# Quantitation of Ventricular Collagen in Male and Female Spontaneously Hypertensive Rats Using Hydroxyproline Analysis

by

Lisa Tofil

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

July, 2010

**THESIS APPROVAL AND RELEASE FORM**

Quantitation of Ventricular Collagen in Male and Female Spontaneously Hypertensive Rats Using Hydroxyproline Analysis

Lisa Tofil

I hereby release this thesis to the public. I understand that 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:

_____

Lisa Tofil, Student Date

Approvals:

_____

Dr. Johanna Krontiris-Litowitz, Thesis Advisor Date

_____

Dr. Diana Fagan, Committee Member Date

_____

Dr. Gary Walker, Committee Member Date

_____

Peter J. Kasvinsky, Dean of School of Graduate Studies and Research Date

# Abstract

Hypertension, or chronically elevated blood pressure, can result in altered cardiac function and structure. The key structural alteration associated with hypertension is left ventricular hypertrophy. This increase in myocardial mass is based upon an increase in myocyte size and collagen deposition in the heart. Hydroxyproline, a nonessential amino acid, is found primarily in collagen. As a result, collagen content can be determined by measurement using a modification of Reddy and Enwemeka's hydroxyproline assay (1996). We tested the hypothesis that the ventricular collagen content in the hypertrophied ventricles of males is greater than in females. The results from the experiment demonstrate that there is a difference in the amount of cardiac collagen content between 18 month male and female spontaneously hypertensive rats (SHRs). The mean ± SEM for the cardiac collagen content in males was found to be 41.178 (± 11.872) µg collagen/mg weight wet ventricle (n= 7) versus 30.196 (± 1.863) µg collagen/wet weight ventricle (n=7) in females.

# Table of Contents

Abstract…… iii

Table of Contents……..... iv

List of Figures……v

List of Tables……..... vi

Chapters

Introduction…….....1

Materials and Methods……...17

Results…….....21

Discussion…….....34

References…….....38

Appendix……43

# List of Figures


| Figure | | Page No. |
|---|---|---|
| Figure #1. | Effect of NaOH on hydroxyproline standard curve | 22 |
| Figure #2. | Typical hydroxyproline standard curve | 27 |
| Figure #3. | Comparison of ventricle weight/body weight ratio | 29 |
| Figure #4. | Comparison of total protein/ventricular weight | 30 |
| Figure #5. | Comparison of µg OH-Pro/mg protein | 31 |
| Figure #6. | Comparison of µg collagen/mg protein | 32 |
| Figure #7. | Comparison of µg collagen/mg wet weight ventricle | 33 |

# List of Tables


| Table | | Page No. |
|---|---|---|
| Table #1. | Hydroxyproline assay slope and $r^2$ values | 24 |
| Table #2. | Effect of rehydration buffer on hydroxyproline assay | 25 |
| Table #3. | Hydroxyproline standard curve concentrations and absorbance values | 26 |

# Introduction

## Collagen Function and Location in the Extracellular Matrix

Collagen is a structural molecule located in the extracellular matrix (Brodsky and Persikov, 2005). This family of proteins consists of three alpha polypeptide chains which, when assembled together, form a right-handed triple helix (Eghbali and Weber, 1990). Collagen can be divided into two classes, fibrillar and nonfibrillar based on its ability to form fibrils. There are currently 27 different types of human collagen that have been identified (Brodsky and Persikov, 2005). Each type of collagen performs a specialized function in various tissues of the body. The major types of collagen are the fibrillar types I, II, and III and the minor types include types V and XI (Brodsky and Persikov, 2005). These different collagen types provide tensile strength to bones, cartilage, skin, tendons, ligaments and other tissues. About 25% of the total body protein in humans is comprised of collagen, making it the most abundant protein found in the human body (Brodsky and Persikov, 2005).

The arrangement of myocardial collagen consists of three components. The entire myocardium is enclosed by a connective tissue sheath called the epimysium. This connective tissue sheath protects cardiac myocytes and their sarcomeres from being overstretched beyond their highest force production (Eghbali and Weber, 1990). Groups of myocytes are surrounded by the perimysium and individual myocytes are surrounded by the endomysium. Therefore, the perimysium connects the epimysium to the endomysium. Collagen types I and III can be found in both the perimysium and endomysium of the myocardium. The collagen fibers in the myocardium consist of densely packed fibrils that have a diameter between 30-80nm (Debessa et al., 2001). In humans, collagen I has the appearance of thick strong fibers, as opposed to type III fibers which appear thinner and weaker (De Souza, 2002).

The interstitial collagen matrix performs numerous important functions in the myocardium. One of its main functions is to enclose and support cardiac myocytes (Debessa et al., 2001). Myocytes account for 25% of all the cells within the myocardium and occupy 70% of the myocardial mass (Zak, 1973). The collagen matrix also maintains the structural design of the heart throughout the cardiac cycle, as well as myocyte alignment by joining myocytes to one another and to adjacent capillaries. In addition to its structural and supportive roles, the collagen matrix is also a determinant of ventricular diastolic function and myocardial stiffness (Debessa et al., 2001) (De Souza, 2002).

**Regulation of Collagen Formation with Age**

Collagen is the only protein showing definite age changes (Debessa et al., 2001). During normal aging, a certain degree of interstitial fibrosis can be seen in the heart. This is due to a decrease in collagen degradation, which results in increased amounts of interstitial collagen (Lakhan and Harle, 2008). This excess in fibrous connective tissue results in thickening of the heart valves from improper growth of cardiac fibroblasts. In pathological conditions, such as hypertension, myocardial fibrosis is one of the components of myocardial remodeling that occurs in response to reduced vascular compliance (Lakhan and Harle, 2008). Studies have shown that angiotensin II plays a critical role in myocardial fibrosis (Campbell et al., 1995). Angiotensin II induces proliferation of fibroblasts, stimulates aldosterone, and affects collagen turnover, resulting in increased amounts of collagen and fibrosis (Campbell et al., 1995).

