group). Bars with different letters are significantly different ($p<0.05$). *Abbreviations: BB, black bean; CTL, control; ERK, extracellular signal-regulated kinase; LEN, green lentil; NB, navy bean; p, phosphorylated; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

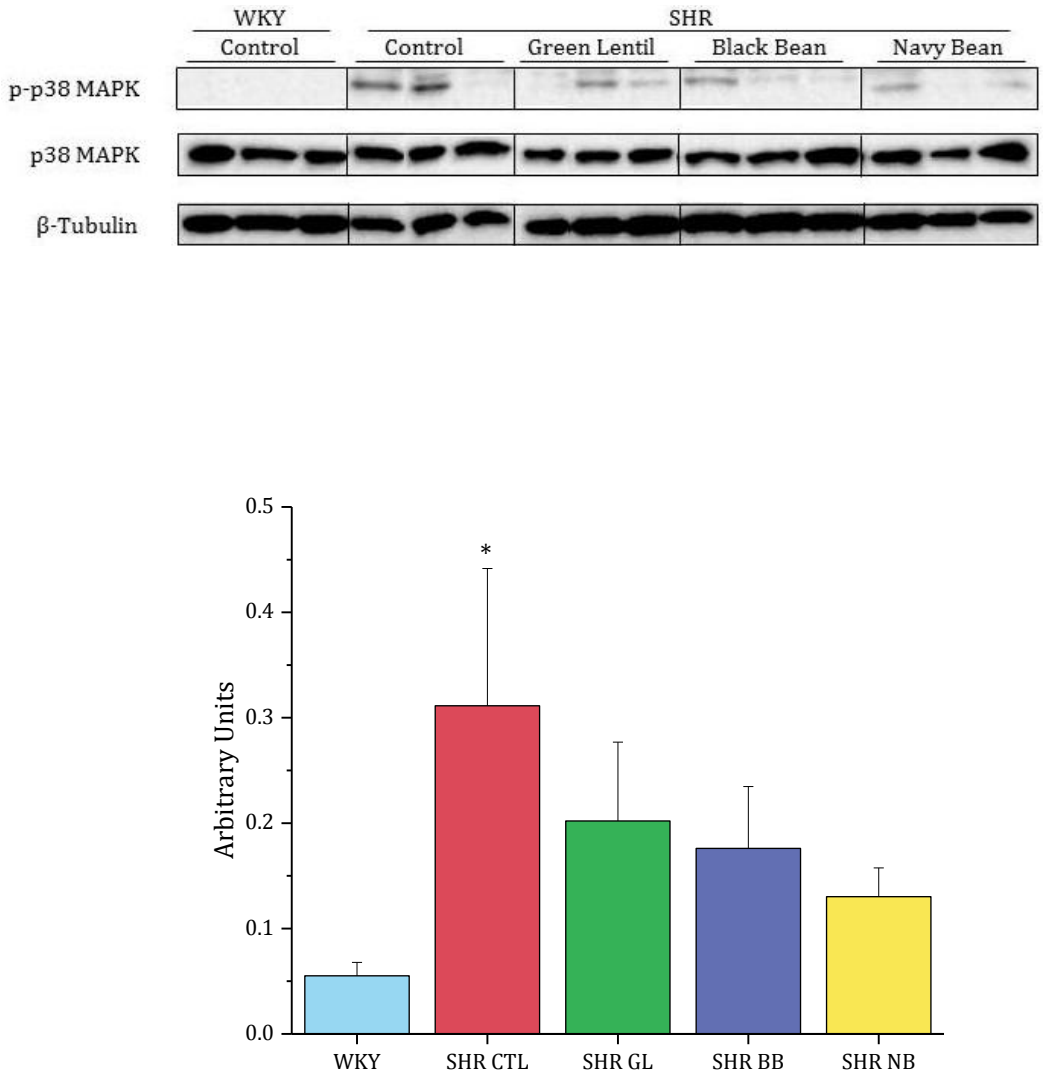


Figure 8. Levels of p-p38 MAPK in Aorta

Aorta lysates were subjected to Western blotting analysis using specific antibodies against p-p38 MAPK and p38 MAPK. p-p38 MAPK and p38 MAPK antibodies were applied at a concentration of 1:1000 and incubated overnight. Rabbit secondary was applied in a 1:10000 concentration and incubated for 1 hour. Relative protein levels for each sample were calculated as the ratio of raw signal strength for band intensity of p-p38 MAPK/p38 MAPK. Data are expressed as mean ± SE (n = 3–4/group). The asterisk indicates a statistical trend (p=0.0902) for a difference from the WKY rats. *Abbreviations: BB, black bean; CTL, control; LEN, green lentil; p38 MAPK, p38 mitogen activated protein kinase; NB, navy bean; p, phosphorylated; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

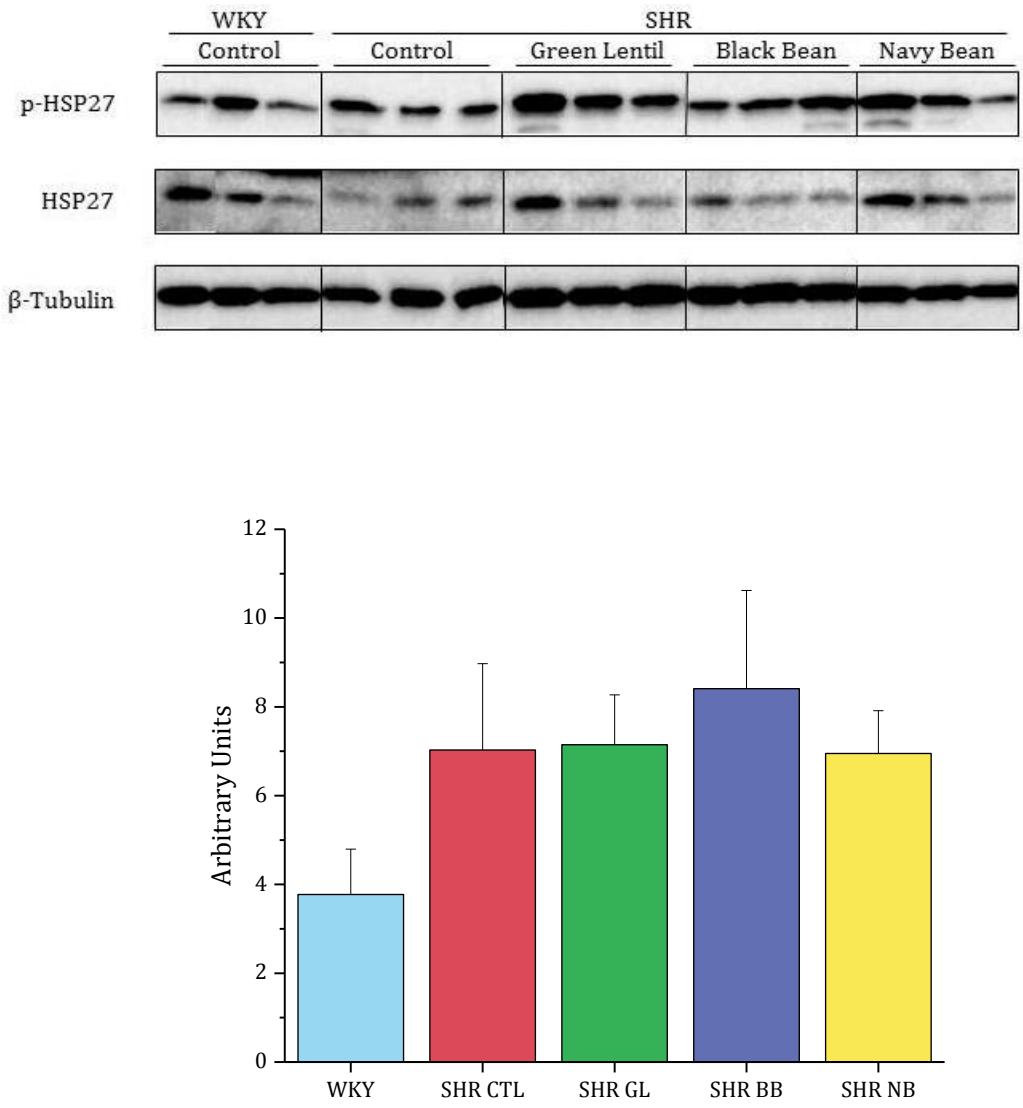


Figure 9. Levels of p-HSP27 in Aorta

Aorta lysates were subjected to Western blotting analysis using specific antibodies against p-HSP27 and HSP27. p-HSP27 and HSP27 antibodies were applied at a concentration of 1:1000 and incubated overnight. Rabbit (p-HSP27) or goat (HSP27) secondary was applied in a 1:10000 concentration and incubated for 1 hour. Relative protein levels for each sample were calculated as the ratio of raw signal strength for band intensity of p-HSP27/HSP27. Data are expressed as mean ± SE (n = 4/group). The absence of letters indicates that the ANOVA model was not statistically significant ($p>0.05$). *Abbreviations: BB, black bean; CTL, control; HSP27, heat shock protein 27; LEN, green lentil; NB, navy bean; p, phosphorylated; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

