2D-PAGE Analysis of Myocardial Collagen in Male and Female Spontaneously Hypertensive Rats

by

Benjamin L. Fulton

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Biological Sciences Program

YOUNGSTOWN STATE UNIVERSITY

August, 2008
2D-PAGE Analysis of Myocardial Collagen in Male and Female Spontaneously Hypertensive Rats

by

Benjamin L. Fulton

I hereby release this thesis to the public. I understand this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

______________________________
Benjamin L. Fulton, Student       Date

Approvals:

______________________________
Dr. J. Krontiris-Litowitz, Thesis Advisor       Date

______________________________
Dr. R. Leipheimer, Committee Member       Date

______________________________
Dr. G. Walker, Committee Member       Date

______________________________
Peter J. Kasvinsky, Dean of Graduate Studies & Research       Date
ABSTRACT

Each year, nearly 250,000 women will die of coronary heart disease, the leading cause of death for women in America. A major contributing factor to coronary heart disease is the onset of hypertension, or high blood pressure. Hypertension is characterized as pertaining to a blood pressure of 140/90 mmHg or greater, 90-95% cases of which have no directly identifiable cause. It has been estimated that 6% of the world’s deaths can be attributed to the effects of hypertension; these effects are systemic, and include damage to the brain, blood vessels, heart and kidneys. Most of these effects, however, often occur before the symptoms of hypertension actually arise, including arterial lesions of the brain, heart and kidneys, hyperinsulinemia, dyslipidemia, and elevated hematocrit levels. Much valuable research has been conducted in the past pertaining to hypertension, but with a deficiency in the area of gender studies with most research being performed exclusively on male test subjects. The present study simultaneously compares the effects of hypertension on both male, female, and ovariectomized female subjects, using the Spontaneously Hypertensive Rat (SHR) as a model. Collagen, which is the main focus of this study and the major ultrastructural protein in the heart, is quantitated using 2D-PAGE to analyze the differences in collagen density in male and female SHR hearts. It is our hypothesis that given the difference between male and female physiology, specifically the presence or absence of estrogen, that hypertensive effects will manifest differently between the sexes, in terms of collagen deposition and density in the cardiac architecture.
ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor, Dr. Krontiris-Litowitz, for imparting her knowledge, time, and guidance throughout this research project. I would also like to thank Dr. Walker for teaching me the art of proteomics, and Dr. Leipheimer for his continued support and insight into this project.

I would also like to thank the Cushwa family and the Youngstown State University Foundation for awarding me the Cushwa/Commercial Shearing Fellowship, which has made this project possible. I also thank the Youngstown State University Biology department for the opportunity and facilities needed to earn my Masters of Science degree.

I would like to offer special thanks to Drew and Carol Hirt of Material Research Laboratories in Struthers, Ohio, for furnishing me with an internship during my course of study at Youngstown State University.

Finally, I would like to express my deep and heartfelt thanks to my family and friends who have supported me throughout my academic career. Their constant support and encouragement has made this project possible in ways too numerous to mention.
# TABLE OF CONTENTS

ABSTRACT...........................................................................................................iii

ACKNOWLEDGEMENTS....................................................................................iv

TABLE OF CONTENTS......................................................................................v

LIST OF FIGURES.............................................................................................vi

INTRODUCTION.................................................................................................1

MATERIALS AND METHODS.............................................................................18

RESULTS...........................................................................................................22

DISCUSSION......................................................................................................42

REFERENCES.....................................................................................................49

APPENDIX..........................................................................................................54
LIST OF FIGURES

Figure 1A. Collagen III gel.........................................................25
Figure 1B. Composite Collagen III gel.................................25
Figure 2A. Collagen I gel.........................................................27
Figure 2B. Composite Collagen I gel.................................27
Table 1. pI and Rf values for spots in collagen I and III composite gels…29
Figure 3A. 2D Gel Profile Male..................................................31
Figure 3B. 2D Gel Profile Female................................................31
Figure 3C. 2D Gel Profile OVX Female........................................31
Figure 4. Mean protein content in CNBr digest of SHR hearts.........33
Figure 5A. 2D Gel Profile of pooled Male...............................37
Figure 5B. 2D Gel Profile of pooled Female...............................37
Figure 5C. 2D Gel Profile of pooled OVX Female.......................37
Table 2. pI and Rf values for spots in male, female, and OVX female pooled sample composite gels........................................39
INTRODUCTION
Hypertension is characterized by blood pressure of 140/90 mmHg or greater. Primary hypertension, which accounts for 90 – 95% of cases has no directly identifiable cause, while secondary hypertension, which accounts for 5 – 10% of all cases, does have identifiable causes. One study has calculated that 6% of the world’s deaths can be attributed to hypertension and its effects. Although hypertension is frequent, it is called the “silent killer” because symptoms do not arise until after the blood vessels, heart, brain, and kidneys are damaged. Arterial lesions of the heart, brain, and kidneys have been observed in many studies involving spontaneously hypertensive rats. Adverse health conditions which accompany hypertension are hyperinsulinemia, dyslipidemia, and elevated hematorit levels (prevalent in male hypertensives). Hypertension can result from the chronic elevation of blood volume, and consequently blood pressure, due to the release of renin and the conversion of angiotensin I to angiotensin II. By altering the volume of blood in the circulatory system, the body can compensate for an abnormal peak or trough in blood pressure. Blood volume and pressure are directly proportional, so that when one increases, the other does as well. In this manner dietary habits, such as high salt intake, can elevate blood pressure. Angiotensin plays a key role in the development of hypertension. When blood pressure falls, the kidneys release renin into the bloodstream, which catalyzes a cascade in which renin activates angiotensinogen released from the liver, to form angiotensin I. Angiotensin I is converted by angiotensin converting enzyme (ACE) to form angiotensin II (Danser et al., 1999). Angiotensin II, binding to AT$_1$ and AT$_2$ receptors, constricts blood vessels, increasing blood pressure and, in turn, increasing the rate of renal perfusion. Angiotensin II acts directly on the
kidney by increasing sodium ion reabsorption by activating a sodium-potassium ion pump in the proximal convoluted tubule cells. Angiotensin II also stimulates the release of aldosterone, a mineralocorticoid hormone, from the adrenal cortex. Aldosterone works directly on the kidneys to regulate blood pressure by causing the kidneys to retain sodium ions while simultaneously causing the excretion of potassium ions. Water retention is also induced by aldosterone via osmosis which acts as homeostatic regulation of blood pressure. By increasing sodium absorption by the kidneys, aldosterone causes an increase in water movement into the bloodstream and an elevation in blood pressure (Gronholm et al., 2004).

Hypertrophy is an increase in myocardial mass due in part to increased myocyte diameter. In an attempt to alleviate stress, caused by a volume overload or by pressure overload, the left ventricle hypertrophies, or enlarges, until the stress inflicted on the heart has been compensated. In the case of an eccentric hypertrophy, which results from volume overload, new sarcomeres are added in-series, causing a lengthening of the myocytes. Adding sarcomeres in this manner allows the ventricle to dilate, while keeping the wall thickness proportional to the ventricular chamber (Olivetti et al., 2000). Non-pathogenic eccentric hypertrophy is common among athletes as their hearts must enlarge to compensate for an increased volume load, and is reversible. Other forms of eccentric hypertrophy are characterized by an eventual thinning of the ventricular wall, and a whitish color due to myocardial fibrosis. The myocardium may also hypertrophy due to a lateral addition of sarcomeres to the myocytes; this is known as concentric hypertrophy, which is caused by pressure overload on the ventricles. Concentric
hypertrophy results in a thickening of the ventricular wall with little or no dilation of the ventricular chamber (Olivetti et al., 2000).