In humans it has been shown that collagen content and crosslinking increases with age (De Souza, 2002). The collagen fibers in young hearts are composed of fibrils that are loosely packed with a diameter ranging from small to medium to large (Debessa et al., 2001). Whereas,

in older hearts the collagen fibers are densely packed and are composed of fibrils that have a large diameter (Debessa et al., 2001). A study conducted on human hearts ranging from 20-87 years of aged showed a significant increase in collagen content in the left ventricular myocardium from adulthood to old age (Debessa et al., 2001). In young hearts the collagen content was 3.92 ± 0.8% and the collagen content obtained in old hearts was 5.86 ± 0.81% (Debessa et al., 2001). Studies in rats also provide consistent evidence of an increase in myocardial collagen associated with aging (Debessa et al., 2001). As the heart ages, collagen accumulates in the ventricle walls (De Souza, 2002). This accumulation of collagen may be due to the loss of myocytes that occurs during aging, since myocytes are postmitotic cells and are, therefore, not replaced as they die (Debessa et al., 2001). Another mechanism for collagen accumulation with age could be inhibition of collagen degradation (Debessa et al., 2001). This may be due to an increase in systolic blood pressure resulting from age related structural changes that occur in the walls of blood vessels (Debessa et al., 2001). This rise in systolic blood pressure with age may be a contributing factor to the age related hypertrophy observed in the left ventricle (Fleg, 1986).

**Collagen Composition in the Heart**

The fibrillar collagen types I and III, the major components of the heart's collagen matrix, are produced by cardiac fibroblasts (Eghbali and Weber, 1990). Type I collagen is the most common type of collagen. Its main function is to provide resistance to tension. It represents about 85% of the total collagen protein in the myocardium (De Souza, 2002). It is mainly distributed in the capsules of organs, in the tendons, dermis, bone, and fibrous cartilage and is produced by fibroblasts, osteoblasts, and odontoblasts (Eghbali and Weber, 1990). Type III

collagen represents 11% of the total collagen protein in the myocardium and is most abundant in the uterus, arteries, and muscle (De Souza, 2002). This type of collagen provides structural maintenance in expansible organs and is produced by smooth muscle cells, fibroblasts, and liver cells (Eghbali and Weber, 1990).

**Collagen Structure**

The primary structure of collagen consists of three alpha (α) polypeptide chains (two α 1 chains and one α 2 chain). These chains coil around each other to form a right-handed triple helix. Each collagen α chain has a repeating Gly-X-Y sequence. Of this triplet, glycine is found in every third position due to its small side chain, which is small enough to fit the space in the center of the triple helix. Proline is often found in the X-position and hydroxyproline (OH-Pro) is found in the Y-position. Gly-Pro-OH-Pro is the most rigid and most stabilizing sequence of collagen (Brodsky and Persikov, 2005).

**Collagen Synthesis**

The synthesis of collagen occurs in two main steps. The first step is the intracellular synthesis and secretion of procollogen. In this step, the proteins of the 3 alpha-chains (2 alpha-1 and 1 alpha-2 chains) undergoes transcription, translation, and post-translational modifications in the rough endoplasmic reticulum (RER). These post-translational modifications include glycosylation and hydroxylation. In glycosylation the carbohydrates galactose and glucose are added to hydroxylysine by hydroxylysyl galactosyltransferase. Lysine and proline are hydroxylated to 4-hydroxyproline and hydroxylysine by prolyl 4- hydroxylase and lysyl hydroxylase. Hydroxylation requires vitamin C, ascorbic acid, as a cofactor. A deficiency in

vitamin C can lead to a condition known as scurvy. Scurvy can lead to inadequate collagen production resulting in blood vessels, tendons, and skin to become fragile due to the loss of the structural support that collagen provides (Brodsky and Persikov, 2005). This is due to the degradation of non-hydroxylated procollagen within the cell. Mannose is added to the N- and C-terminal propeptides and disulfide bonds between these propeptides align the three chains to form the triple helix. Procollagen then goes to the Golgi apparatus and exits the cell by exocytosis.

The second step in collagen assembly is the extracellular cleavage to tropocollagen and the formation of collagen fibrils. In this step procollagen peptidases cleave the N- and C-terminal propeptides now forming the triple helix known as tropocollagen. Crosslinking then occurs, which stabilizes the fibrils by connecting them to neighboring fibrils, making the collagen fibers unable to be easily degraded (McCormick and Thomas, 1998). Crosslinks between collagen fibers are initially transient links between two collagen molecules. With time they are replaced by covalent non-reducible bonds in a reaction catalyzed by lysyl oxidase. Lysyl oxidase requires copper as a cofactor during the development of the crosslinks (McCormick and Thomas, 1998). Studies have shown that copper deficient newborn rats displayed reduced crosslinking in the myocardium and also developed an increase in soluble collagen, leading to the formation and rupture of a ventricular aneurysm. This demonstrates that normal crosslinking of fibrils is essential to the growth of normal tissues (McCormick and Thomas, 1998) (De Souza, 2002). Abnormal crosslink formation leads to aortic rupture in Ehlers Danlos syndrome type IV, which occurs as a result of abnormal crosslinking due to mutations in type III collagen. Abnormal crosslinking also leads to osteogenesis imperfect (OI). OI causes bones to become

brittle as a result of mutations in type I collagen, which is the main structural protein in bone (Brodsky and Persikov, 2005).

**Specificity of Hydroxyproline as a Measure for Collagen**

Collagen is the only protein that contains a high content of hydroxyproline (Brodsky and Persikov, 2005). Research has shown that in vertebrate animals most of the hydroxyproline can be found in collagen. Collagen removed from skin was shown to contain 10-14g hydroxyproline/100g protein for various species (Edwards and O'Brien, 1980).