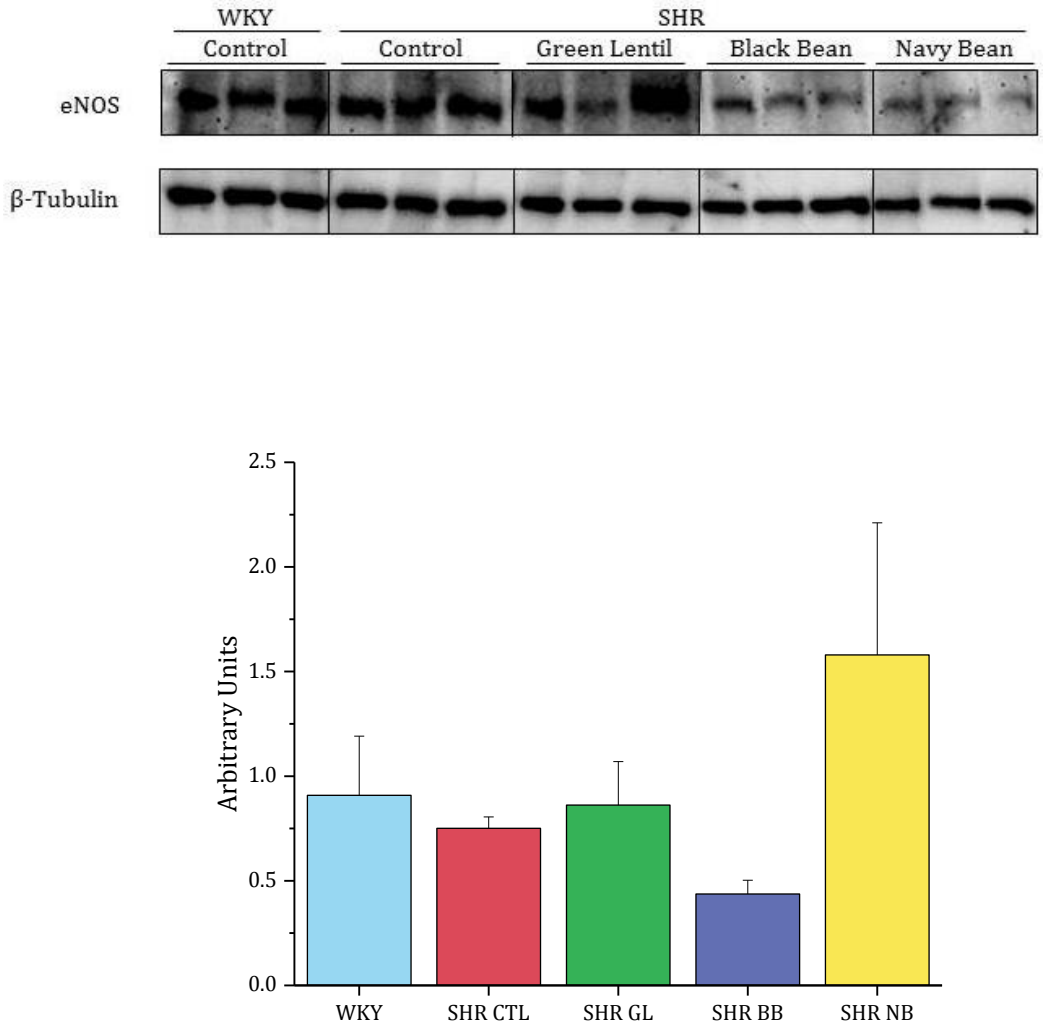


Figure 10. Levels of eNOS in Aorta

Aorta lysates were subjected to Western blotting analysis using specific antibodies against eNOS and β-tubulin (loading control). eNOS and β-tubulin antibodies were applied at a concentration of 1:1000 and incubated overnight (eNOS) or for 1 hour (β-tubulin; loading control). Rabbit secondary was applied in a 1:10000 concentration and incubated for 1 hour. Relative protein levels for each sample were calculated as the ratio of raw signal strength for band intensity of eNOS/β-tubulin. Data are expressed as mean ± SE (n = 4/group). The absence of letters indicates that the ANOVA model was not statistically significant ($p>0.05$). *Abbreviations: BB, black bean; CTL, control; eNOS, endothelial nitric oxide synthase; LEN, green lentil; NB, navy bean; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Discussion

The primary objectives of this study were to determine the effects of an eight-week feeding intervention with black or navy beans on blood pressure, PWV, vascular remodelling, and cell-signalling pathways associated with vascular structure and function in SHR. The main findings of this study were as follows.

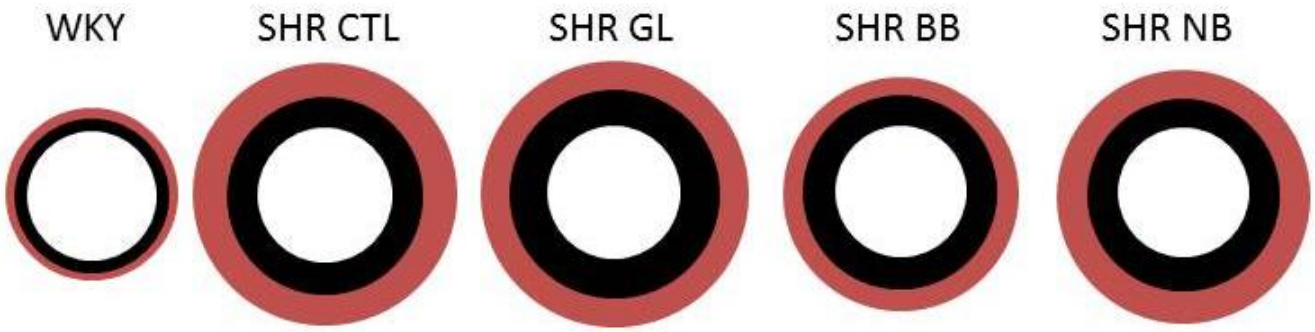
At the end of the eight-week feeding trial, none of the pulse-containing diets attenuated the rise in blood pressure that occurs in the ageing SHR. The SHR fed black beans, navy beans, or green lentils did not have significantly different blood pressure or PWV compared to control SHR. As expected, the blood pressures of all SHR groups were significantly higher than WKY rats indicating the presence of hypertension. However, vascular stiffness as determined by PWV was not different among the SHR and WKY rats, which was unexpected.

With regards to vascular structure of the aortae, significant differences among all SHR and WKY rats were observed, where SHR had thicker media, greater media CSA, and larger media/lumen ratios compared to WKY rats. Thus, all SHR groups experienced vascular remodelling relative to WKY rats. However, when examining the structure of the aorta among the SHR groups, it is evident that both the black and navy beans mitigated vascular remodelling to some degree even though differences in vascular structure did not reach statistical significance compared to SHR control (Figure 11). For example, the media/lumen ratios of the control- and green lentil- fed SHR were, respectively, 146% and 145% greater than that of the WKY rats. On the other hand, the media/lumen ratios in the black bean-

and navy bean-fed SHR relative to WKY rats were increased by only 135% and 136%, respectively. When taking into account other structural changes relative to WKY rats, such as media thickness and media CSA, the overall improvement in blood vessel structure was more apparent in the black bean-fed SHR than in the navy bean-fed SHR. Interestingly, black beans were able to mitigate compositional changes to the medial layer of the aorta in SHR, given the absence of significant differences in elastin content relative to media area compared to WKY rats. Evidence from Western blotting suggests that the vascular remodelling in the SHR groups may have been due to ERK1/2 and p38 MAPK activation. Scleraxis may have also played a role in the remodelling process in the SHR. However, upregulation of this protein in the WKY rats relative to SHR was surprising and seemingly unusual, which deters conclusions regarding the role of this protein in vascular remodelling in the SHR.

Feed intake did not differ among the SHR groups suggesting that the dose of ingested pulses was not different between the treatment groups and would not explain the unique benefit of the black beans to attenuate compositional changes to the aorta. In addition, no differences were seen among SHR groups in terms of body weight or composition or serum lipids, although these were significantly different from WKY rats. Some differences for serum indicators of liver function were evident among the animal groups where pulse-fed SHR may have experienced a decline in liver function.

The following sections will discuss these aforementioned results in more detail and attempt to put them into context with the study hypothesis and the relevant literature.