Accompanying hypertension is myocardial fibrosis, which is the addition to or remodeling of the heart’s collagen ultrastructure. Three major patterns of cardiac fibrosis have been identified (Weber et al., 1989). In the first pattern of myocardial fibrosis, resulting from pressure overload hypertrophy, the amount of collagen produced is directly proportional to the increase in myocardial mass, even though there is generally not a significant increase in fibroblast proliferation until after collagen has already begun to be synthesized. Fibroblast proliferation does not need to occur until after the advent of collagen synthesis because, in the short term, hypertrophy is a normal, and often reversible, compensation for a drop in blood pressure, thus additional fibroblasts should not be needed in beginning stage hypertrophy. It is only as the hypertrophy becomes more chronic that additional fibroblasts are needed to produce collagen matrix in response to myocardial necrosis. The preexisting collagen matrix is augmented during overload hypertrophy, thickening the established matrix and making it denser (Weber et al., 1989). Throughout the progression of hypertension, the thickening and strengthening of the preexisting collagen matrix continues, producing a myocardial ultrastructure of increasing tensile strength and rigidity. The space in between cardiac myocytes is also filled with collagen at this time, contributing to the interstitial fibrosis of the heart. The initial stages of cardiac hypertrophy are not in themselves pathological but rather serve to make the heart a more forceful pump (Weber et al., 1989). Also seen in the early stages of myocardial fibrosis are thick collagen fibers amassing around intramyocardial blood vessels. These perivascular collagen enlargements, called septae, then radiate out into the
surrounding myocardial tissue, adding another dimension to the fibrosis (Weber et al., 1989; Doering et al., 1988).

Of the three major patterns of myocardial fibrosis identified, a second pattern of fibrosis, documented by Weber et al. completely alters the ultrastructure of the normal collagen matrix, creating a new design. This form of fibrosis is categorized as a reactive fibrosis rather than reparative. This second pattern can be generated in a rat model after administration of isoproterenol, a synthetic catecholamine that serves as a β-agonist, stimulating the release of angiotensin II and causing increased heart rate (Masuoka et al. 1995; Leenen et al., 1998). Thin collagen (III) fibers originating in the intermuscular space transit across the myofibrils at a 90° angle to their long axis. Later, thick collagen (I) fibers form a lattice, or grid with the previously established thin collagen fibers along the length of the muscle cells, effectively creating a blanket, or mat around the myocytes, a feature not seen in the previous pattern of myocardial fibrosis (Weber et al., 1989). The thick collagen fibers continue to grow in dimension crowding out the thin fibers. As muscle fibers are enveloped by the ever thickening mat of collagen, diastolic stiffness is increased to the point that the myocytes cannot effectively generate force.

A third type of myocardial fibrosis has been identified in conjunction with cell necrosis due to pressure overload hypertrophy, and appears to be reparative. In this third pattern of fibrosis, collagen is used to fill the gaps left behind by necrotized myocytes, and form perpendicular to the long axis of the muscle. Thin collagen fibers become enmeshed in thick fibers running parallel to the muscle cells, forming a weave of collagen between the myocytes. Unlike the second pattern of fibrosis, the third pattern serves to increase systolic stiffness, as well as diastolic stiffness (Weber et al., 1989).
Increased collagen deposition, or fibrosis, in the extracellular matrix increases the rigidity of the heart, contributing to hypertrophy, cell necrosis, and continued fibrosis to replace necrotic tissue. Fibrosis, and increased collagen concentrations in heart tissue are significant factors contributing to myocardial rigidity, pressure overload hypertrophy, and other left ventricular cardiomyopathies (Badenhorst et al., 2002).

**Spontaneously Hypertensive Rats**

Spontaneously hypertensive rats (SHR) are an inbred, albino strain that have long been used as a model for studying hypertension in humans (Kawamura et al., 1976). In the life cycle of SHR is seen a progressive pathology of hypertension marked by increasing cardiocyte hypertrophy and connective tissue (collagen) deposition throughout the ventricular myocardium. Studies show that SHR achieve hypertrophy due to a malfunction in the autonomic (sympathetic) nervous system thereby creating an ideal model for genetic hypertension. In the SHR model, hypertension is sustained throughout life including specific hypertensive pathologies such as cardiac hypertrophy and interstitial fibrosis of the myocardium. The pathologies associated with SHR hypertrophy are due to chronic pressure overload hypertrophy (Conrad et al., 1995).

An extensive study by Kawamura et al., in 1976 demonstrates the progressive morphology of hypertrophy that SHR display with distinct pathologies for each stage of life (Kawamura et al., 1976). At three weeks of age, SHR do not display any pathologies associated with hypertension, including interstitial fibrosis. Any collagen proliferation at this stage of development is believed to be associated with myofibrillogenesis. At five and nine weeks of age, SHR showed no difference in cardiac pathology than SHR at three weeks. The papillary muscles in SHR, however, were larger than that of controls.
Beginning at eleven weeks of age SHR manifest the early stages of hypertrophy. The diameter of cardio myocytes in eleven week SHR surpasses that of the controls in both papillary muscle and ventricular walls. The first signs of interstitial fibrosis are also seen at eleven weeks of age. Also, filamentous structures can be seen beginning to form around cardio myocytes and in subsarcolemmal areas, and are thought to be associated with hypertrophying cardiocytes and myofibrillogenesis. By fifteen weeks of age, SHR show marked signs of hypetrophy, including a sustained increase in myocyte diameter when compared to controls with respect to the papillary muscle and the ventricular walls. Hypertrophy is also seen to be more developed at fifteen weeks of age, as well as interstitial fibrosis. The subsarcolemmal, dense, filamentous mats observed at eleven weeks of age had grown in thickness. Numerous fibroblasts with large amounts of granular endoplasmic reticulum were observed in areas of dense collagen aggravating the interstitial fibrosis. Moreover, some areas of atrophied cardiocytes were also observed where subsarcolemmal dense mats had increased in size. At twenty-one weeks of age to one year of age, SHR show an increase in cardiomyocyte diameters in the lateral ventricular walls, exceeding the diameter of cardiomyocytes in the papillary muscles. Interstitial collagen and fibroblasts had proliferated greatly in the cardiac tissue, even to the exclusion of most myocytes in some areas.

Even as a genetic model for hypertrophy, the state of hypertension and myocardial fibrosis within SHR is reversible. Several studies have implied that not only hypertrophy, but also interstitial collagen deposition can be reversed with the aid of pharmaceuticals in the SHR model. The pharmacological agents employed in the study
of hypertension are Angiotensin Converting Enzyme (ACE) inhibitors, Angiotensin Type-1 Receptor Blockers (ARB), and Vasopeptidase inhibitors (VPI).