Hydroxyproline, which is critical for collagen stability, is an amino acid which is not present in other proteins (Brodsky and Persikov, 2005). It is formed as a post-translational product from the hydroxylation of proline by the enzyme prolyl-4-hydroxylase. Studies have shown a decrease in collagen stability when hydroxylation of proline is absent (Perret et al., 2001). The presence of prolyl-hydroxylase inhibitors results in a decrease in stability when unhydroxylated collagen is synthesized (Sakikabara et al., 1973).

The main type of hydroxyproline found in collagen is 4-hydroxyproline and it is found in the Y position of the Gly-X-Y repeating sequence (Reddy and Enwemeka, 1996). Studies have shown that the presence of 3-hydroxyproline in place of 4-hydroxyproline destabilizes the collagen triple helix (Jenkins et al., 2003). When hydroxyproline is in the X position instead of the Y position, it does not assume a triple-helical conformation (Inouye et al., 1982). Hydroxyproline in the Y position has also been shown to have a higher stabilizing effect on the triple helix, as opposed to proline, demonstrating this stabilization to be steriospecific and dependent on the location of hydroxyproline (Burjanadze, 1982). Hydrogen bonding of hydroxyproline through hydration networks is a critical part of triple helix stabilization.

Hydroxyl groups on hydroxyproline molecules are linked by water molecules to a glycine carbonyl group of the same chain and to hydroxyproline carbonyl groups of the adjacent chain. This creates a water network that enhances stability (Brodsky and Ramshaw, 1997). Due to the unique specificity of hydroxyproline localization in collagen, the hydroxyproline content has been used to estimate collagen content (Colgrave et al., 2008).

**Collagen Estimation with Hydroxyproline**

There are various methods for determining the total collagen content based on the measurement of hydroxyproline. Some of the various methods include mass spectrometry, high performance liquid chromatography with separation of amino acids, and spectrophotometry without preseparation of amino acids (Ignat'eva et al., 2007). Research has shown that spectrophotometric techniques without preseparation of amino acids are of the most interest. Methods that utilize this technique give the most accurate results, are reliable, inexpensive, and simplify analysis (Ignat'eva et al., 2007). One example of this type of procedure includes the interaction of ninhydrin (1,2,3-trioxiondane hydrate) with the analyzed tissue samples. In this reaction, compounds containing this amino group interact and generate colored products. The main disadvantage of this procedure is that ninhydrin reacts with all of the amino acids. Specificity is improves when ninhydrin is initially treated with sodium nitrite, which oxidizes the amino groups. However, when ninhydrin and sodium nitrite are present together the reaction cannot distinguish between hydroxyproline and proline. This results in an inaccurate measurement of total hydroxyproline content, as it may contain various amounts of proline (Ignat'eva et al., 2007).

Another procedure for measuring hydroxyproline content is based upon the interaction of Ehrlich's reagent (p-dimethylamino benzaldehyde) with hydroxyproline. This technique is based upon three main steps: hydrolysis, oxidation, and development of a chromophore which gives color to certain organic compounds (Reddy and Enwemeka, 1996). Hydrolysis is usually carried out at 120$^{o}$C and the oxidizing agent is chloramine-T (N-chloro-4-toluenesulfonamide, sodium salt) (Ignat'eva et al., 2007). In the oxidation process, the pyrrolidine ring of hydroxyproline undergoes oxidative dehydrogenation to a pyrrole ring (Ignat'eva et al., 2007). This effect usually occurs within 20 minutes at room temperature. Addition of Ehrlich's reagent produces a quinoid compound that is colored. The development of the chromophore is obtained within 20-25 minutes of incubation at 60-65$^{o}$C (Reddy and Enwemeka, 1996). The absorbance is read at 550-570 nm within three hours of chromophore development. After three hours the chromophore starts to break down. This results in decreased absorbance values (Edwards and O'Brien, 1980).

Inexpensive and reliable methods for determining the collagen content based on the hydroxyproline content are of great importance because of the need to monitor the amount of collagen in various pathological conditions, as well as in relation to age (Reddy and Enwemeka, 1996). A few of these pathological conditions that result in aterations in collagen comoposition include hypertension, chronic ulcers, rheumatoid arthritis, tumor invasion and metastasis, muscular dystrophy, and diabetes mellitus (Reddy and Enwemeka, 1996).

**Causes and Characteristics of Hypertension**

Hypertension, or chronically elevated blood pressure, is the leading cause of cardiovascular disease, which is the primary cause of death for both men and women in the United States (McBride et al., 2005). Hypertension does not produce symptoms in patients and is

therefore referred to as the silent killer. A person may be unaware they have hypertension unless their blood pressure is checked often. If left untreated, hypertension can lead to a myocardial infarction, sudden cardiac death, and congestive heart failure (Wallen et al., 2000). Factors that may influence high blood pressure include diet, smoking, body weight, family history, alcohol abuse, and gender (McBride et al., 2005).

Systolic pressure between 120-139 mmHg and diastolic pressure between 80-89 mmHg is known as pre-hypertension. Pre-hypertension usually develops into hypertension which results in consistently high blood pressure over 140/90 mmHg, compared to normal blood pressure of 120/80 mmHg. Hypertension can be divided into three groups based on the severity of the rise in blood pressure. Mild hypertension is associated with a systolic blood pressure less than 175 mmHg (Shapiro and McKenna, 1984). Systolic blood pressure between 176-200 mmHg is considered to be moderate and above 201 mmHg is characterized as severe hypertension (Shapiro and McKenna, 1984). When systolic pressure is gradually elevated above the normal range of 120, there is an increase in myocardial mass, myocyte size, and interstitial collagen leading to left ventricular hypertrophy (Yang et al., 1997). Weber and coworkers (Weber et al., 1987) reported that during hypertrophy cardiac myocytes increase by 49%, the capillaries by 36%, and the interstitium by 15%.