	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Lumen Diameter	100%	102%	101%	99%	100%
Lumen CSA	100%	104%	103%	102%	101%
Media Thickness	100%	153%	146%	134%	136%
Media CSA	100%	148%	155%	136%	147%
Media/Lumen Ratio	100%	146%	145%	135%	136%
Elastin Content	100%	127%	135%	125%	124%
Elastin/Media Ratio	100%	86%	87%	92%	82%

Figure 11. Aorta Cross-Sections Drawn to Scale and Vessel Morphometry Relative to WKY Rats
For SHR groups, aorta cross-sections (diagrams) were drawn relative to WKY rats. The morphological parameters were calculated as a percentage relative to WKY rats (table). In the diagrams, the media is represented by the black and red areas where the black specifically represents elastin and red represents other components of the media. *Abbreviations: BB, black bean; CSA, cross-sectional area; CTL, control; GL, green lentil; NB, navy bean; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.*

Feed Intake, Body Weight, and Body Composition

While no diet-related effects of the pulses on feed intake, body weight, and body composition were observed among the SHR groups, the significant differences in these measured outcomes between the SHR and WKY rats gives insight into the unique metabolic characteristics of these two strains.

The lower body weight but higher feed intake of the SHR compared to WKY rats indicates that SHR may have a higher basal metabolic rate. Given that total lean mass was not different between the SHR and WKY rat groups but that WKY rats had greater body weight and fat mass, it is evident that SHR utilize their fat stores for energy and thus accumulate less fat. This phenomenon is not typical in humans who experience hypertension, and thus highlights a difference in the disease progression.

In addition to being a model of essential hypertension, the SHR are also a model for Attention Deficient Hyperactivity Disorder (ADHD) and thus experience hyperactivity compared to WKY rats (Sagvolden, 2000). This genetic predisposition to increased energy expenditure caused by their hyperactivity would explain the differences in body composition and feed intake seen in our study. However, energy expenditure and basal metabolic rate were not measured in our study. Nonetheless, the absence of significant differences in feed intake among the SHR groups suggests that the dose of pulses consumed were similar among the treatment groups. Thus, any differences observed with other measured outcomes are unlikely to have been caused by variances in intake of the pulse constituents.

Serum and Urine Biochemistry

Serum lipids were measured to evaluate whether pulse consumption could alter lipid metabolism, since it is purported that lipid levels can potentially alter endothelial function and thus blood pressure. Our study showed that serum lipids, namely triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol, were significantly higher in WKY rats compared to SHR. This difference in serum lipids between these rat strains has been previously reported (Hanson et al., 2016b; Hanson et al., 2014). Conversely, it has also been reported that pulses reduce serum cholesterol levels in both animal and human subjects, but the exact mechanism is unknown (Arnoldi et al., 2015). In our study, the black beans lowered total cholesterol compared to control SHR, although no differences in triglycerides, HDL cholesterol, or LDL cholesterol were evident among SHR groups. The absence of significant HDL and LDL cholesterol lowering by pulses was unexpected given that this has been observed previously (Hanson et al., 2016b; Hanson et al., 2014). Although, in either case, rodents are not good models of human cholesterol metabolism and so these findings do not appropriately represent the lipid modulating behaviour of pulse consumption in humans.

Kidney function is known to decline when hypertension is present, and can be measured by the presence of creatinine and urea in serum and urine (Manttari et al., 1995). Creatinine and urea are metabolic waste products that are normally excreted by the kidneys, and thus higher levels in serum and lower levels in urine indicate a failure of the kidneys to effectively remove them from the circulation.

Assessment of serum and urine kidney markers indicated that there were no differences among WKY rats or SHR groups at baseline or week eight. The exception was baseline serum urea concentrations, which were higher in SHR compared to WKY rats (with the exception of the green lentil group), although by week eight, these differences in serum urea were abolished.

Liver function was determined by serum ALT and AST. At baseline, serum ALT and AST were significantly higher in SHR groups compared to WKY rats with no differences among SHR groups. By week eight, the pulse-fed SHR had significantly higher serum ALT and AST compared to control SHR and WKY rats indicating that liver function may have been impaired in the pulse-fed SHR. The only exception was for green lentil-fed SHR that did not have significantly different serum AST from control SHR at week eight. These differences in liver function are likely independent of blood pressure given that previous research has reported no correlation between serum ALT or AST and incident hypertension (Gupta et al., 2013). Since the liver is considered the processing site for circulating toxins, it is possible that the SHR fed pulses experienced liver injury when processing the pulse-derived metabolites. On the other hand, a mesquite bean extract was shown to inhibit acetaminophen-induced liver toxicity in Wistar rats suggesting that legumes do not reduce liver function and could be protective (Asadollahi et al., 2014). Furthermore, liver injury caused by the consumption of cooked pulses has not been previously reported to this author's knowledge. However, future studies with pulses should continue to monitor these liver enzymes.

Blood Pressure, PWV, and Vessel Morphology

In this study, blood pressure rises were not attenuated by the green lentil, black bean, or navy bean diets in the SHR. This conclusion is made with the observation that the control SHR blood pressures were not significantly different from the pulse-fed SHR, and all SHR had significantly higher blood pressures than the normotensive WKY rats. On the other hand, explanations for the lack of effect can be provided focusing on the animal models, the blood pressure detection method, and the pulses used in our study.

First, the usefulness of the control-fed SHR and WKY rats to act as hypertensive and normotensive control groups, respectively, in our study can be debated. Unexpectedly, the control SHR group experienced a decline in blood pressure over the eight-week intervention period (observed in 8/9 animals in this group). To this author's knowledge, no previously published study utilizing the SHR has shown the blood pressure of these animals to decrease over time unless administered an antihypertensive treatment. This anomaly begs the question, are the control SHR used in this study a suitable hypertensive control?

The design for this study was based on reports from two previous studies showing that lentil-based diets attenuated rises in blood pressure and vascular remodelling in SHR (Hanson et al., 2016b; Hanson et al., 2014). For comparison, the blood pressure measurements recorded at baseline, week four, and week eight for the control SHR groups of these two dietary intervention studies are presented in Table 12 along with the results from our study. It is evident from Table 12 that the

control SHR used in our study had higher baseline blood pressures and experienced a decline to levels much lower than what were observed at week four or week eight in the previous studies' control SHR whereas SBP in the previous studies increased to >200 mmHg at weeks four and eight.

Table 12. Comparison of Blood Pressure Measurements in Three Studies by the Tail-Cuff Method in SHR and WKY Rats Fed the AIN-93G Diet

	Baseline			Week 4			Week 8		
	SBP	DBP	MAP	SBP	DBP	MAP	SBP	DBP	MAP
SHR[1]									
Hanson *et al.* (2014)	175	124	140	201	156	171	NA	NA	NA
Hanson *et al.* (2016)	183	138	153	211	168	182	207	156	174
Loader (2017)	197	149	165	188	140	156	184	138	153
WKY[1]									
Hanson *et al.* (2014)	133	83.6	102	132	81.5	98.3	NA	NA	NA
Hanson *et al.* (2016)	129	78.9	95.3	148	95.6	113	147	90.1	109
Loader (2017)	153	98.7	116	153	103	119	142	94.4	110

Abbreviations: AIN-93G, American Institute of Nutrition-93 Growth diet; DBP, diastolic blood pressure; MAP, mean arterial pressure; NA, not applicable; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat, WKY, Wistar Kyoto.

[1]WKY rats and SHR were sixteen weeks old at baseline, twenty-weeks at week four, and twenty-four-weeks at week eight. Results are expressed as mean (n = 7–10/group).

Likewise, the WKY rats used in our study had higher baseline blood pressure measurements compared to previously tested WKY rats (Table 12). However, at week four and week eight, the WKY rats in our study had blood pressure measurements that were similar to the previous two studies. On the other hand, it has been previously reported that SBP of 130–140 mmHg is considered to be higher than normal for WKY rats (Okamoto et al., 1963). Our WKY rats had SBP measurements of 142–153 mmHg. Thus, the WKY rats used in our study were not normotensive. In agreement, WKY rats from Charles River have been previously reported to exhibit higher blood pressures compared to WKY rats from other breeders (Kurtz et al., 1987). We presented Charles River with our data to inquire on this disparity and discovered that Charles River does not monitor blood pressure of their WKY rat strain to confirm that these animals have remained normotensive. Considering that the SHR spontaneously developed from the WKY rats, it is not unreasonable to suggest that this phenomenon could occur again. Thus, the suitability of the WKY rat as an appropriate normotensive control for the SHR is debatable. Furthermore, without appropriate hypertensive and normotensive controls, it is difficult to make conclusions regarding the effects of the pulse intervention on blood pressure and hypertension status, in part because the closer blood pressures between strains would reduce the sensitivity of our measurements and thus impair our ability to detect any changes resulting for the interventions.

The tail-cuff method for measuring blood pressure can also be critiqued for its precision and accuracy. In a previous study, researchers compared blood pressure measured by the indirect tail-cuff method and the direct strain gauge

method and found differences in the readings (Resurreccion et al., 1978). Intriguingly, SBP recorded by the tail-cuff method was higher than for the strain gauge method and led to erroneous significant differences (Resurreccion et al., 1978). The strain gauge method for detecting blood pressure is considered to be the gold standard as it is an invasive method using a catheter or radio-telemetric implant inserted into the blood vessel to directly measure the pressure in that vessel. It has been suggested that any blood pressure data collected by the indirect tail-cuff method should be verified by direct methods (Resurreccion et al., 1978).