ACE inhibitors block the effects of angiotensin II in the myocardium, and much research has been performed to examine the effects of ACE inhibitors, ARBs, and VPIs on SHR. As a hormone, angiotensin II stimulates thirst and elevates blood pressure. Angiotensin II also stimulates the secretion of aldosterone by the adrenal cortex and Anti-diuretic hormone (ADH) of the posterior pituitary gland. Aldosterone and ADH act to conserve both water and salt loss at the kidneys. Angiotensin II also acts as a growth factor by regulating cardiac fibroblast function by manipulating growth factors, increasing fibroblast proliferation, apoptosis, and collagen turnover (Gronholm et al., 2004). Aniotensin II may induce myocyte growth and fibrosis in the heart without hemodynamic changes if synthesized locally. These effects by angiotensin II are mediated by AT$_1$ receptors. Angiotensin II also induces the expression of Connective Tissue Growth Factor (CTGF) in the heart, as well as mediating production of extracellular matrix production via a transformation of Growth Factor-β (TGF-β) (Gronholm et al., 2004). Alpha-methyldopa has also been implicated in having influence on cardiac fibrosis. Myocardial collagen has been seen to increase in conjunction with alpha-methyldopa levels (Sen and Bumpus, 1979; Motz et al., 1982; Brilla et al., 1991). These findings suggest that the renin-angiotensin system (RAS) may be a major contributor in both remodeling and alteration of the myocardial collagen matrix, contributing both to the pathology of cardiac fibrosis, and the reversal of cardiac fibrosis. It has also been suggested that angiotensin II may also play a role in cardiac hypertrophy and fibrosis via a phosphatidylinositol (PI) pathway. PI turnover results in the production of inositol-
1,4,5-triphosphate (IP₃) and s-1,2-diacylglycerol (DAG). IP₃ stimulates the release of intracellular Ca²⁺ from the endoplasmic reticulum, and DAG activates Ca²⁺ dependent protein kinase C (PKC), which can phosphorylate other proteins generating multiple biological cascades (Kawaguchi et al., 1996). Elevated levels of PI and IP₃ kinase activity have been reported in SHR given no prior stimulus. It has also been reported that neonatal cardiac myocytes and fibroblasts have responded to angiotensin II induced PI turnover (Sadoshima and Izumo, 1993).

ACE inhibitors such as Captopril and Isinopril have been studied to determine their effect on hypertrophy and collagen deposition in SHR. Captopril was shown to not only reverse hypertrophy in SHR, but also reverse the deposition of collagen I and III in the extracellular matrix, in the experimental model where there had been an abnormal deposition, but had no significant effect on normotensive rats (Mukherjee and Sen, 1990). When tested side-by-side, Captopril and Isinopril proved to reduce hypertrophy, blood pressure and collagen deposition in the experimental model, while not effecting collagen deposition in normotensive rats. Captopril, however, proved to reduce the collagen type I and III ratio in SHR, while Isinopril did not (Yang et al., 1997). These data suggest that hypertensive pathology in SHR is influenced not only by the quantity of collagen in, but on the quality of collagen. Other ACE inhibitors, such as Cilazapril, work in the same manner as Captopril, reducing blood pressure and left ventricular mass, but differ in the way the myocardial ultrastructure is altered post hypertension. Cilazapril seems to have a higher affinity for ACE and angiotensin II than does Captopril (Vulpis et al., 1995). In untreated SHR with established hypertension, multiple intracellular and extracellular defects, such as irregular mitochondrial distribution, malformed mitochondrial cristae,
myofibrils of varying size interspersed among the cell, abnormal intercalated discs, and irregular cellular junctions (Vulpis et al., 1995). Post Cilazapril treated SHR show no difference in intracellular or extracellular composition with age-matched WKY controls. Still other studies have been done where an ACE inhibitor is coupled with an angiotensin II receptor blocker (ARB). The premise for such studies is that during the occurrence of hypertrophy, volume and pressure overload are not the only factors at play. Accompanying volume or pressure overload is the activation of the sympathetic nervous system and the renin-angiotensin system to create the hypertrophy. Shikata et al. (Shikata et al., 2003) studied the effects of delapril, an ACE inhibitor, and cadesartan, an ARB on SHR. When used together delapril and cadesartan lowered blood pressure, and reduced heart weight in ovariectomized SHR, although heart rate was not lowered compared to WKY. Delapril and cadesartan also were shown to reduce fibrosis in treated SHR. The amount of collagen I, III, and V decreased in the extracellular matrix as a result of delapril treatment.

Vasopeptidase inhibitors (VPI) block the activity of ACE and neutral endopeptidase (NEP) and exhibit anti-hypertensive and anti-fibrotic effects in SHR. By targeting ACE, angiotensin I cannot be converted into angiotensin II, thereby depriving AT1 receptors of activation and inhibiting the production of transforming growth factor β (TGF-β), which helps to mediate the proliferation of extracellular matrix in SHR. TGF-β possesses a downstream effector called connective tissue growth factor (CTGF), which has been implicated in cases of cardiac fibrosis, and apparently corresponds not only to the presence of fibrosis, but also to the quantity, or degree of fibrosis present in the myocardium (Gronholm et al., 2004).
Collagen

Collagen is the main component of most connective tissues, infiltrating every tissue in vertebrate systems. Collagens form networks, fibers, and filaments which anchor cells and contribute to the structural integrity of the extracellular matrix. Of the twenty known types of collagen, five (types I, II, III, V, and XI) are fibrillar, and form striated fibers in various tissues, including areolar tissue, tendons, ligaments, bone, cartilage, and cardiac interstitium. Collagens compose roughly one-third of the proteins found in vertebrate species (Brown and Timpl, 1995). Fibrillar collagens are used as scaffolding proteins and used for cellular migration, cellular attachment and cellular differentiation, as well as transmission of loads, and redistribution of weight.

Each fibrillar collagen chain (~ 1000 amino acids) contains three hundred, or more, Gly-X-Y motifs with non-triplet telopeptides (~ 40 amino acids) at the end (Kreis and Vale 2000; Silver et al., 2002). The Glysine(Gly) molecule of the Gly-X-Y motif is a small amino acid that allows the superstructure of the alpha chain to bend. The Gly side chains are integral to collagen structure. If Gly is replaced by a larger side chain, the mutation results in an abnormal coil that cannot fold into a superhelix. Hydroxyproline residues are specific to collagen and are found on the Y position of the Gly-X-Y motif. Propyl residues at the Y position of the collagen Gly-X-Y motif are post-translationally hydroxylated into 4-hydroxyproline by propyl 4-hyroxlase, significantly increasing the thermal denaturation temperature of the triple helix (Kreis and Vale, 2000). Alternating sites of increased flexibility can be found along the length of the molecule (type I collagen) at locations (N-terminus to C-terminus) 30-45, 90-105, 50-157.5, 210-217.5, and 270-277.5nm. These more flexible locations on the collagen I molecule correspond
with areas containing no proline or hydroxyproline residues, which tend to inhibit flexibility (Hoffmann et al., 1984). A $D$-period of collagen consists of 12, positively staining (charged amino acid residues) regions which form a flexible point in the molecule, alternating with 13 inflexible regions (uncharged amino acid residues) (Silver et al., 2002). The polar amino acid residues visible on positive staining $D$-periods, as seen on electron micrographs, are thought to be the main location of elastic energy storage on the collagen molecule, especially in collagen I, which is thought to store energy during locomotion (Silver et al., 2002). Studies suggest that given the rigidity of collagen I, the main component of the collagen matrix in the myocardium, that myocardial stiffness due to a proliferation of collagen I and its inflexible nature will only compound the effects of chronic hypertension.

The collagen molecule is a triple-helix formed by three alpha chain subunits containing a (Gly-X-Y)$_n$ repeated sequence motif. Each alpha chain is coiled into an extended, left-handed, polyproline II helix. Three alpha chains are then twisted together into a right-handed superhelix which is surrounded by a shell of hydration that provides added stability (Kreis and Vale, 1999). Myocardial fibrillar collagens I and III possess distinctly different structures. Collagen I is a heterotrimer and may consists of two different alpha chains; two $\alpha$-1 chains and one $\alpha$-2 chain. Collagen III is a homotrimer, consisting of three coiled $\alpha$1(III) chains.