**Hypertension Leading to Cardiac Hypertrophy**

Cardiac hypertrophy is an adaptive response to chronic pressure overload that allows the ventricular chamber to accommodate excessive pressure load while maintaining its ability to deliver oxygen to the metabolizing tissues (Weber et al., 1987). This remodeling is a result of the enlargement of sarcomeres in cardiac myocytes, causing the cells to increase in width, which

increases wall thickness (Thrainsdottir et al., 2003). A study measuring left ventricular mass in hypertensive patients demonstrated that healthy patients not suffering from hypertension had an average left ventricular mass of 185 ± 50 cm. In patients, left ventricular mass increased to 203 ± 44 cm in patients with mild hypertension, 289 ± 47 cm in moderate hypertension, and 528 ± 205 cm in severe hypertension (Shapiro and McKenna, 1984). If elevated systolic tension is not accommodated by hypertrophy then an increasing pressure greater than 100 mmHg is enough to break fibrillar collagen and an intraventricular pressure greater than 500 mmHg can cause ventricular rupture (Weber, 2001).

**The Phases of Hypertension**

Hypertension can be characterized by three phases. The first phase is the evolutionary phase in which the myocardium begins to undergo remodeling of its various compartments. However, oxygen delivery to the tissues and ventricular pump function remain unaltered (Weber et al., 1987). Following the evolutionary phase of hypertrophy is the physiologic phase. In this phase protein synthesis and breakdown in the myocardium is balanced. Therefore, oxygen delivery to the tissues still exceeds oxygen demand (Weber et al., 1987). The final phase in the hypertrophic process is referred to as the pathologic phase. In this phase myocardial remodeling is no longer balanced, ventricular pump function is abnormal, and oxygen delivery to the tissues is impaired (Weber et al., 1987). As a result of the imbalance between oxygen delivery and demand, hypertrophy is no longer considered adaptive when at the pathologic state (Weber et al., 1987). Furthermore, the remodeled myocardium cannot return to its normal state when the excessive pressure overload is removed, as opposed to the physiologic phase which can return to its normal state (Weber et al., 1987).

**Hypertension in Rat Models**

The spontaneously hypertensive rat (SHR) is generally used for studies in hypertension and cardiovascular disease. SHRs possess several qualities that make them ideal experimental models of hypertension. They are low in cost, small in size, and have a short life span (Trippodo and Frohlich, 1981). SHR models are genetically predisposed to have naturally occurring hypertension and heart failure that is similar to hypertension in humans (Trippodo and Frohlich, 1981). SHRs were first developed in the 1960s by breeding Wistar-Kyoto (WKY) rats with high blood pressure (Okamoto, 1963). In studies where SHRs are used, WKY rats are often used as the control.

Systolic blood pressure in SHRs rapidly increases between five and ten weeks of age and cardiac hypertrophy develops between nine and twelve weeks of age (Shimamoto et al., 1982). At 40-50 weeks the systolic pressure in SHRs increases to the point that the rat becomes hypertensive (Conrad, 1995). In male SHRs the mean arterial pressure averages around 190-200 mmHg (Trippodo and Frohlich, 1981). Studies in SHRs have shown that cardiac collagen content increases as hypertrophy progresses. A study conducted by Yang et al., (1997) demonstrated that during the initial phase of hypertrophy in SHRs, the collagen concentration of the heart was not altered even though hypertension and hypertrophy were present. However, at 40 weeks of age, during the chronic phase of hypertrophy, the collagen concentration increased. At 65 weeks of age the collagen concentration increased further from $10 \pm 0.5$ mg/g wet tissue to $23.7 \pm 0.5$ mg/g wet tissue.

## Development and Maintenance of Hypertension

One of the leading factors associated with the development and maintenance of hypertension is the Rein-Angiotensin-Aldosterone System (RAAS). When there is a loss in blood pressure, arterial pressure, or blood volume this system is activated in order to regulate cardiac and vascular function and arterial pressure (McBride et al., 2005). When blood volume is low, the juxtaglomerular apparatus in the kidneys secretes rennin which converts angiotensinogen to angiotensin I. Angiotensin I is then converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II causes vasoconstriction of blood vessels resulting in an increase in blood pressure. The increase in blood pressure promotes the release of aldosterone from the adrenal cortex, which increases sodium and water reabsorption in the kidneys. As a result, the volume of fluid in the body increases, causing an increase in blood pressure (Klabunde, 2005).

## Influence of Gender on Hypertension

In addition to the RAAS system, gender has also been shown to influence hypertension. Males have been shown to have a higher incidence of hypertension and transition into heart failure earlier than females. Females exhibit greater concentric remodeling of the ventricle and better maintenance of the left ventricle (Douglas et al., 1998). Recent studies have shown that estrogen, platelet aggregation during hemostasis, and the RAAS system are important determinants of hypertrophy in males and females (McBride et al., 2005).

Males have been shown to be at a greater risk than premenopausal women for hypertension due to the female sex hormones, estrogen and progesterone. However, postmenopausal women have a greater incidence of left ventricular hypertrophy than both men

and premenopausal women (Orshal and Khalil, 2004). Recent studies have shown that estrogen can block p38 MAP kinase phosphorylation, which is usually activated in the failing human heart (McBride et al., 2005). However, after menopause females tend to have an increase in left ventricular hypertrophy, almost similar to left ventricular hypertrophy in males (Forman et al., 1997). This study demonstrated that cardiac remodeling may be affected by estrogen.

Studies have shown that male SHRs have larger, thinner, and more fibrotic hearts compared to females. Due to the testosterone in males, male platelets tend to be more reactive causing them to aggregate. Less reactive platelets and platelet inhibition in females, due to estrogen, may provide women with protection from hypertension and atherosclerosis (McBride et al., 2005). Testosterone has also been shown to increase RAAS in male SHRs, which also have increased rennin and ACE activity compared to females, whereas estrogen in females has been shown to suppress ACE activity (Radin et al., 2002).