Reasons for inaccuracies with the tail-cuff method relate to the cuff width and the position of the cuff on the tail of the rats, the temperature of the rats, and the use of anesthetized vs. unanesthetized rats (Resurreccion et al., 1978). While the use of a heating platform to warm the rats encourages blood flow to the tail enabling a blood pressure reading, some researchers have expressed concern that externally applied heat could deceptively elevate blood pressure in these animals (Bunag et al., 1982). In addition to the heating platform, the use of restraints may contribute to false high blood pressure readings. As previously mentioned, the SHR are hyperactive and they exert themselves much more than WKY rats. In our study, it was observed that the SHR experienced great amounts of stress when they were restrained. Indeed, it has been discussed that restraint and thermal stress are activators of the cardiovascular system causing increases in blood pressure(Kurtz et al., 2005). Using anesthesia can reduce the stress experienced by the rat, but the anesthesia itself may impact the cardiovascular system resulting in inaccurate blood pressure recordings (Kato et al., 2016). Acclimating the rats to become familiar with

the tail cuff method procedure is recommended to reduce stress, although it is often unsuccessful (Kurtz et al., 2005).

In addition, the time of day chosen to conduct study measurements could have contributed to rises in blood pressure. In our study, blood pressure and the other study outcomes were recorded during human daytime, which is the middle of the night for rats as they are nocturnal in nature. Studies have shown that reduced sleep quality and quantity are associated with incident hypertension (Calhoun et al., 2010a). Thus, interrupting the sleep of the animals used in our study could have contributed to an inaccurate rise in their blood pressure. Furthermore, the tail-cuff method records blood pressure at only one time of the day. Using an alternative means to collect ambulatory blood pressure measurements would be more reflective of true blood pressure.

Aside from these aforementioned criticisms, some positive comments on the tail-cuff method can be presented as well. For example, the same two researchers performed the blood pressure measurements for the duration of our study, the room was dimly lit, and cloths were placed on the rats if they appeared anxious. These aforementioned strategies are recommended to help reduce the stress of the animal. In addition, blood pressure was measured at the same time on the same day of the week for each animal and time point. Lastly, one study comparing indirect and direct methods of blood pressure measurement found good correlation between the methods, and no significant differences in blood pressure detected among a group of SHR or among a group of WKY rats (Ibrahim et al., 2006).

The final explanation for the unsuccessful treatment of high blood pressure by the pulses in our study is related to the particular pulses that were utilized. In previous studies, a mixed lentil diet attenuated the rise in blood pressure in SHR (Hanson et al., 2016b; Hanson et al., 2014). Furthermore, Hanson *et al.* (2016) showed that a green lentil diet reduced arterial remodelling and stiffness in mesenteric arteries of SHR. Based on these aforementioned studies, the green lentil-fed SHR in our study were meant to act as a positive control. However, this group did not experience the expected vascular improvements as previously observed. This may have been due to differences in the year that the lentils were grown, as our lentils were from a more recent crop year. Crops grown in different years have been shown to vary in their phenolic content, due to differences in sunlight exposure, temperature, soil nutrition, drought, and atmospheric carbon dioxide levels (Treutter, 2010).

On the other hand, it is possible that the dose of ingested pulses in the previous studies were higher than in ours. These aforementioned studies did not report feed intake and their animals were fed *ad libitum*. Indeed, some end of study differences in body weight are evident, where the SHR in our study appear to weigh slightly less (range of mean body weight for all SHR 370–379 g) than in the Hanson *et al.* (2016) study (range of mean body weight for all SHR: 384–399 g).

However, it should be pointed out that while the previously examined mixed lentil diet had blood pressure lowering effects, the green lentils alone were not capable of depressing blood pressure to levels lower than control SHR (Hanson et

al., 2016). Thus, the absence of a blood pressure regulating effect by the green lentils in our study is in agreement with Hanson *et al.* (2016).

With regards to the beans, it was hypothesized that the navy beans would not attenuate blood pressure given their lower phenolic contents compared to darker coloured beans. Thus, this hypothesis was shown to be true in our study. On the other hand, Hanson *et al.* (2014) showed that SHR fed a mixed bean diet had 13% lower SBP than control-fed SHR after a four-week intervention. While these results were not statistically significant, they indicated that there could be potential for a bean intervention to provide blood pressure-regulating effects if different bean types and longer intervention periods were examined. Although the black bean-fed SHR did not experience mitigated blood pressure rises, other vascular benefits in these animals were observed and are discussed below.

Vascular remodelling occurred in each SHR group relative to WKY rats, and while no statistically significant differences in vascular structure among SHR groups were present, it is evident that remodelling of the aorta was reduced in the black bean- and navy bean-fed SHR when compared to the control SHR (Figure 11). However, it is also apparent that the black beans were better able to attenuate vascular remodelling than the navy beans. This is supported by the compositional results of the aorta, where it was found that the black bean-fed SHR maintained the elastin content relative to media area to levels statistically similar to WKY rats.

Elastin is a protein found in the medial layer of blood vessel wall. The majority of the aortic vessel wall thickness is attributed to the media, which is

composed of VSMC and ECM (primarily collagen and elastin). When vascular remodelling takes place, collagen production is upregulated and/or VSMC undergo hypertrophy and/or hyperplasia leading to a thickening of the medial layer (Lee et al., 1995; Xu et al., 2014). An increase in these other materials in the media contributes to the reduction in elastin content relative to media area. This can occur with or without a change to the actual elastin content in the vessel.

In our study, the total aortic elastin levels were not significantly different among all groups. However, given that the media were significantly larger in SHR vs. WKY rats, alterations of other media component(s) would thus explain the difference in size. This is supported by the result that the levels of elastin relative to media area were significantly lower in the control-, green lentil-, and navy bean- fed SHR compared to WKY rats. The similar elastin contents but different ratios of elastin to vessel wall area confirm that these SHR groups had higher levels of other media components, which could have been collagen and/or VSMC.

Given that the black bean-fed SHR did not have significantly different elastin levels relative to media area compared to WKY rats, it can be suggested that the black beans mitigated the production/growth of these other materials and thus attenuated vascular remodelling in the SHR. This would be the first study, to this author's knowledge, that has reported an improvement in SHR blood vessel structure by black beans.

On the other hand, PWV, a measure of arterial stiffness that is dependent on elastin and collagen content, was not improved by the black bean diet. However, it

should be acknowledged that PWV was not different from baseline to week eight in all groups indicating that arterial compliance did not significantly decrease over time. In addition, PWV for all SHR were not significantly different from WKY rats suggesting that either arterial stiffness was not present in the SHR or that the WKY rats had arterial stiffness. The former is more likely to be true given that PWV measurements for SHR were lower in our study compared to Hanson *et al.* (2016) where PWV was higher. Furthermore, this could indicate that the altered media thickness in the SHR may not be due to collagen accumulation, a stiff protein that would increase PWV, but perhaps due to increase VSMC size or number. Indeed, Hanson *et al.* (2016) did not find aortic collagen levels to be different in WKY rats compared to control- or green lentil-fed SHR whereas VSMC number was significantly increased in both SHR groups relative to WKY rats.

On the other hand, it would be expected that the improvements in vascular remodelling in the black bean-fed SHR would parallel reductions in blood pressure through indirect effects, which was not observed in our study. However, speculation for this dichotomy can be provided. It is plausible that the pathway(s) causing the rise in blood pressure may be different from the pathway(s) leading to vascular remodelling in the SHR. Indeed, researchers have speculated that vascular remodelling and blood pressure rises are separate events (Christensen et al., 2001; Mitchell, 2014). Furthermore, in the study by Hanson *et al.* (2016), the green lentils attenuated vascular remodelling without improving blood pressure. Thus, the black beans may have only been capable of interfering with the vascular remodelling pathway. Cellular pathways leading to vascular remodelling in hypertension have

been associated with the activation of MAPKs by ROS, AngII, and ET-1 (Schiffrin, 2012; Touyz, 2004; Touyz et al., 2004). Therefore, the black beans may have interfered with such vascular remodelling pathways. Mechanisms for this intervention are proposed in the following section.

Protein Analysis

Scleraxis

Scleraxis is a protein that has been previously associated with vascular ageing and arterial stiffness. In mice deficient in the anti-aging gene, Klotho, aortic scleraxis levels were found to be upregulated (Chen et al., 2015). The increase in scleraxis was mediated by aldosterone, whose production and circulation increased as a consequence of the Klotho deficiency (Chen et al., 2015). The upregulation of aortic scleraxis led to vascular remodelling as observed by altered ECM protein levels with increased collagen and decreased elastin (Chen et al., 2015). Interestingly, there was no rise in blood pressure in these animals (Chen et al., 2015). Administration of eplerenone (an aldosterone receptor blocker) caused a reduction in scleraxis and prevented vascular remodelling (Chen et al., 2015). This implies that high circulating levels of aldosterone can initiate vascular remodelling, which can occur independently of or prior to a blood pressure rise.