The architecture of the heart is maintained by an elaborate extracellular matrix containing a three-dimensional network of collagen. The cardiac collagen network exists at every level of myocardial organization. First, the heart is surrounded by a collagenous sheath called the pericardium. At the next level of organization, bundles of myofibrils
are surrounded by a collagen sheath called a perimysium. Finally, individual myofibrils are surrounded by an endomysium that also connects myofibrils. The perimysium and endomysium are composed primarily of type I and type III collagen, collagen I being the most abundant (Janicki and Matsubara, 1994). The perimysial infrastructure can take on one of two forms. One form of perimysial structure is a woven sheet of collagenous tissue encompassing bundles of myofibers, the other being coiled collagenous fibers running parallel along the length of the myofiber. Endomysial connective tissue exists as a lattice network and collagen struts. The collagen lattice network and the struts work together to protect cardiomyocytes from overstretching during cardiac systole and cushion the same cardiomyocytes during cardiac diastole (Janicki and Matsubara, 1994). The struts connect myocytes together, by way of an integrin insertion into the basal lamina via the sarcomere’s Z-band (Janicki and Matsubara, 1994), while the lattice network holds and links struts to the myoctes and large collagen fibers. Cardiomyoctes are also connected to capillaries in the same manner as myoctye to myocte connctcetions by collagen struts (Janicki and Matsubara, 1994).

**Estrogen**

Estrogen is a female sex hormone produced by the ovaries, and is responsible for female reproductive development and the presence of secondary sex characteristics. Estrogen has long been thought to play a protective role in certain pathologies, including cardiovascular disease and hypertension. This assertion is supported by research done using SHR. When heart failure was evaluated in the SHR model, males are shown to have heart failure starting a 16 months of age, while females present as asymptomatic at 22 months of age (Tamura et al., 1999; Du et al., 2006). Myocyte hypertrophy and left
ventricular dysfunction are also shown to be greater in males than in females (Tamura et al., 1999). While the exact mechanism of estrogen protection is unknown, several theories have been investigated. Some researchers believe that estrogen causes an elevation of high density lipoprotein (HDL) in the blood stream, and that estrogen promotes healthy functioning of endothelial cells (Paranjape et al., 2005; Mendelsohn and Karas, 1999; Barret-Connor, 1997). Still other studies report that estrogen may aid in cardiac recovery. Xu et al., in 2005 conducted a study aimed at the recovery aspect of estrogen in the case of cardiac ischemia and reperfusion, and proposed that estrogen works thorough its effects on tumor necrosis factor α (TNFα). The results were that ovariectomized rats that were given estrogen replacement therapy were more likely to recover post ischemia than ovariectomized female rats who were not give estrogen replacement therapy. This study suggests that estrogen does have a protective, though not exactly prophylactic effect (Xu et al., 2005).

Estrogen has also been thought to slow or reverse the effects of myocardial fibrosis by inhibiting collagen synthesis and fibroblast proliferation. Serum induced proliferation of fibroblasts and collagen synthesis has been inhibited by 17β-estradiol according to the concentration administered (Dubey et al., 1998; Du et al., 2006). Estrogen also acts to inhibit the effects of TGF-β by inhibiting collagen production initiated by the growth factor in murine mesangial cells (Lei et al., 1998; Silbiger et al., 1998; Du et al., 2006).

Estrogen replacement therapy has also been used in a laboratory setting to study the possible protective effects of estrogen. Research conducted on rats that have undergone aortic constriction to produce overload hypertrophy show that hypertrophy
was aggravated by ovariectomy, whereas estrogen replacement therapy reversed the damage (Skavadahl et al., 2005; van Eickels et al., 2001; Du et al., 2006). Studies performed on ovariectomized rats with volume overload hypertrophy caused by aorta-caval fistula, show a marked increase in left ventricular dilation and heart failure. Intact female rats, however, show that left ventricular function was retained after volume overload hypertrophy (Brower et al., 2003; Du et al., 2006).

Besides estrogen, androgens also have an affect on hypertension. The Y chromosome in male SHR has been shown to increase blood pressure (Ely & Turner, 1990). In this experiment, SHR and normotensive Wistar-Kyoto (WKY) rats were bred and blood pressure readings taken on the F1 generation from eight to twenty weeks of age. Male rats with a WKY mother and an SHR father displayed a significantly higher blood pressure than male rats with an SHR mother and a WKY father from 12 to 20 weeks of age. Likewise, the F2 generation of males that possessed the SHR Y chromosome displayed the same significantly different blood pressure when compared to male rats with the WKY Y chromosome (Ely & Turner, 1990). Similar studies featuring backcrossing of the F2 SHR Y chromosome into an autosomal WKY strain for the presence of the androgen receptor also showed a significant increase in blood pressure (Ely et al., 1993). These data suggest that androgens increase blood pressure and promote hypertension in the male rat.

**Sex-related Cardiovascular Pathology**

Cardiovascular disease is the greatest cause of morbidity and mortality in the world today (Wenger, 1995; Champney, 2005). Most, if not all of the research done on hypertension, as well as studies of many other disease processes, has consistently used
only male test subjects. Until a decade ago, little or no research had been done on the
effects of hypertension in females. The need for such studies is necessary due to the
number of women who are being affected by cardiovascular disease, of which
hypertension is a significant factor. It has been estimated that in the United States alone,
that there are 32 million women who present some form of cardiovascular disease (Bello
and Mosca, 2004). It is our hypothesis that given the difference between male and
female physiology, specifically the presence or absence of estrogen, that hypertensive
effects will manifest differently between the sexes, in terms of collagen deposition and
cardiac architecture.

Many studies have been conducted detailing sex related differences concerning
cardiovascular morbidity and mortality (Du et al., 2006-review). Clinical trials have
stated that men and post-menopausal women have an elevated risk for cardiovascular
disease and other cardiomyopathies, while the risk is decreased in pre-menopausal
women. These data suggest that there is a direct sex related difference in cardiovascular
pathology between men and women, specifically the presence or absence of estrogen,
which is widely believed to be protective (Du et al., 2006; Mendelsohn & Karas, 1999;
Dubey & Jackson, 2001b; Babiker et al., 2002-review). Women tend to express a lower
survival rate post-infarction than men, however, men are more likely to manifest heart
failure to a greater degree than in women (Du et al., 2006; Vaccarino et al., 2001).
Cardiac hypertrophy also manifests differently between the sexes. In clinical studies, in
women who exhibit pressure overload hypertrophy, the myocardium prefers concentric
hypertrophy with preserved left ventricular function. Men, however, tend to express
eccentric hypertrophy with a dialated left ventricle (Du et al., 2006; Aurigemma &
Gaasch, 1995). The incidence of myocardial fibrosis and abnormal collagen composition is also seen to be greater in male hearts than in female hearts (Villari et al., 1995; Du et al., 2006). There are also studies that demonstrate that suggest a gender difference in the chronic phase of cardiac pathology. Female mice that suffered from chronic myocardial infarction possess less remodeled left ventricular structure, and retained better myocardial function post-infarct than did males (Cavasin et al., 2003, 2004; Wu et al., 2003; Wakatsuki et al., 2004; Du et al., 2006). These data suggest a strong correlation between gender and the presence of estrogen in the pathological dimorphism of cardiovascular disease and other myocardial myopathies.

The purpose of this study is to characterize the collagen profiles for male, female, and ovariectomized female SHR, and the differences in collagen profile as pertains to gender. The myocardial collagen profile of SHR will be analyzed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Little or no research has been performed to exact a collagen profile in the second dimension.

We hypothesize that the collagen content and profile of SHR is different according to gender between male and female SHR, and that the collagen profile of ovariectomized females will be similar if not identical to that of males.
MATERIALS AND METHODS
The seven male, seven female and seven ovariectomized female spontaneously hypertensive animals used in this study were bred at Youngstown State University, housed in enriched cages with bedacob and shredded aspen bedding, given standard rat chow and water *ad libitum*, and placed on a standard 12 hour, reverse light and dark cycle. Seven female SHR were ovariectomized at 8 weeks of age. All animals in this experiment were euthanized at twelve months of age. The treatment and protocol of the animals used in this experiment complied with all IACUC guidelines.