**Regulation of Collagen Synthesis and Degradation during Hypertrophy**

The myocardium responds to changes in systolic pressure through structural and biochemical changes in rates of protein synthesis and degradation (Weber et al., 1987). Regulation of collagen synthesis and degradation is essential to normal crosslinking in the development of the myocardium (Eghbali et al., 1990). It has been shown that collagen degradation is equal to synthesis in the myocardium (De Souza, 2002). This steady state is regulated by an equal balance between stimulator and inhibitor signals which regulate collagen turnover (Weber, 2001). Stimulators include angiotensin II and aldosterone which create a state designed for growth that can lead to adverse structural remodeling (Weber, 2001). Inhibitors include bradykinin and nitric oxide which have the opposite effects of stimulators (Weber,

2001). An imbalance between stimulator and inhibitor production leads to cardiac remodeling in normal and pathological states (Eghbali et al., 1989) (Weber, 2001). Studies have shown that an excess of stimulators promotes fibrosis and thereby pathologic hypertrophy (Weber, 2001). For example, following a myocardial infarction there is an overproduction of angiotensin II which contributes to formation of the infarct scar and interstitial fibrosis (Weber, 2001). Collagenases, which belong to a family of zinc containing proteins called matrix metalloproteinases (MMPs), are responsible for degrading collagen and are essential in order to remodel the cardiac extracellular matrix (Eghbali and Weber, 1990). It has been reported that during cardiac hypertrophy collagen degradation increases in order to repair the damage cardiac tissue (Eghbali and Weber, 1990). If arterial hypertension is reduced during hypertension this will not promote the regression of established fibrosis. Therefore, the proteolytic enzymes MMPs are required to degrade the fibrillar collagen and aid in cardioreparation (Weber, 2001). MMPs are located in the myocardium and upon activation, can be inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Sellers and Reynolds, 1977). TIMPs are able to form complexes with metalloproteinases and inhibit MMP activity (Welgus et al., 1985).

**Treatments for Hypertension**

Hypertrophy and hypertension can be treated using many antihypertensive drugs. Angiotensin converting enzyme (ACE) inhibitors such as captopril and linsinopril have been shown to be the most effective class of drugs (Yang et al., 1997). ACE inhibitor drugs not only regress hypertrophy but also reduce the total collagen content which is important in cardioreparation. ACE inhibitor drugs are effective in treating hypertension for the reason that they prevent angiotensin II formation, thereby causing blood vessels to relax resulting in

decreased blood pressure. Angiotensin II may play a role in increasing myocyte size and increasing myocardial collagen content during hypertrophy (Yang et al., 1997). Badenhorst et al., (2003) reported that myocardial stiffness correlates with collagen concentration. Increased total collagen content in the myocardium has been linked to abnormal cardiac function as well as diastolic and systolic stiffness, which are characteristic changes in pressure overload hypertrophy and heart failure (Conrad et al., 1991). The use of antihypertensive drugs to reduce the myocardial collagen concentration in SHRs has been shown to prevent the development of left ventricular remodeling and reverse remodeling in human heart failure (Tsotetsi et al., 2001), both of which are associated with enhanced collagen crosslinking (Tsotetsi et al., 2001). Yang et al., (1997) revealed that both captopril and hydrazine were effective in lowering blood pressure in SHRs. However, only captopril resulted in both regression of hypertrophy and collagen content in the heart. Both captopril and linsinopril have been shown to effectively reduce blood pressure from 189 mmHg to 126 mmHg and 116 mmHg (Yang et al., 1997). Furthermore, both ACE inhibiting drugs also reduced the total collagen content from 13 ± 0.4 mg/g in untreated SHRs to 8.8 ± 0.5 mg/g with captopril and 10.2 ± 0.4 mg/g when treated with linsinopril (Yang et al., 1997).

**Purpose of the Study**

Previous investigations have linked cardiac hypertrophy to an increase in ventricular collagen content, demonstrating that the ventricular collagen content in the hypertrophied ventricles of males is greater than in females, resulting in an earlier transition to heart failure in males. This study was designed to quantitate cardiac collagen in male and female spontaneously hypertensive rats by developing a hydroxyproline assay and to demonstrate that a gender-based

difference in collagen content exists between male and female SHRs that could potentially contribute to gender-based differences in cardiac function.

# Materials and Methods

## Study Group

Seven male and seven female 18 month Spontaneously Hypertensive Rats (SHR) were used in this study. The animals were euthanized and their hearts harvested. The atria were removed and the ventricles were washed, dried, and weighed. A midline incision in the ventricle was made and the superior section was frozen on dry ice and stored at -80$^{o}$C for hydroxyproline analysis.

## Tissue Preparation to Eliminate Insoluble Protein

The cardiac tissue was minced and homogenized (Kontes homogenizer) on ice with 5 ml of cold phosphate buffered solution and protease inhibitor (PBS/PI). An aliquot of homogenate (100 µl) was saved for total protein analysis. The homogenate was centrifuged (Sorvall RC 5B Plus centrifuge) at 15,000 rpms for 25 minutes at 4$^{o}$C and the supernatant was discarded. The pellet was resuspended in 4 ml of a 2% sodium dodecyl sulfate (SDS) solution to denature proteins, rehomogenized at room temperature, centrifuged at 15,000 rpms at room temperature for 10 minutes, and the supernatant was discarded. The procedure was repeated for a total of 3 times. The pellet was then homogenized with 4 ml of PBS to remove the SDS and any water soluble proteins, centrifuged at 15,000 rpms for 10 minutes at room temperature, and the supernatant was discarded for a total of 3 times. The remaining pellet was then homogenized with 5 ml of acetone at room temperature using a tapered spatula, centrifuged at 15,000 rpms for 10 minutes, and the supernatant was discarded. The pellet was dried under air for 30 minutes and resuspended in 4.0 ml of 70% formic acid. Cyanogen bromide (20 mg/ml) was added to the solution with nitrogen and incubated for 16-18 hours on an orbital shaker. The digest was

centrifuged for 30 minutes at 15,000 rpms. The supernatant was dialyzed against water for 3-4 hours changing the water every 60 minutes. The cyanogens bromide dialysate was then lyophilized (Labconco freeze dryer) and resuspended with rehydration buffer to the appropriate concentration (50 mg tissue per 10 µl) for hydroxyproline analysis.