Under normal circumstances, aldosterone is secreted in response to AngII to assist in RAS-mediated blood pressure rises. Chronic activation of the RAS, as observed by high circulating levels of AngII, has been implicated in the progression

of hypertension in the SHR (Bolterman et al., 2005). In addition, circulating aldosterone levels are typically higher in SHR than WKY rats (Wexler et al., 1982). Given this knowledge, one would hypothesize that scleraxis would be upregulated in the SHR compared to WKY rats. However, our study showed scleraxis levels to be upregulated in WKY rats relative to SHR. While unexpected, plausible explanations for this result can be provided.

The increase in scleraxis protein levels in the WKY rats compared to SHR may have been due to the differences in body fat mass of these two rat strains. Given the role of aldosterone in the upregulation of scleraxis, it is interesting to note that circulating aldosterone has been positively correlated with obesity (Calhoun et al., 2010b). In our study, the WKY rats had greater fat mass and body weight compared to SHR. A greater fat mass, which is associated with an increase in adipocyte size and/or number, may have caused an increase in aldosterone levels in the WKY rats. As reviewed by Calhoun & Sharma (2010), adipocytes have been shown to produce Ang II, aldosterone, and adipokines that stimulate aldosterone release. However, whether or not plasma aldosterone was higher in the WKY rats in our study cannot be confirmed, as aldosterone was not measured. In addition, it would be expected that the SHR would also have high levels of circulating aldosterone, yet these animals did not experience increases in scleraxis levels. Thus, another mechanism may more likely explain the upregulation in scleraxis in the WKY rats.

Leptin, an adipokine produced from adipocytes, is found in high concentrations in obese compared to lean individuals and has been associated with

vascular remodelling independent of blood pressure, smoking, and insulin sensitivity (Ciccone et al., 2001). It was shown that leptin acts through the PI3K-Akt pathway to increase gene expression and proteins levels of Transforming Growth Factor-β (TGF-β) that acts in VSMCs to upregulate ECM protein production (Martinez-Martinez et al., 2014). Interestingly, TGF-β activation of mothers against decapentaplegic homolog 3 (SMAD3) has been shown to induce scleraxis expression (Bagchi et al., 2012; Espira et al., 2009). Furthermore, WKY rats have been shown in genetic analysis to be predisposed to obesity and also express the genes for leptin signalling in obesity (Zhang-James et al., 2013). Thus, the possibly higher leptin levels in the WKY rats may have contributed to the greater scleraxis levels observed in our study.

Hence, the increased adiposity in the WKY rats may explain the upregulation of scleraxis, mediated either by aldosterone or leptin. Future analysis of WKY rats should include measurements of circulating aldosterone and leptin, as well as assessment of proteins involved in scleraxis activity, namely TGF-β and SMAD3.

Alternatively, the differences in scleraxis levels among SHR groups parallels nicely with the results for vascular remodelling. The Western blot results showed that the black bean group had attenuated rises in scleraxis relative to control SHR. Given the role of scleraxis in vascular remodelling, lower levels of this protein in the black bean group may have partially contributed to the better vascular composition at the end of the study.

Mitogen-Activated Protein Kinases (MAPKs)

The protein, p38 MAPK can be activated by ROS, ET-1, and AngII in VSMC and can contribute to vascular remodelling in hypertension. Although not significant, there was a trend for increased levels of phosphorylated p38 MAPK in control SHR compared to WKY Conversely, no differences for phosphorylated p38 MAPK in pulse-fed SHR relative to WKY rats were observed. Given that p38 MAPK activation and vascular remodelling are connected events, the activation of this protein may have been involved in vascular remodelling in the control SHR group in this study. On the other hand, this interpretation does not help to explain the cause of vascular remodelling in the pulse-fed SHR.

Similar to p38 MAPK, the activation of ERK1/2 in VSMC by ET-1 and AngII, has been thoroughly demonstrated, and shown to initiate VSMC hypertrophy, hyperplasia, and collagen production (Bouallegue et al., 2013; Chen et al., 2009; Kubo et al., 1998; Kyaw et al., 2001; Lambers et al., 2013; Luo et al., 2006; Romero et al., 2010; Yogi et al., 2007). In our study, there were significantly higher levels of phosphorylated ERK1/2 in all SHR groups compared to WKY rats, especially in the green lentil and navy bean groups. Conversely, the activation of ERK1/2 in the black bean group was not as great suggesting that a compound in black beans may have partially attenuated activation of ERK1/2 in addition to p38 MAPK.

Ginsenosides, the bioactive compounds in ginseng, were recently shown to mitigate hypertension and vascular remodelling while also inhibiting the activation of p38 MAPK, ERK1/2, and JNK in rats with pulmonary hypertension (Qin et al.,

2016). The findings of this aforementioned study confirm that bioactive compounds derived from foods can intervene with multiple MAPK pathways. Furthermore, Hanson *et al.* (2016) also showed lentils inhibited aortic p38 MAPK in SHR, which was postulated to be a function of their antioxidant capacity.

It should be noted that Western blotting results for p38 MAPK were only available for n = 3–4/group, therefore it is possible that the ability to detect significant differences in our study was limited especially given the high variability among samples. On the other hand, the absence of a similar pattern for the activation of HSP 27, a protein downstream to p38 MAPK, suggests a disconnect in these results, although, a larger sample size may have prevented this problem. Furthermore, p38 MAPK activates many other downstream proteins, which may have better represented p38 MAPK activity in our study.

In the end, the mechanism by which the black beans prevented changes to the composition of the vessel remains to be elucidated. However, the overall lower levels of scleraxis, phosphorylated p38 MAPK, and phosphorylated ERK1/2 compared to other SHR groups suggests that black bean compounds may have acted through multiple pathways to improve vascular structure. Production and/or activity of AngII and ET-1 are increased in hypertension and both of these proteins can initiate a multitude of cell-signalling events that lead to MAPK activation, which triggers vascular remodelling. In addition, upregulation of scleraxis has been shown to be involved in collagen production and vascular stiffening. Thus, directing future work to clarifying the mechanism(s) through which black beans act to attenuate

vascular remodelling would contribute further to the understanding of how black beans could improve vascular health in hypertensive individuals.

Summary and Conclusions

Summary of Major Findings

- Neither black beans, navy beans, nor green lentils attenuated the rise in blood pressure in the SHR; however, the navy bean-fed SHR experienced the greatest rise in SBP over time.
- Black beans, but not navy beans or green lentils, partially attenuated vascular remodelling. This was evident by the lack of significant differences in aortic elastin levels relative to media area compared to WKY rats.
- Vascular remodelling in the SHR groups may have been due to an upregulation of phosphorylated ERK1/2 (in all SHR), phosphorylated p38 MAPK (in control SHR), and scleraxis (in control, green lentil, and navy bean SHR), which were lower in the black bean groups and thus may partially explain the improved vascular structure in these animals.

Conclusions

In agreement with the hypothesis, black beans were more beneficial than navy beans at attenuating vascular remodelling in the SHR. This was evident by the lack of differences in the elastin content relative to media area of the aorta between the WKY rats and black bean-fed SHR, whereas the navy bean-fed SHR had significantly lower ratios. While blood pressure was not different between the black bean- and navy bean-fed SHR, the rise in SBP of the navy bean group was significantly greater compared to the SBP change experienced by the WKY rats.

Furthermore, the change in SBP in the black bean group was not significantly different from the WKY rats. Thus, black beans were overall more superior to navy beans with regards to improving hypertension in the SHR.

If black beans are truly capable of attenuating vascular remodelling, future studies should further examine this pulse type for its therapeutic applications in human hypertension. Presently, there is no cure for hypertension and current pharmacological treatments aim to reduce high blood pressure rather than heal the root causes. Black beans are a healthful Canadian crop that may treat the underlying contributors to high blood pressure. Incorporating black beans into the Canadian diet may ultimately lead to improvements in overall population health.

Strengths and Limitations

Strengths

- This study employed the SHR, which is a model of essential hypertension and thus exhibits pathological similarity to human essential hypertension unlike other animal models of hypertension.
- This is the first study to examine the vascular effects of a black bean *vs.* navy bean diet in the SHR.
- While the inclusion of pulses in the diets was high, these levels could still reasonably be consumed at the human level. Furthermore, the use of cooked pulses in this study is representative of human consumption methods and would inactivate anti-nutritional components.
- The diets used in this study were isocaloric with similar macronutrient profiles, eliminating the effect of body weight gain on vascular outcomes in the SHR.
- The diets used in this study were isonitrogenous, eliminating the effects of protein on blood pressure in the SHR.
- All SHR consumed the same amount of feed suggesting there were no differences in the intake of the pulse components, and the diets neither promoted nor reduced feed intake.
- The SHR began this study when they had established hypertension, and so the results of the pulse intervention are reflective of what would occur if a

pulse intervention were initiated in a patient newly diagnosed with hypertension.