All animals were euthanized and their hearts removed. The animals were deeply anesthetized with ketamine (50mg/100kg) and xylazine (8mg/20kg), and the heart harvested. The heart was perfused with saline solution, and the aorta, atria, and extra tissues removed, and the ventricles weighed. The ventricles were then cut transverse at the midline and the inferior half weighed, flash frozen and stored at -80°C for collagen analysis.

In preparation for collagen extraction, the ventricular tissue was minced and homogenized on ice in solution of cold phosphate buffered saline (PBS). The homogenate was then centrifuged at 15,000 rpm for 25 minutes at 4°C and the pellet washed 3 times with of 2% SDS by centrifuging at room temperature at 15,000 rpm for 10 minutes. The pellet was then washed 3 times in PBS and centrifuged at room temperature at 15,000 rpm for 10 minutes. Finally the pellet was washed in acetone, centrifuged at room temperature at 15,000 rpm for 10 minutes, and dried under air. The pellet was then suspended in 70% formic acid and cyanogen bromide and (CNBr) (25mg per mL), and incubated for 18 hours and then centrifuged for 30 minutes at 15,000 rpm. The supernatant was dialyzed and lyophilized. The lyophilized samples were
resuspended in rehydration buffer (8M urea, 50 mM DTT, 4% CHAPS, 0.0002% bromphenol blue) at a final concentration of 50mg wet weight of tissue / 10uL and stored at -20°C or used for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The reconstituted sample was brought up to a final volume of 125uL with modified sample buffer (2M thiourea, 7M urea, 4% CHAPS, 1% DTT) and 0.1% bromphonol blue, loaded onto a 7cm, 3 – 10pH Bio-Rad IPG strip, incubated overnight in the refrigerator, and focused the next day using a Bio Rad Protean IEF Cell at 20,000 volt hours, 4,000 volts rapid. The IPG strips were then equilibrated with equilibration buffer I (6M urea, 2%SDS, 0.375M Tris-HCl pH8.8, 20% glycerol, 800mg DTT) for 10 minutes while shaking gently. This was followed by a second 10 minute equilibration in equilibration II buffer (6M urea, 2%SDS, 0.375M Tris-HCl pH8.8, 20% glycerol, 1g iodoacetamide). The IEF strip was then laid onto a 12% SDS polyacrylamide gel and electrophoresed with tris-glycine buffer (tris, glycine, SDS) at constant current, 500 volts, 20 milli-amps per gel. The gels were stained with Coomasie Blue overnight and destained for 2-3 hours. The destained gel was then imaged using a scanner and on PDQuest (for master gels).

The collagen I 2D gel profile was characterized using rat tail collagen (Sigma #C7661). The collagen III 2D gel profile was characterized using bovine skin collagen (Chemicon #CC078). Collagen standards were prepared for electrophoresis as described above. Three gels were prepared in triplicate for collagen I and collagen III. These gels were imaged and compiled into a digital master gel for collagen I (300µg), and another for collagen III (300µg). These composite gels served as collagen I and collagen III standards.
In the third experiment, protein content in the combined samples was analyzed using the Bradford assay, and the samples normalized for protein concentration. The combined samples were derived from the same samples used in the second experiment, as detailed in the previous paragraph. Combined sample gels were run in triplet for each sample at a concentration of 70μg of protein per gel.

The triplet gels were then digitized using PDQuest imaging software. After the gels had been digitized, the images were cropped. The cropped images were then analyzed to set the parameters for the new match set. A faint spot, a small spot, and the largest cluster of spots were selected on the first cropped image, and those parameters applied to the subsequent two cropped gels to assemble a statistical master gel for the sample. Master gels were made in this fashion for pooled male samples, pooled female samples, and pooled OVX female samples. The coordinate system used in this study to denote particular spots on the master gels was devised by using a two point reference characterized by estimated pI and by calculated Rf values. Relative pI (Rpl) was also used to verify the location of a particular spot. Rpl was calculated by measuring from the pI origin along the x coordinate plane to the nearest edge of the spot, divided by the total distance from the furthest positive point of unfocused protein to the furthest negative point of unfocused protein.

All statistical analysis was performed using Sigma Plot software. A one-way ANOVA on ranks was performed on the results from the Bradford assay done after the second experiment. Post hoc testing was done according to Dunn’s method.
RESULTS
Characterizing the 2D-gel electrophoresis profile of collagen I and collagen III

In the first set of experiments, 2D-PAGE profiles of collagen I and collagen III were characterized. Each profile (Figures 1A, B and 2A, B) containing 300μg, was run in triplicate, and digitally merged into one collagen I master gel (Figure 2B), and one collagen III master gel (Figure 1B). The 2D gel electrophoresis profile of collagen I contained a total of sixteen distinctive spots (Figure 2). The pI and Rf values of these spots are shown in Table 1. The 2D gel electrophoresis profile of collagen III yielded a total of seven distinctive spots (Figure 1). The pI and Rf values of each spot are also shown in Table 1. Four spots in collagen III profile, A, B, E, and F (Figure 1B), are present in the collagen I profile (Figure 2B) suggesting that these spots represent the collagen α1 chain. Ten spots in the collagen I profile (g, h, i, j1, j2, k, l1, m, and n) were not found in the collagen III profile and potentially correspond to the collagen α2 chain.

Characterizing the 2D-PAGE of SHR myocardial collagen normalized for wet weight

The 2D gel electrophoresis profile of cardiac collagen was examined for male, female and OVX female SHR. The equivalent of 150mg (wet weight) cardiac tissue was processed and loaded onto each gel. As seen in Figure 3 the collagen profile of female SHR myocardial collagen was visibly lighter than the gels of either male or the OVX female SHR. In a similar manner, the collagen profiles of male and OVX gels were practically indistinguishable from each other, while both male and OVX female profiles were visibly darker than intact females. Protein analysis confirmed that the collagen-associated protein loaded onto the 2-D gel of females (53.3μg) was significantly less (p<0.05) than on 2D gels of males (95.0μg) or OVX females (142.3μg) (Figure 4).
Figure 1A. 2D-PAGE collagen III profile (300mg) stained with Coomasie Blue stain.

Figure 1B. Collagen III digital composite gel. Each spot on the composite gel was assigned a letter corresponding to specific pI and Rf values (see Table 1).
Figure 1A
Collagen III gel (300μg)

Figure 1B
Composite Collagen III Gel
**Figure 2A.** 2D-PAGE collagen I profile (300mg) stained with Coomasie Blue stain.

**Figure 2B.** Collagen I digital composite gel. Each spot on the composite gel was assigned a letter corresponding to specific pI and Rf values (see Table 1).
Figure 2A
Collagen I (300μg)

Figure 2B
Composite Collagen I Gel
Table 1. pI and Rf values for lettered spots in collagen I and collagen III composite gels.
Table 1

<table>
<thead>
<tr>
<th>Spot</th>
<th>Collagen I</th>
<th>Collagen III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pl</td>
<td>Rᵣ</td>
</tr>
<tr>
<td>A</td>
<td>5.8</td>
<td>0.67</td>
</tr>
<tr>
<td>B</td>
<td>6.0</td>
<td>0.68</td>
</tr>
<tr>
<td>C</td>
<td>6.8</td>
<td>0.59</td>
</tr>
<tr>
<td>D</td>
<td>7.6</td>
<td>0.62</td>
</tr>
<tr>
<td>E</td>
<td>7.5</td>
<td>0.27</td>
</tr>
<tr>
<td>F</td>
<td>7.5</td>
<td>0.16</td>
</tr>
<tr>
<td>g</td>
<td>9.5</td>
<td>0.14</td>
</tr>
<tr>
<td>h</td>
<td>6.9</td>
<td>0.26</td>
</tr>
<tr>
<td>i</td>
<td>8.0</td>
<td>0.24</td>
</tr>
<tr>
<td>j₁</td>
<td>9.0</td>
<td>0.28</td>
</tr>
<tr>
<td>j₂</td>
<td>9.0</td>
<td>0.24</td>
</tr>
<tr>
<td>k</td>
<td>7.0</td>
<td>0.42</td>
</tr>
<tr>
<td>l</td>
<td>8.0</td>
<td>0.4</td>
</tr>
<tr>
<td>m</td>
<td>8.6</td>
<td>0.43</td>
</tr>
<tr>
<td>n</td>
<td>9.4</td>
<td>0.42</td>
</tr>
<tr>
<td>o</td>
<td>9.5</td>
<td>0.62</td>
</tr>
</tbody>
</table>
Figure 3A. 2D-PAGE profile of male 12 month old SHR, representing the myocardial collagen profile in 150mg of cardiac tissue (wet weight). Collagen associated protein on gel was 92μg as determined by Bradford assay.