The rehydration buffer was prepared by dissolving 24 g of urea, 2 g of CHAPS, 385 mg of DTT, and 100 µl of 0.1% bromphenyl blue in 25 ml of water and then brought up to a final volume of 50ml with water.

**Bradford Protein Assay**

The bradford protein assay was performed using 14 standards that ranged from 1.2-10 µg/ml. The standard solution was prepared using bovine serum albumen (BSA), 1mg/ml, in a final volume of 800 µl. Then a Bio-Rad Protein Assay Solution, 200 µl, (Bio-Rad Laboratories #500-006) was added to the standard and unknown tubes. All tubes were capped, vortexed, and the absorbance was read at 595 nm using a Bio-Rad SmartSpec Plus spectrophotometer.

**Hydroxyproline Assay**

**Preparation of Reagents**

A hydroxyproline standard solution (1 mg/ml) was stored at 5$^{o}$C and diluted 1:10 and then the standard solution (0.1 mg/ml) was diluted 1:20 on the day of the assay.

The acetate-citrate buffer (pH 6.5) was made in advance by dissolving 120 g of sodium acetate trihydrate, 46 g of citric acid, 12ml of acetic acid, and 34 g of sodium hydroxide in distilled water. The pH was then adjusted to 6.5 and brought to one liter.

N-propanol/perchloric acid (2:1) was prepared by mixing 333 ml of n-propanol and 167 ml perchloric acid for a final volume of 500 ml.

Chloramine-T and Ehrlich's reagents were prepared daily before each assay. Chloramine T reagent was prepared by dissolving 0.32 g of chloramine-T in 5 ml of n-propanol and then brought to 25 ml with acetate-citrate buffer. Ehrlich's reagent was prepared by dissolving 3.75 g p-dimethylaminobenzaldehyde in n-propanol/perchloric acid and brought to 25 ml with n-propanol/perchloric acid.

**Assay Procedure**

One hundred and fifty microliter aliquots of the resuspended dialysate (488-1235 mg tissue) were pipetted into 4 ml Matrix reaction vials. An equal volume of 12N hydrochloric acid was added for a final concentration of 6N. The aliquot samples were hydrolyzed for 16-18 at 110$^{o}$C in a Stable-Therm Blue M Oven. Vials were then centrifuged (IEC HN-SII centrifuge) at 10,000 rpms for 30 minutes at room temperature. The supernatant was pipetted into 10x76 mm borosilicate disposable culture tubes and water was added for a final concentration of 3N hydrochloric acid. Tubes were boiled (Thermolyne Dri-Bath typed 17600) using a vacuum at 112$^{o}$C for 1 hour. Tissue samples were then resuspended in 150 µl of water.

The standard curve, ranging from 0.4-20 µg, was prepared by pipetting OH-Pro standard solution and water into 20 small 0.1 ml Thermo Scientific reaction vials for a final volume of 50 µl. Vials were then autoclaved, using a Market Forge Sterilmatic autoclave machine, at 120$^{o}$C, 15lb pressure, for 20 minutes and cooled to 20$^{o}$C. Hydrolyzed samples were then gently mixed with 450 µl of chloramine-T and oxidized at room temperature for 25 minutes. Five hundred microliters of Ehrlich's reagent was added and the vials incubated at 65$^{o}$C for 20 minutes using a

Stabil-Therm Blue M oven. The absorbance was read at 550 nm using a Bio-Rad SmartSpec Plus spectrophotometer. Cardiac tissue samples were analyzed using the same protocol.

**Hydroxyproline Analysis**

The Bradford protein assay was used to determine the protein concentration and the hydroxyproline assay was used to quantitate collagen. Hydroxyproline values were calculated per milligram of tissue protein. The collagen content in male and female ventricular tissue was calculated by multiplying the hydroxyproline content by a factor of 8.2 as described by Mukherjee and Sen (1993).

**Statistical Analysis**

Collagen content is reported as mean and standard error. A t-test was performed to determine significance.

# Results

## Development of the Protocol

During the development of the hydroxyproline assay protocol, two experimental questions were addressed. The first was how to effectively concentrate samples after hydrolysis. The second dealt with the interference of the rehydration buffer with the assay.

## Concentrating Samples after Hydrolysis

In this assay, samples required hydrolysis with 6N hydrochloric acid (HCl). The problem encountered with HCl was that it cannot be put into a lyophilizer or CentriVap because the acid will degrade the equipment. A first solution to this problem was to neutralize the hydrolysate with 10N sodium hydroxide (NaOH). However, this produced two problems. 1.) The NaOH reacted with chloramine-T to produce a precipitate during the assay. 2.) The sensitivity of the assay was decreased. The slope of the standard curve changed from 0.9858 to 0.8022 (Fig. 1). For this reason, neutralization with NaOH was not utilized in this study.