- This study provides *in vivo* data on vascular morphology and protein levels in aortae, which could not be obtained from human subjects in a clinical trial.

Limitations

- The WKY rats and control-fed SHR in our study had questionable blood pressure values and thus might not have been appropriate normotensive and hypertensive controls, respectively. Alternatively, the tail-cuff method for blood pressure measurement is subject to precision and accuracy errors (its usefulness has been previously debated).
- There was incomplete vascular morphology data as only the elastin content of the aorta was determined whereas collagen, cell size, and cell number were not. The reason for the lack of collagen analysis was due to the inability to detect differences between the colours of the collagen stain and of the "other tissues" stain. Moreover, elastin was only determined by staining and not verified by Western blotting. Furthermore, baseline vascular morphological data could not be gathered and given that the randomization did not result in an absolute absence of significant differences for other measured parameters, it is unknown if differences in vascular composition of the black bean-fed SHR aorta were also present at baseline.
- Western blotting for p38 MAPK, HSP 27, and eNOS only had an n = 3–4/group, which could have limited the ability to detect significant differences.

- The exact mechanism by which the black beans exerted a vascular benefit was not determined.
- The bioactive compound(s) in the black beans were not determined.

Future Recommendations

With the closing of this study, many new questions have arisen. Since black beans exerted greater vascular protection than navy beans, future work should continue research with black beans to clarify the effects of this pulse crop on hypertension and vascular remodelling.

Clarifying the specific components of the blood vessel wall that are altered by black beans (i.e. collagen, VSMC size, VSMC number) would provide greater insight into the mechanism through which black beans modulate vascular remodelling. Moreover, the MAPK pathways should be further explored by examining other proteins involved in these pathways, such as the upstream activators, ET-1 and AngII. Examining ^{3}H-Thymidine and ^{3}H-Proline incorporation in VSMC culture after ET-1 or AngII stimulation and black bean treatment can indicate whether cell proliferation or collagen production, respectively, are altered by the black beans.

A future study should also examine different doses of black beans and different durations of intervention. In our study, an eight-week intervention with a diet containing 30% (w/w) black bean powder was sufficient to attenuate changes in vascular composition of SHR aortae. Thus, a lower dose could also exert such effects and could be examined in future studies especially since the current intake of pulses among Canadians is low and therefore a lower dose would be more attainable in a human diet. On the other hand, longer trials with black bean interventions should also be conducted to determine if black beans could eventually impose blood pressure regulating effects in addition to vascular remodelling. The

next animal studies should utilize ambulatory methods for detecting blood pressure or, if using the tail-cuff method, use anesthesia to prevent erroneously high blood pressure measurements. Moreover, SD rats could be used in place of WKY rats as a normotensive control.

Determining the bioactive compound(s) in black beans responsible for their vascular benefit is also imperative for future research. This could be determined by identification of the metabolic compounds present in the serum and urine after the consumption of black beans, and if these compounds are related to blood pressure regulation and/or vascular remodelling. A study on urinary metabolites from SHR was conducted for lentils, but this has not yet been done for black beans, nor has this been done in humans.

Finally, translating this information to humans will not be possible until a clinical trial is conducted. Thus, a clinical trial is the final recommendation for future research with black beans and hypertension-related outcomes. However, this should only be implemented once the effective bioactive compound(s), dose, duration, and mechanism have been determined.

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Appendix A – Proximate Analyses and Dietary Protein Calculations

Table A- 1. Proximate Analysis of Pulse Powders[1,2,3]

	Black Bean	Navy Bean	Green Lentil
Moisture[4]	1.05	1.15	1.00
Dry Matter	99.0	98.9	99.0
Crude Protein[5]	22.8	23.8	27.3
Crude Fibre[6]	4.89	5.39	5.59
ADF[7]	11.7	10.8	9.06
NDF[8]	20.5	18.5	11.3
Carbohydrate[9]	66.7	64.8	62.4
Fat[10]	2.18	2.48	1.59

Abbreviations: ADF = acid detergent fibre; NDF = neutral detergent fibre.

[1]Proximate analysis conducted by Central Testing Labs Ltd., Winnipeg, MB, Canada.

[2]Results are expressed as % of weight, as is.

[3]Pulse powders were prepared as described in the Materials and Methods section.

[4]Moisture was determined by the AOAC 930.15 method.

[5]Crude protein was determined by a modified AOAC 990.03 method.

[6]Crude fibre was determined by the AOCS Ba6a-05 method.

[7]ADF determined by the ANKOM Technology (Macedon, NY, USA) method 08-16-06.

[8]NDF determined by the ANKOM Technology (Macedon, NY, USA) method 08-16-06.

[9]Non-fibre carbohydrate was determined by calculation.

[10]Fat was determined by the AOCS Am 5-04 method.

Calculation for casein adjustment in the black bean diet:

$$200\ g\ casein\ \times\ 0.87\frac{g\ protein}{g\ casein} = 174\ g\ protein\ per\ 1000\ g\ AIN93G\ diet$$

$$174\ g\ protein - (68.28\ g\ protein\ per\ 300\ g\ black\ bean\ powder) = 105.72\ g\ protein\ from\ casein\ required$$

$$105.72\ g\ protein\ \times\ \frac{1}{0.87\frac{g\ protien}{g\ casein}} = 121.5\ g\ casein\ (to\ be\ added\ to\ black\ bean\ diet)$$

Calculation for casein adjustment in the navy bean diet:

$$200\ g\ casein\ \times\ 0.87\frac{g\ protein}{g\ casein} = 174\ g\ protein\ in\ 1000\ g\ AIN93G\ diet$$

$$174\ g\ protein - (71.37\ g\ protein\ per\ 300\ g\ navy\ bean\ powder) = 102.63\ g\ protein\ from\ casein\ required$$

$$102.63\ g\ protein\ \times\ \frac{1}{0.87\frac{g\ protien}{g\ casein}} = 118\ g\ casein\ (to\ be\ added\ to\ navy\ bean\ diet)$$

Calculation for casein adjustment in the green lentil diet:

$$200\ g\ casein\ \times\ 0.87\frac{g\ protein}{g\ casein} = 174\ g\ protein\ in\ 1000\ g\ AIN93G\ diet$$

$$174\ g\ protein - (81.78\ g\ protein\ per\ 300\ g\ green\ lentil\ powder) = 92.22\ g\ protein\ (required)$$

$$92.22\ g\ protein\ \times\ \frac{1}{0.87\frac{g\ protien}{g\ casein}} = 106\ g\ casein\ (to\ be\ added\ to\ green\ lentil\ diet)$$

Appendix B – Solutions

10× PBS

80 g NaCl (Fisher, #BP358-10, lot 140292)
2 g KCl (Fisher, #P217, lot 027298)
14.4 g Na_2HPO_4 (Fisher, #BP332. Lot 090520)
2.4 g KH_2PO_4 (Fisher, #P285, lot 084292)
Dissolve in 600 mL ddH_2O
Adjust to pH 7.4 with 10 N NaOH (Fisher, #55255, lot 130890)
Bring up to 1 L with ddH_2O
Store at 4°C

1× PBS

10 mL 10× PBS
90 mL ddH_2O
Store at 4°C

1 M NaOH

40 g NaOH pellets (Fisher, #S320-500, lot 024703)
Dissolve in 1 L ddH_2O
Store at room temperature

9% NaCl

90 g NaCl (Fisher, #BP358-10, lot 112683)
Dissolve in 1 L ddH_2O
Autoclave
Store at room temperature

0.5 M Na_3PO_4 pH 7.4

30 g Na_2HPO_4 (Fisher, #BP332-1, lot 084296)
4.7 g NaH_2PO_4 (Fisher, #BP329-500, lot 980680)
Dissolve in 250 mL ddH_2O
Adjust to pH 7.4 with HCl
Bring up to 500 mL with ddH_2O
Autoclave
Store at room temperature

1% Paraformaldehyde

630 µL 1 M NaOH
36 mL 9% NaCl
144 mL ddH_2O
Add 3.6 g paraformaldehyde
Warm to 60°C, stirring to dissolve
Cool
Add 108 mL Add 0.5 M Na_3PO_4 pH 7.4, and
72 mL ddH_2O
Stir to dissolve
Store at 4°C

0.5 M Tris-HCl pH 6.8

60.57 g Tris Base (Fisher, #BP152-5, lot 990424)
Dissolve in 750 mL ddH_2O
Adjust pH to 6.8 with HCl
Bring up to 1 L with ddH_2O
Autoclave
Store at room temperature

3× Sample Buffer

67.5 mL 0.5 M Tris-HCl pH 6.8
54 mL 10% sodium dodecyl sulfate (SDS)
54 mL glycerol (Fisher, #BP229-1, lot 112528)
Bring up to 200 mL with ddH_2O
Store at room temperature