Figure 3B. 2D-PAGE profile of female 12 month old SHR, representing the myocardial collagen profile in 150mg of cardiac tissue (wet weight). Collagen associated protein on gel was 65μg as determined by Bradford assay.

Figure 3C. 2D-PAGE profile of OVX female 12 month old SHR, representing the myocardial collagen profile in 150mg of cardiac tissue (wet weight). Collagen associated protein on gel was 181μg as determined by Bradford assay.
**Figure 3A: 2D Gel Profile Male**

150mg cardiac tissue
Collagen associated protein (92μg)

**Figure 3B: 2D Gel Profile Female**

150mg Cardiac tissue
Collagen associated protein (65μg)

**Figure 3C: 2D Gel Profile OVX Female**

150mg cardiac tissue
Collagen associated protein (181μg)
**Figure 4.** Mean protein content in CNBr digest of SHR hearts. All samples were normalized to 50mg wet weight of tissue per 10μL. Female vs male, p<0.05; female vs OVX female, p<0.05 (ANOVA, Dunn’s post hoc test).
Protein (μg/mL)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
<th>140</th>
<th>160</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Female VS Male (p<0.05)
+ Female VS OVX Female (p<0.05)
In the third set of experiments male, female and OVX female samples were combined to generate a two dimensional collagen profile for each group. Each combined sample was run in triplicate (70μg protein per gel) and a composite profile generated. The profiles of the pooled sample gels were virtually identical to that of their counterparts in the previous section (Figure 1, 2, 3A,B,C). When compared to each other, however, these gels presented markedly different profiles.

The composite 2D profile of male SHR exhibited twenty three spots. Four spots, A, B, E and F were similar or consistent with spots A, B, E and F associated with the α1 chain and found in collagen I and collagen III profiles (Figure 1, 2). Three spots, k, l1 and i were specific to the collagen I profile and may represent the α2 chain were found in the male heart profile (Table 2; Figure 5A). Spots h, j1, j2, and k, found in the collagen I profile, are believed to represent the collagen α2 chain are not visible in the composite. The remaining sixteen spots in the male composite cardiac collagen profile could not be correlated to any spot in the collagen I or collagen III profile but were present in either female or OVX female SHR as discussed below.

The composite 2D profile of female SHR exhibited nineteen spots. Three spots, A, B, and E were consistent with spots A, B and E associated with the α1 chain and found in collagen I and collagen III profiles (Figure 1, 2). One spot, h, was specific to the collagen I profile and may represent the α2 chain and was found in the female heart profile (Table 2; Figure 5B). Spots g, i, j1, j2, k, and l1 corresponding to the collagen I α2 chain were not found in the female profile. The remaining fifteen spots in the female
composite gel could not be correlated to the collagen I and collagen III profile but were present in either the male or the OVX female SHR, and will be discussed later.

The composite 2D profile of OVX female SHR exhibited twenty three spots. Four spots, A, B, E and F were similar or consistent with spots A, B, E and F associated with the $\alpha_1$ chain and found in collagen I and collagen III profiles (Figure 1, 2). Four spots, h, i, k, and l, which appear in the collagen I profile and may represent the collagen I $\alpha_2$ chain are also found in the OVX female heart profile (Table 2; Figure 5C). The remaining fifteen spots could not be correlated to the collagen I and collagen III profile but were present in either male or female SHR.

A comparison of composite male, female and OVX female collagen profiles showed that a core of peptide fragments could be identified in all gels. Spots A, B and E were found in males, females and OVX females, however, spots A and B, were slightly larger and darker in OVX females as compared to males and females. Spot F in males and OVX females were similar to spot F found in collagen I and collagen III, however, spot F does not appear in the 2D gel profile of the pooled female sample. Spots m and n were present in all pooled samples. Spot m corresponded to spot m of the collagen I $\alpha_2$-chain and spot n (males; pI = 8.7; R$_f$ = 0.39), (females; pI = 8.9; R$_f$ = 0.38), (OVX females; pI = 9.0; R$_f$ = 0.36) were similar to spot m of the collagen I $\alpha_2$ chain (pI = 9.4; R$_f$ = 0.42).

Some spots in the 2D gel profiles of pooled samples were similar but not equivalent to those found in the profiles of the collagen I and collagen III standards. For example, spots C and D in males (C$_M$: pI = 7.0; R$_f$ = 0.49) (D$_M$: pI = 8.1; R$_f$ = 0.51),
Figure 5A. Male, 12 moth old, SHR digital composite gel representing 70μg protein. Each spot on the composite gel was assigned a letter corresponding to specific pI and Rf values (see Table 2).

Figure 5A. Female, 12 moth old, SHR digital composite gel representing 70μg protein. Each spot on the composite gel was assigned a letter corresponding to specific pI and Rf values (see Table 2).

Figure 5A. OVX female, 12 moth old, SHR digital composite gel representing 70μg protein. Each spot on the composite gel was assigned a letter corresponding to specific pI and Rf values (see Table 2).
Figure 5A

2D Gel Profile of pooled Male (70 μg protein)

Figure 5B

2D Gel Profile of pooled Female (70 μg protein)

Figure 5C

2D Gel Profile of pooled OVX female (70 μg protein)
Table 2. pI and Rf values for lettered spots in male, female and OVX female pooled sample composite gels.
Table 2