An alternative solution was to boil off the HCl rather than to neutralize it. Since HCl is an azeotrope at the concentration used in hydrolysis, when it reaches its boiling point of 110$^{o}$C, it produces a vapor with the same composition as its mixture (hydrogen chloride and water) preventing it from being boiled off. Therefore, the concentration of HCl had to be adjusted in order to eliminate the azeotrope. Since the concentration of HCl at 3N was dilute enough to boil off the HCl without affecting the assay, we elected to reduce the concentration of HCl used in the assay to 3N. The mean value ± standard error (SEM) for the slope of the standard curve using 3N HCl was 0.0278 (± 0.0014) compared to 0.0256 (± 0.0042) for the slope of the standard curve without using HCl (Table 1). The $r^2$-value ± SEM of the standard curve using 3N HCl was

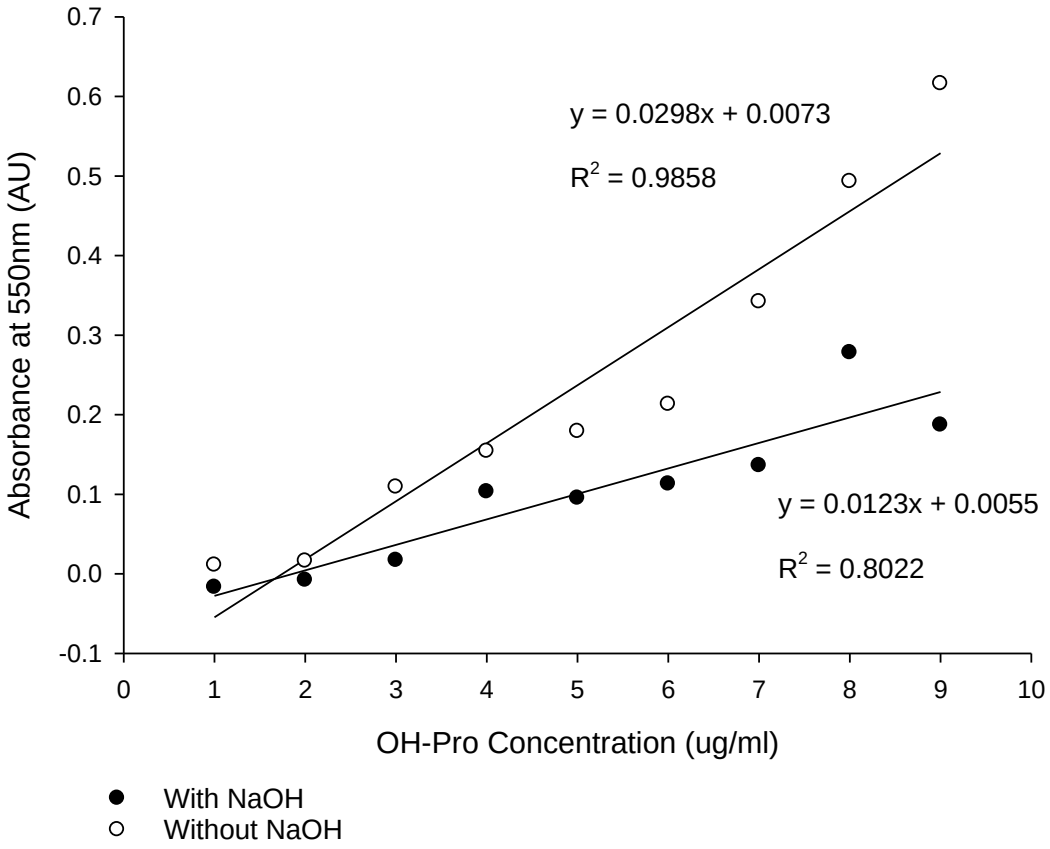


**Figure 1.** Hydroxyproline standard curve with and without the use of NaOH. Each data point represents duplicates of the concentration.

0.8110 (± 0.1430) compared to 0.9690 (± 0.0055) for the $r^2$-value of the standard curve without using HCl (Table 1). Since there was no significant difference between either the $r^2$-values ($p = 0.295$) or the slopes ($p = 0.629$) for the standard curve (Table 1), we diluted all hydrolyzed samples to a final concentration of 3N HCl.

**Evaluating the Effect of the Rehydration Buffer on the Assay**

Cardiac tissue samples were resuspended in rehydration buffer containing urea, dithiothreitol, CHAPS, and bromphenyl blue for each hydroxyproline assay. Some of these reagents might interfere with the assay. For this reason, the rehydration buffer was tested at 150 µl which was equal to that used in the tissue samples. The absorbance values for the rehydration buffer ranged from -0.023 to 0.006 AU (Table 2), which was typically lower than the lowest detectable standard (Table 3). The mean value ± SEM of rehydration buffer was –0.5 (± 0.4) µg (Table 2), also less than the lowest detectable standard. As a result, the rehydration buffer was not considered as a factor that would contribute or affect the absorbance values of the cardiac tissue samples when analyzed using this assay.

**Hydroxyproline Assay: Standard Curve**

The standard curve generated from the hydroxyproline assay was linear from 0.4 to 20 µg/ml of hydroxyproline when absorbance was measured at 550 nm. Data from a typical assay is shown in Table 3 and Fig. 2. Hydroxyproline concentrations above 20.0 µg/ml did not yield linear absorbance values and were considered to exceed the limits of the assay. Hydroxyproline concentrations less than 0.4 µg/ml did not yield consistently positive absorbance values and were considered too low to read.

**A Comparison of Slope and $r^2$ values for the Hydroxyproline Assay**

| 6N HCl | | | 3N HCl | | |
|---|---|---|---|---|---|
| **Assay** | **Slope** | **$R^2$-value** | **Assay** | **Slope** | **$R^2$-value** |
| 1 | 0.0341 | 0.9784 | 1 | 0.0291 | 0.9462 |
| 2 | 0.0409 | 0.9849 | 2 | 0.0216 | 0.9552 |
| 3 | 0.0269 | 0.9763 | 3 | 0.0314 | 0.9728 |
| 4 | 0.0201 | 0.9482 | 4 | 0.0272 | 0.9518 |
| 5 | 0.0151 | 0.9628 | 5 | 0.0295 | 0.9435 |
| 6 | 0.0166 | 0.9635 | 6 | 0.0281 | 0.9570 |
| **Mean** | 0.0256 | 0.9690 | | 0.0278 | 0.9540 |
| **SEM** | 0.0042 | 0.0055 | | 0.0014 | 0.0042 |