20% Acrylamide

100 mL 40% acrylamide (Fisher, #BP1408-1, lot 159040)
100 mL ddH_2O
Store at 4°C

10% Ammonium Persulfate (APS)

1.5 g APS (Fisher, #BP179-100, lot 082634)
Dissolve in 15 mL ddH_2O
Store at -20°C

10% SDS

800 mL ddH_2O
Heat to a near boil then remove from heat
Add 100 g SDS (Sigma, #L3771, lot 091M02031V), stir to dissolve
Bring up to 1 L with ddH_2O
Store at room temperature

1.5 M Tris-HCl pH 8.8

181.32 g Tris base (Fisher, #BP152-5, lot 990424)
Dissolve in 750 mL ddH_2O
Adjust pH to 8.8 with HCl
Bring up to 1 L with ddH_2O
Store at room temperature

5× Running Buffer

60 g Tris base (Fisher, #BP152-5, lot 990424)
288 g glycine (Fisher, #BP381-5, lot 153722)
Dissolve in 200 mL 10% SDS
Bring up to 4 L with ddH_2O
Store at room temperature

5× Transfer Buffer

75.6 g Tris base (Fisher, #BP152-5, lot 990424)
245.6 g glycine (Fisher, #BP381-5, lot 153722)
Dissolve in 4 L ddH_2O
Store at 4°C

5 M NaCl

876.8 g NaCl (Fisher, #BP358-10, lot 140292)
Dissolve in 3 L ddH_2O
Autoclave
Store at room temperature

1 M Tris-HCl pH 7.4

242.34 g Tris base (Fisher, #BP152-2, lot 990424)
Dissolve in 1.8 L ddH_2O
Adjust pH to 7.4 with HCl
Bring up to 2 L with ddH_2O
Autoclave
Store at room temperature

5× TBST

600 mL 5 M NaCl
400 mL 1 M Tris-HCl pH 7.4
10 mL Tween 20 (OmniPure, Calbiochem, #9480, lot 0262C529)
Bring up to 4 L with ddH_2O
Store at 4°C

1× TBST

100 mL 5× TBST
400 mL ddH_2O
Store at room temperature

3% BSA-TBST

30 g BSA (Roche, #0735094001, lot 70505421)
Dissolve in 200 mL 5× TBST
Bring up to 1 L with ddH_2O
Store at 4°C

1% BSA-TBST

10 g BSA (Roche, #0735094001, lot 70505421)
Dissolve in 200 mL 5× TBST
Bring up to 1 L with ddH_2O
Store at 4°C

Appendix C – Staining and Measurement of Aorta Sections

C.1. Staining Protocol

1. Slides were thawed to room temperature and then placed in 1% PFA for eight minutes.
2. Slides were washed in 1× PBS for ten minutes.
3. Slides were hydrated in double-distilled water (ddH_20) for fifteen minutes.
4. Slides were placed in a working elastin stain solution (20 mL hematoxylin solution, 3 mL ferric chloride solution, 8 mL Weigert's iodine solution, and 5 mL ddH_20) for ten minutes.
5. Slides were rinsed in ddH_20.
6. Slides were differentiated in working ferric chloride solution (3 mL ferric chloride solution and 37 mL ddH_20) for three minutes.
7. Slides were rinsed in ddH_20.
8. Slides were rinsed in 95% ethanol.
9. Slides were rinsed in ddH_20.
10. Slides were stained with Van Gieson Stain for ninety seconds.
11. Slides were rinsed in 95% ethanol.
12. Slides were dehydrated in xylene for fifteen minutes.
13. Slides were mounted using Vecta Mount and left in the fumehood until dry.

C.2. Assessment of Vessel Morphometry by ImageJ

1. Slides were imaged on an EVOS microscope with a 20× objective.
2. Digital images were captured and saved as TIFF images.
3. Images were opened on ImageJ software.
4. The *Freehand selections* tool was selected from the ImageJ toolbar and using a pen tablet, the inner and outer circumferences of the vessel were manually traced.
5. After tracing, *Measure* was selected from the dropdown *Analyze* menu and ImageJ generated measurements on the lumen circumference, lumen CSA, outer media circumference, and whole vessel CSA.

6. Measurements from ImageJ were exported to Microsoft Excel.
7. Calculations were done in Excel to determine: lumen diameter, media thickness, media CSA, and media/lumen ratio (as shown in Appendix C.3).
8. Two non-consecutive slides per animal were analyzed, and the average of the two was used in statistical analysis via SAS.

C.3. Calculations for Vessel Morphology

Calculation for lumen diameter:

$$Lumen\ Diameter\ = \frac{Lumen\ Circumference}{\pi}$$

Calculation for media thickness:

$$Media\ Thickness\ = \left(\frac{Media\ Circumference}{2\pi}\right) - \left(\frac{Lumen\ Diameter}{2}\right)$$

Calculation for media CSA:

$$Media\ CSA\ = \pi {r_{media}}^2 - \pi {r_{lumen}}^2$$

Calculation for media/lumen ratio:

$$Media/Lumen\ Ratio\ = \frac{Media\ Thickness}{Lumen\ Diameter}$$

C.4. Assessment of Vessel Elastin Content by ImageJ

1. Slides were imaged on an EVOS microscope at 4× objective.
2. Digital images were captured and saved as TIFF images.
3. Images were opened on ImageJ software.
4. The *Freehand selections* tool was selected from the ImageJ toolbar and using a pen tablet, the entire media area was traced.
5. After tracing the media, the outside area was removed by selecting *Clear Outside* from the *Edit* menu in ImageJ.

6. Elastin was stained black, and so to calculate the level of elastin in the media the area of black was determined.
7. Black was highlighted in the media by opening the *Image* menu, selecting *Adjust*, then selecting *Threshold*.
8. In the *Threshold* window, Color space was set to YUV and the Y maximum bar was adjusted until only elastin (black) was highlighted. The *Select* button was then selected.
9. To determine the area of elastin, *Measure* was selected from the dropdown *Analyze* menu and ImageJ generated the area measurement based on the highlighted area.
10. To determine the area of the media, the *Threshold* window was opened; the Y maximum bar was adjusted to 250-255 until the whole vessel was highlighted. The *Select* button was then selected.
11. *Measure* was selected from the dropdown *Analyze* menu and ImageJ generated the area measurement based on the highlighted area.
12. All measurements were exported to Microsoft Excel.
13. The elastin content of the vessel wall was calculated as the %blackness relative to vessel area.
14. Two non-consecutive slides per animal were analyzed, and the average of the two was used in statistical analysis via SAS.

Appendix D – Feed Intake, Body Weight, and Body Composition

Table D- 1. Average Daily Feed Intake by Week (g) in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Week 1	17.6 ± 0.6 b	20.9 ± 1.5 ab	24.0 ± 2.8 a	21.3 ± 1.7 ab	18.4 ± 1.8 ab
Week 2	19.0 ± 1.2	20.8 ± 1.7	27.1 ± 4.2	26.5 ± 3.8	21.5 ± 2.3
Week 3	21.0 ± 2.1	24.8 ± 2.4	28.6 ± 4.5	30.7 ± 4.1	23.4 ± 2.7
Week 4	20.6 ± 1.4 c	29.3 ± 3.6 abc	31.1 ± 3.9 ab	35.9 ± 3.8 a	24.8 ± 3.2 bc
Week 5	21.9 ± 1.7 b	30.3 ± 3.3 ab	33.9 ± 4.3 a	35.6 ± 4.4 a	30.6 ± 3.5 ab
Week 6	22.0 ± 1.8 b	35.6 ± 3.2 a	36.7 ± 4.0 a	40.4 ± 3.7 a	31.7 ± 4.2 ab
Week 7	22.5 ± 2.0b	39.4 ± 2.6 a	37.6 ± 4.4 a	37.3 ± 4.0 a	32.9 ± 4.5 ab
Week 8	24.8 ± 2.5 b	43.5 ± 2.7 a	39.9 ± 3.5 a	39.2 ± 4.5 a	34.9 ± 4.1 a
Total Intake	1180 ± 76 b	1710 ± 117 a	1810 ± 198 a	1870 ± 184 a	1530 ± 163 ab

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained with an electronic scale for each week. Results are expressed as the mean of 4 consecutive day's intake ± SE (n = 9–10/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Table D- 2. Weekly Body Weight (g) in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Baseline	336 ± 6	324 ± 6	334 ± 5	335 ± 4	338 ± 3
Week 1	346 ± 7	337 ± 6	341 ± 6	340 ± 4	350 ± 3
Week 2	358 ± 7	347 ± 6	348 ± 6	348 ± 5	355 ± 3
Week 3	370 ± 7	353 ± 6	359 ± 7	353 ± 6	361 ± 3
Week 4	378 ± 7 a	356 ± 7 b	363 ± 7 ab	357 ± 6 ab	368 ± 3 ab
Week 5	386 ± 7 a	362 ± 6 b	368 ± 7 b	364 ± 6 b	372 ± 4 ab
Week 6	394 ± 8 a	364 ± 6 b	374 ± 7 b	368 ± 4 b	376 ± 4 b
Week 7	403 ± 7 a	365 ± 6 b	377 ± 6 b	372 ± 6 b	378 ± 4 b
Week 8	407 ± 7 a	370 ± 5 b	379 ± 7 b	373 ± 6 b	379 ± 5 b
Weight Gain	76.8 ± 4.5 a	41.5 ± 3.4 b	44.0 ± 4.3 b	37.3 ± 4.3 b	39.3 ± 3.9 b