<table>
<thead>
<tr>
<th>Spot</th>
<th>Male</th>
<th>OVX Female</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pI</td>
<td>Rf</td>
<td>pI</td>
</tr>
<tr>
<td>A</td>
<td>5.8</td>
<td>0.66</td>
<td>5.8</td>
</tr>
<tr>
<td>B</td>
<td>6.1</td>
<td>0.66</td>
<td>6.2</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>0.49</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>8.1</td>
<td>0.51</td>
<td>8.1</td>
</tr>
<tr>
<td>E</td>
<td>7.7</td>
<td>0.26</td>
<td>7.8</td>
</tr>
<tr>
<td>F</td>
<td>8</td>
<td>0.16</td>
<td>8</td>
</tr>
<tr>
<td>g</td>
<td>8.6</td>
<td>0.17</td>
<td>8.9</td>
</tr>
<tr>
<td>h</td>
<td>X</td>
<td>X</td>
<td>7.1</td>
</tr>
<tr>
<td>i</td>
<td>8.1</td>
<td>0.28</td>
<td>8.1</td>
</tr>
<tr>
<td>k</td>
<td>7.4</td>
<td>0.41</td>
<td>7.5</td>
</tr>
<tr>
<td>l₁</td>
<td>8</td>
<td>0.39</td>
<td>8</td>
</tr>
<tr>
<td>l₂</td>
<td>X</td>
<td>X</td>
<td>8.1</td>
</tr>
<tr>
<td>m</td>
<td>8.6</td>
<td>0.45</td>
<td>8.5</td>
</tr>
<tr>
<td>n</td>
<td>8.7</td>
<td>0.39</td>
<td>9</td>
</tr>
<tr>
<td>p</td>
<td>5.5</td>
<td>0.41</td>
<td>5.8</td>
</tr>
<tr>
<td>q</td>
<td>5.7</td>
<td>0.77</td>
<td>5.5</td>
</tr>
<tr>
<td>r</td>
<td>7.7</td>
<td>0.78</td>
<td>7.8</td>
</tr>
<tr>
<td>s</td>
<td>8.7</td>
<td>0.82</td>
<td>8.9</td>
</tr>
<tr>
<td>t</td>
<td>5.7</td>
<td>0.58</td>
<td>5.8</td>
</tr>
<tr>
<td>u</td>
<td>7.5</td>
<td>0.66</td>
<td>7.5</td>
</tr>
<tr>
<td>v</td>
<td>8.1</td>
<td>0.55</td>
<td>8.1</td>
</tr>
<tr>
<td>w</td>
<td>6.7</td>
<td>0.54</td>
<td>6.6</td>
</tr>
<tr>
<td>x</td>
<td>7.8</td>
<td>0.54</td>
<td>X</td>
</tr>
<tr>
<td>y</td>
<td>8.6</td>
<td>0.58</td>
<td>X</td>
</tr>
<tr>
<td>α</td>
<td>8.2</td>
<td>0.53</td>
<td>X</td>
</tr>
<tr>
<td>β</td>
<td>X</td>
<td>X</td>
<td>7.2</td>
</tr>
<tr>
<td>γ</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>δ</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
females (CF: pI = 7.0; Rf = 0.48) (DF: pI = 8.1; Rf = 0.51), and OVX females (COVX: pI = 7.0; Rf = 0.47) (DOVX: pI = 8.1; Rf = 0.51) were similar to spots C and D of collagen I (CI: pI = 6.8; Rf = 0.59) (DI: pI = 7.6; Rf = 0.62) and collagen III (CIII: pI = 6.5; Rf = 0.61) (DIII: pI = 8.1; Rf = 0.61) standards, but varied slightly with regards to Rf. Some spots were found in the 2D gel profiles of pooled samples but were not visible in the gels of collagen I and collagen III standards. Spots p, q, r and s, which fell into this category, were found in all pooled samples and appeared to be a cluster of two or three proteins of similar pI and Rf value. Spot q in males (pI = 5.7; Rf = 0.77), and OVX females (pI = 5.5; Rf = 0.79) were located in the same place in both pooled samples, however, in females spot q (pI = 5.0; Rf = 0.85) migrated further in the gel. Spot w is also common in all pooled samples but varies somewhat among groups. In females and OVX animals, the pI and Rf of spot w are similar (wF: pI = 6.5; Rf = 0.45) (wOVX: pI = 6.6; Rf = 0.42) (wM: pI = 6.7; Rf = 0.54), however, in males, spot w has the same pI as females and OVX females, but the Rf is larger suggesting a lower molecular weight.

There are also a series of spots in the pooled sample gels that are visible in only two of the three groups. For example seven spots, F, i, k, l1, t, u, and v are visible in males and OVX females, but not in intact females. Spot F, as discussed previously, represents one of the core peptide fragments common to both collagen I and collagen III. Spot i corresponded to spot i in the collagen I α2-chain. Spot k in males, pI = 7.4; Rf = 0.41 and OVX females, pI = 7.5; Rf = 0.39, is comparable to spot k in collagen I a2-chain (pI = 7.0; Rf = 0.42), however, the pI is a little greater in males and OVX females than in collagen I. Spot l1 correspond to l1 in collagen I α2-chain. Finally, spots t, u, and v,
which are found in males and OVX females, are not found on either collagen I or collagen III profiles. Spot t in males also appears larger than spot t in OVX females.

There are a second group of peptide fragments that are present in females and OVX females, but are absent in males. One of these fragments, spot h, has the same characteristics as spot h in the collagen I ā2-chain. A second peptide fragment, spot l2 is found in females and OVX females only and does not have any counterpart in the collagen standards. Spots l1 and l2 are in a similar location on the gel, however, the larger pI value coupled with the presence of l1 and l2 together in OVX females, suggests that l2 is a different protein, and is not related to either collagen I or collagen III.

Some peptide fragments are found only in males and females and not in OVX animals. Spots x and y in males (x: pI = 7.8; Rf = 0.54) (y: pI = 8.6; Rf = 0.58) and females (x: pI = 7.8; Rf = 0.52) (y: pI = 8.8; Rf = 0.56) are similar to each other, but do not correspond to any spots on the OVX female, collagen I or collagen III gels.

Lastly, there are also spots that are unique to a single group and do not correspond to any spot on collagen I or collagen III standards. Spots α in males (pI = 8.2; Rf = 0.53), β in OVX females (pI = 7.2; Rf = 0.28), and γ and δ in females (γ: pI = 6.7; Rf = 0.24) (δ: pI = 6.0; Rf = 0.73) are unique to their respective groups.
In the first experiment which provided 2D gel profiles for collagen I and collagen III standards one is able to identify spots that correspond to the $\alpha_1$ and $\alpha_2$ chains of their respective proteins. Since collagen III is a homotrimer, comprised of three $\alpha_1$ chains, all of the spots represent CNBr generated peptide fragments of $\alpha_1$ chain. The spots, A, B, E, and F are comparable to collagen I and collagen III gels, and are therefore considered the common collagen core of the $\alpha_1$ chain that exists within both collagen I and collagen III. The remaining spots observed only in the collagen I master, g, h, i, j1, j2, k, l, m, n, and o represents the elements of the collagen I $\alpha_2$ chain of the collagen I heterotrimer ($2\alpha_1$ chains, $1\alpha_2$ chain). These observations are consistent with previous experiments (Kreis and Vale, 1999). The spot observed between fragments C and D in the collagen III profile is not seen in either the collagen I profile and is presumed to be a collagen III-associated protein, and can be considered unique to collagen III as that it is not visible in collagen I. Spots C and D themselves are thought to be part of the common collagen core of the $\alpha_1$ chain, but could not be verified as such by calculation of relative pI (Rpi), or the percentage of distance a spot’s pI is from the origin in relation to the total distance of focused protein. Post-translational modifications may also account for the discrepancy in Rpi as it differs from the collagen I gel.

The second experiment, which compared collagen profiles of 150mg of heart tissue revealed that the intact female possessed significantly lesser concentration of collagen than either males or OVX females. The profile of all male gels and all OVX female gels were virtually indistinguishable with regards to visual density, indicating that the cardiac collagen content in these animals is similar. In comparison, the collagen profile of intact female gels was lighter, the spots smaller, and total number of spots
present were fewer. These data suggest that there was not an equivalent amount of collagen on each gel despite the fact that each gel contained an equivalent amount of cardiac tissue. The female gels possessed considerably less collagen and collagen associated protein than either the male or OVX female gels. The male gels and OVX female gels appear identical in visual density and profile, except for a few key spots (detailed in the third experiment). These data also suggest the protective role of estrogen during hypertension by reducing the amount of collagen produced in the female SHR, and attenuating myocardial fibrosis (Dubey et al., 1998; Du et al., 2006). The extent of hypertrophy seen in female SHR may also be reduced by the presence of estrogen, given the presence of lighter and fewer spots compared to male and OVX female animals (Brower et al., 2003).

In the third experiment, the CNBr digests of each group were pooled to form three composite samples, a pooled male sample, a pooled female sample, and a pooled OVX female sample. A comparison of collagen I and collagen III master gels and male, female, and OVX female masters suggests that collagen I and collagen III are present in all of the pooled samples. Four distinctive spots, A, B, E and F are found in all of the pooled samples (except in females where there is no spot F), corresponding α1 chain found in collagen I and collagen III. Therefore, collagen III has been identified in all of the pooled samples. Spot E is larger and more visually dense in OVX females than in either males or intact females, suggesting that the absence of estrogen may bring about change in the amount of peptide cleaved by CNBr along the α1 chain, or a higher translational effect upon removing estrogen. Spot F is present in males and OVX
females, but is absent in females suggesting that estrogen may play a role in the cleaving of this peptide fragment.

Moreover, spots corresponding to the $\alpha_2$ chain in collagen I have been identified in all of the pooled samples. These $\alpha_2$ chain spots vary between groups, and represent sex-related differences in the quality, such as possible post-translational modifications (methylation, glycosylation, or addition of R-groups) of collagen. Males and OVX female gels possess spots $g$, $i$, $k$, $l_1$, and $m$, of the collagen I $\alpha_2$ chain. Females gels are missing spots $i$, $k$, and $l_1$ of the collagen I $\alpha_2$ chain. Spot $g$ and spot $m$ which are present in all groups, with no great variance in pI or R$_f$ value, are therefore not considered to be a sex difference, however, spots $h$ and $l_2$ are seen only in intact females and OVX females suggesting that these peptide fragments may represent a sex-based difference, but not necessarily from the removal of an estrogen source. Spots $h$ and $l_2$ may be more a function of female protein expression and not necessarily due to the removal of estrogen. Spots $h$ and $l_2$ being present only in intact females and OVX females, may be estrogen-dependent in its formation (pre-OVX), but may not need estrogen for maintenance. Spots $i$, $k$, and $l_1$ corresponding to the collagen $\alpha_2$ chain, are found only in males and OVX females suggesting that the expression of these protein fragments is estrogen-dependent, and that with the removal of estrogen the female profile can be converted into a profile that more resembles a male. The presence of both $\alpha_1$ and $\alpha_2$ chain spots in male and OVX female combined samples also suggest that collagen I is being produced in a greater quantity than in the intact females, as there are fewer spots corresponding to collagen $\alpha_2$ in intact females. These data suggest that collagen composition may also be dependent on estrogen (Villari et al., 1995; Du et al., 2006).
Four peptide fragments, \( p \), \( q \), \( r \), and \( s \) are found in all of the composite group gels, but absent from either collagen standard. These four spots of high molecular weight peptides are similar in male and OVX female. Spot \( w \) in males has a greater Rf and therefore a larger molecular weight, suggesting there is a sex-related difference, however, that difference may be due to male protein expression and not to a lack of estrogen, as spot \( w \) is present in OVX females, while smaller than spot \( w \) in intact females, has a lower Rf value than that in the male sample. Spot \( q \) in intact female samples possesses a slightly larger molecular weight and lower pI than males or OVX females. This may be due to a post-translational modification unique to the estrogen bearing animal for that particular protein fragment.

Some spots are found in only two of the combined samples, but not in the third. Spot \( F \), part of the \( \alpha_1 \) core is present in males and OVX females, but not in the combined female sample. Spots \( i \) and \( j \) in males and OVX females are part of the collagen I \( \alpha_2 \) chain. Spot \( k \) in males and OVX females corresponds to the collagen I \( \alpha_2 \) chain, with a slightly greater pI in the animal samples than in the collagen I standards, due possibly to a post-translational modification to the \( \alpha_2 \) chain. Spot \( l_1 \) corresponding to the collagen I \( \alpha_2 \) chain, also is present in males and OVX females, but not in intact females. These data reiterate the idea presented previously that there is a greater amount of collagen I made in male and OVX females, as the presence of more \( \alpha_2 \) chain fragments can be seen in male and OVX animals, but also that there are qualitative differences that manifest between the sexes after the removal of the normal estrogen source.

Spots \( t \), \( u \) and \( v \) are also present in male and OVX female profiles, but not in the intact female profile, nor are they seen in either collagen I or collagen III standards.
While there is no link between either the collagen $\alpha_1$ or $\alpha_2$ chain and these three spots, they may constitute a sex difference made when estrogen is removed from the female animals. It is also possible that spots t, u and v represent other acid soluble proteins. Spots x and y are only present in male and intact females, but not in OVX females, or either collagen I or collagen III standards, which may suggest that the removal of estrogen from the female SHR produced a lack in these fragments, however, the presence of x and y in males does not suggest this is due to the absence of estrogen.

In summary, it has been shown that both collagen I and III $\alpha_1$ chain (spots A, B, E and F) and the collagen I $\alpha_2$ chain (spots h, i, k, l) can be found in male, female and OVX female 2D-PAGE profiles. It has also been shown that in 150mg of cardiac tissue, intact females have less collagen concentration than do males or OVX females. Moreover, the male and OVX female 2D-PAGE profiles were visibly darker, and possessed a spot pattern that was virtually indistinguishable from each other, while markedly darker and distinct from the profile of females. Finally, it has been shown that when normalized for protein concentration (70\,$\mu$g per gel), the pooled male profile and the pooled OVX female profile were more visibly similar than male compared to female profiles, or OVX female compared to intact female profiles. Male and OVX female pooled profiles possessed more of the same spots than each group compared to the intact female profile. Other spots that cannot be seen in the collagen I and collagen III standard profiles were observed in the animal profiles and may be attributed to acid soluble proteins found in the SHR myocardium, or collagen associated proteins unique to the SHR myocardium. Other spots possessed by the SHR profiles that can be seen in the collagen I and collagen III standard profiles, but differ slightly with regards to $pI$ and $R_f$.
value may be attributed to post-translational modification of those $\alpha_1$ and $\alpha_2$ chain proteins.
References


Carabello B, MD, FACC. Concentric Versus Eccentric Remodeling 2002. *J. of Cardiac Failure* 8(6); S258-S263.


Conrad C, MD, PhD, Brooks W, DSc, Hayes J, MD, Sen S, PhD, DSc, Robinson K, MT, Bing O, MD. Myocardial Fibrosis and Stiffness With Hypertrophy and Heart Failure in the Spontaneously Hypertensive Rat 1995. *Circulation* 91;161-170.


Ely DL, Turner ME. Hypertension in the spontaneously hypertensive rat is linked to the Y chromosome 1990. *Hypertension* 16; 277-281.


Forman D, MD, FACC, Cittadini A, MD, Azahar G, MD, Douglas P, MD, FACC, Wei J, MD, PhD, FACC. Cardiac Morphology and Function in Senescent Rats: Gender-Related Differences 1997; *JACC 30*(7); 1872-1877.


Janicki J, Matsubara B. Myocardial Collagen and Left Ventricular Diastolic Dysfunction 1994; In: *Left Ventricular Diastolic Dysfunction and Heart Failure*; 125-137.

Kannel W B, Schatzkin A. Sudden death: lessons from subsets in population studies 1985. *J Am Coll Cardiol 5*; 141B-149B.


Wednesday, August 29, 2007

Dr. Krontiris-Litowitz
Biology Department
UNIVERSITY

Re: IACUC Protocol # 06-07
Title: Characterizing Gender-Based Differences in Ventricular Hypertrophy of SHR

Dear Dr. Krontiris-Litowitz:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled "Characterizing Gender-Based Differences in Ventricular Hypertrophy of SHR" and determined it should be unconditionally approved for the period of 5/21/07 through its expiration date of 5/21/2010.

This protocol is approved for a period of three years; however, it must be updated twice via the submission of an Annual Update form prior to its annual expiration date 5/21/08 and 5/21/09. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kasvinsky
Associate Provost for Research
Research Compliance Officer

PJK:dk

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
    Dr. Robert Leipheimer, Chair IACUC, Chair Department of Biological Sciences
    Dawn Amolsch, Animal Tech., Biological Sciences