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained *in vivo* with an electronic scale. Results are expressed as mean ± SE (n = 9–10/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Table D- 3. Additional Body Composition Results for WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Total Water (g)					
Baseline	204 ± 3	211 ± 7	208 ± 5	211 ± 6	214 ± 5
Week 8	213 ± 2	209 ± 3	212 ± 4	209 ± 3	216 ± 3
Baseline vs. Week 8	8.36 ± 2.91	-2.61 ± 6.74	4.23 ± 6.65	-1.94 ± 6.33	1.86 ± 4.06
Total Water (g/100 g Lean Mass)					
Baseline	83.1 ± 0.2 b	83.9 ± 0.5 ab	83.5 ± 0.4 b	84.6 ± 0.3 a	83.5 ± 0.4 b
Week 8	82.9 ± 0.1	83.1 ± 0.3	83.1 ± 0.4	82.7 ± 0.5	82.6 ± 0.3
Free Water (g)					
Baseline	0.77 ± 0.05	0.71 ± 0.09	0.79 ± 0.08	0.70 ± 0.06	0.77 ± 0.10
Week 8	0.77 ± 0.06 a	0.60 ± 0.07 ab	0.62 ± 0.05 ab	0.60 ± 0.06 ab	0.56 ± 0.04 b
Baseline vs. Week 8	-0.00 ± 0.06	-0.11 ± 0.09	-0.17 ± 0.10	-0.10 ± 0.09	-0.21 ± 0.10
Free Water (g/100 g Total Water)					
Baseline	0.38 ± 0.03	0.34 ± 0.06	0.38 ± 0.03	0.33 ± 0.02	0.36 ± 0.04
Week 8	0.36 ± 0.03 a	0.29 ± 0.03 ab	0.29 ± 0.02 ab	0.29 ± 0.03 ab	0.26 ± 0.02 b

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained *in vivo* with a QMR instrument. Results are expressed as mean ± SE (n = 9–10/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Appendix E – Serum Biochemistry

Table E- 1. Fasting Serum Lipids in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Triglycerides (mmol/L)					
Baseline	1.45 ± 0.18 a	0.58 ± 0.10 b	0.53 ± 0.05 b	0.60 ± 0.08 b	0.64 ± 0.05 b
Week 8	1.41 ± 0.10 a	0.59 ± 0.06 b	0.70 ± 0.08 b	0.62 ± 0.07 b	0.69 ± 0.09 b
Baseline vs. Week 8	-0.12 ± 0.14	-0.04 ± 0.09	0.18 ± 0.09	-0.03 ± 0.12	0.04 ± 0.09
Total Cholesterol (mmol/L)					
Baseline	2.56 ± 0.02 a	1.63 ± 0.05 b	1.61 ± 0.06 b	1.63 ± 0.03 b	1.69 ± 0.05 b
Week 8	3.51 ± 0.09 a	1.68 ± 0.08 b	1.66 ± 0.05 bc	1.50 ± 0.04 c	1.58 ± 0.06 bc
Baseline vs. Week 8	0.92 ± 0.11 a	-0.01 ± 0.08 b	0.03 ± 0.06 b	-0.13 ± 0.03 c	-0.14 ± 0.08 bc
HDL Cholesterol (mmol/L)					
Baseline	2.01 ± 0.05 a	1.34 ± 0.03 b	1.32 ± 0.03 b	1.31 ± 0.03 b	1.32 ± 0.03 b
Week 8	2.57 ± 0.06 a	1.24 ± 0.06 b	1.19 ± 0.04 b	1.15 ± 0.04 b	1.17 ± 0.07 b
Baseline vs. Week 8	0.53 ± 0.11 a	-0.12 ± 0.06 b	-0.13 ± 0.04 b	-0.15 ± 0.03 b	-0.30 ± 0.14 b
LDL Cholesterol (mmol/L)					
Baseline	0.40 ± 0.03 a	0.34 ± 0.03 ab	0.33 ± 0.02 ab	0.31 ± 0.03 b	0.31 ± 0.02 b
Week 8	0.77 ± 0.05 a	0.40 ± 0.04 b	0.35 ± 0.03 b	0.32 ± 0.01 b	0.32 ± 0.04 b
Baseline vs. Week 8	0.37 ± 0.07 a	0.04 ± 0.03 b	0.01 ± 0.02 b	0.03 ± 0.02 b	0.01 ± 0.03 b

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained using a CIII Cobas auto-analyzer. Results are expressed as mean ± SE (n = 8–9/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Table E- 2. Fasting Serum Lipid Ratios in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Total/HDL Cholesterol					
Baseline	1.28 ± 0.03	1.22 ± 0.04	1.22 ± 0.02	1.24 ± 0.03	1.28 ± 0.03
Week 8	1.37 ± 0.03	1.35 ± 0.03	1.40 ± 0.05	1.31 ± 0.03	1.36 ± 0.05
Baseline vs. Week 8	0.09 ± 0.04	0.11 ± 0.04	0.17 ± 0.05	0.05 ± 0.04	0.08 ± 0.06
LDL/HDL Cholesterol					
Baseline	0.19 ± 0.01 b	0.25 ± 0.02 a	0.25 ± 0.01 a	0.23 ± 0.02 ab	0.23 ± 0.01 ab
Week 8	0.30 ± 0.02	0.32 ± 0.02	0.30 ± 0.02	0.28 ± 0.01	0.26 ± 0.03
Baseline vs. Week 8	0.10 ± 0.02	0.05 ± 0.02	0.04 ± 0.01	0.06 ± 0.02	0.04 ± 0.02
Triglycerides/HDL Cholesterol					
Baseline	0.69 ± 0.09 a	0.44 ± 0.08 b	0.40 ± 0.03 b	0.46 ± 0.07 b	0.49 ± 0.04 ab
Week 8	0.55 ± 0.04	0.48 ± 0.06	0.60 ± 0.08	0.54 ± 0.06	0.52 ± 0.06
Baseline vs. Week 8	-0.21 ± 0.09 b	0.01 ± 0.06 ab	0.21 ± 0.08 a	0.04 ± 0.10 ab	0.14 ± 0.13 ab

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained using a CIII Cobas auto-analyzer. Results are expressed as mean ± SE (n = 8–9/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.

Appendix F – Blood Pressure

Table F- 1. Blood Pressure Measurements in WKY Rats and SHR

	WKY	SHR			
	Control	Control	Green Lentil	Black Bean	Navy Bean
Systolic Blood Pressure (mmHg)					
Baseline	153 ± 7 b	197 ± 6 a	182 ± 7 a	187 ± 4 a	181 ± 8 a
Week 4	153 ± 4 b	188 ± 5 a	196 ± 4 a	194 ± 5 a	194 ± 7 a
Week 8	142 ± 6 b	184 ± 5 a	192 ± 6 a	199 ± 6 a	199 ± 6 a
Baseline vs. Week 8	-11.1 ± 7.3 bc	-12.9 ± 5.2 c	10.1 ± 10.4 abc	12.5 ± 8.5 ab	17.9 ± 7.4 a
Diastolic Blood Pressure (mmHg)					
Baseline	98.7 ± 4.1 b	149 ± 7 a	138 ± 6 a	140 ± 5 a	134 ± 8 a
Week 4	103 ± 5 b	140 ± 4 a	148 ± 6 a	142 ± 7 a	145 ± 7 a
Week 8	94.4 ± 4.3 b	138 ± 4 a	143 ± 7 a	144 ± 7 a	146 ± 9 a
Baseline vs. Week 8	-4.2 ± 4.2	-11.4 ± 6.1	5.0 ± 10.0	4.1 ± 11.4	11.0 ± 10.7
Mean Blood Pressure (mmHg)					
Baseline	116 ± 5 b	165 ± 6 a	152 ± 7 a	155 ± 5 a	149 ± 8 a
Week 4	119 ± 4 b	156 ± 4 a	163 ± 5 a	159 ± 6 a	161 ± 7 a
Week 8	110 ± 5 b	153 ± 4 a	159 ± 7 a	162 ± 6 a	163 ± 8 a
Baseline vs. Week 8	-6.7 ± 5.2	-11.8 ± 5.6	6.7 ± 10.1	7.1 ± 10.4	13.0 ± 9.2

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Measurements presented were obtained *in vivo* with a Coda (Kent Scientific) instrument. Results are expressed as mean ± SE (n = 8–10/group). For means within the same row, different letters represent significant differences ($p<0.05$). An absence of letters indicates no statistical differences.