**Table 1.** This table compares the effect of 3N HCl and 6N HCl on the hydroxyproline assay. For each assay, the slope and $r^2$-values were calculated from 9 data points of a standard curve. P = 0.629 for the slopes; p = 0.295 for the $r^2$-values.

| Hydroxyproline Assay: Rehydration Buffer | | |
|---|---|---|
| **Assay** | **Absorbance (AU)** | **Calculated µg Rehydration Buffer** |
| 1 | -0.023 | -0.5 |
| 2 | -0.006 | 0.5 |
| 3 | -0.020 | -0.4 |
| 4 | -0.003 | 0.04 |
| 5 | 0.006 | -0.1 |
| 6 | 0.006 | -2.4 |
| **Mean** | -0.007 | -0.5 |
| **SEM** | 0.005 | 0.4 |

**Table 2.** Evaluation of the interference of rehydration buffer with hydroxyproline assay. Absorbance values (at 550 nm) for the rehydration buffer and µg rehydration buffer for six hydroxyproline assays.

| Hydroxyproline Assay: Standard Curve | |
|---|---|
| **OH-Pro (µg/ml)** | **Absorbance (AU)** |
| 0.4 | 0.011 |
| 0.4 | 0.008 |
| 1.0 | 0.016 |
| 1.0 | 0.030 |
| 2.0 | 0.059 |
| 2.0 | 0.040 |
| 4.0 | 0.113 |
| 4.0 | 0.142 |
| 6.0 | 0.164 |
| 6.0 | 0.194 |
| 8.0 | 0.204 |
| 8.0 | 0.321 |
| 12.0 | 0.383 |
| 12.0 | 0.373 |
| 16.0 | 0.538 |
| 16.0 | 0.405 |
| 20.0 | 0.634 |
| 20.0 | 0.608 |

**Table 3.** Typical standard curve for hydroxyproline assay. Hydroxyproline concentrations and absorbance values (at 550 nm).

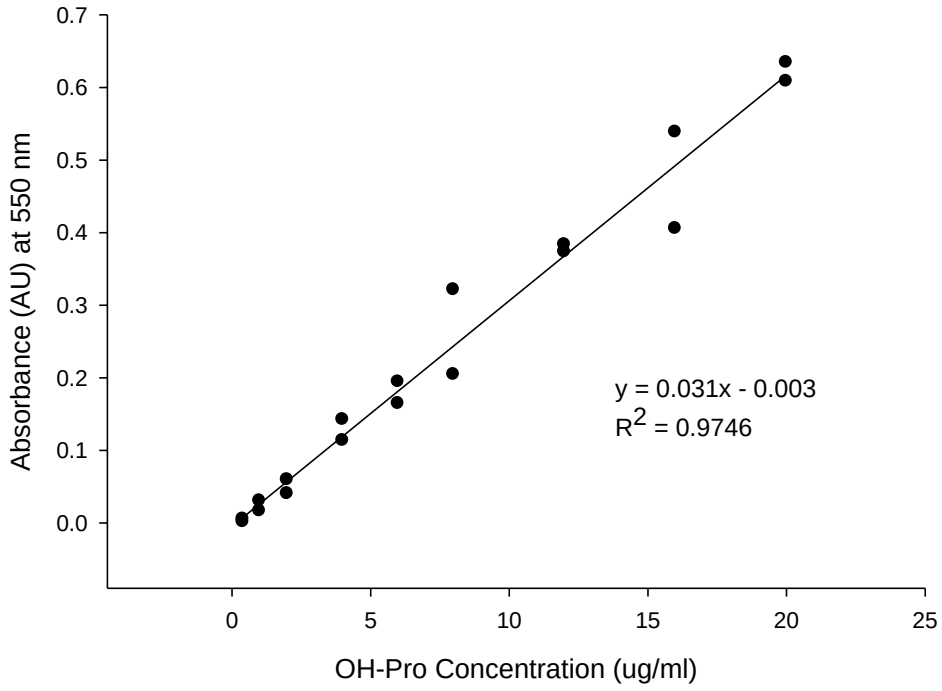


**Figure 2:** A typical standard curve for the hydroxyproline assay.

**Cardiac Collagen Tissue**

The ventricular weights for the seven 18 month male SHR's ranged from 1.422 to 1.956 g and the mean ± SEM of the ventricle weights was 1.619 (± 0.0706) g. Ventricular weights for the seven female SHRs ranged from 0.975 to 1.058 g and the mean ± SEM of the ventricle weights was 1.012 (± 0.0302) g. For males, the ventricular weight/body weight ratio ± SEM was 0.00405 (± 0.000140) g and for the females 0.00408 (± 0.000092) g (Fig. 3). There was no significant difference between the male and female ventricular weight/body weight ratio ($p = 0.859$).

The mean ± SEM mg protein/g ventricle weight for the male SHRs was 518.0 (± 69.0) and 371.0 (± 29.2) for the female SHRs (Fig. 4). There was no significant difference between the male and female total protein/ventricle weight ($p = 0.073$).

Cardiac collagen was analyzed at three different levels. In the first analysis, the µg OH-Pro/mg tissue protein was determined for each animal. The mean ± SEM of the µg OH-Pro/mg protein for the males and females was 9.4 (± 1.9) and 10.3 (± 1.0.), respectively (Fig. 5). In the second analysis, the µg collagen/mg protein was determined and the mean ± SEM of the µg collagen/mg tissue protein for the male SHRs was 77.1 (±15.7) and 84.1 (± 8.2) for the female SHRs (Fig. 6). There was no significant difference between either hydroxyproline content or collagen content of the males and females. Finally, the µg collagen/mg wet weight ventricle tissue was determined and the mean ± SEM of the µg collagen/mg wet weight ventricle tissue for males was 41.2 (± 11.9) and 30.2 (± 1.9) for females (Fig. 7). There was no significant difference between the male and female µg collagen/mg wet weight ventricle tissue ($p = 0.379$).

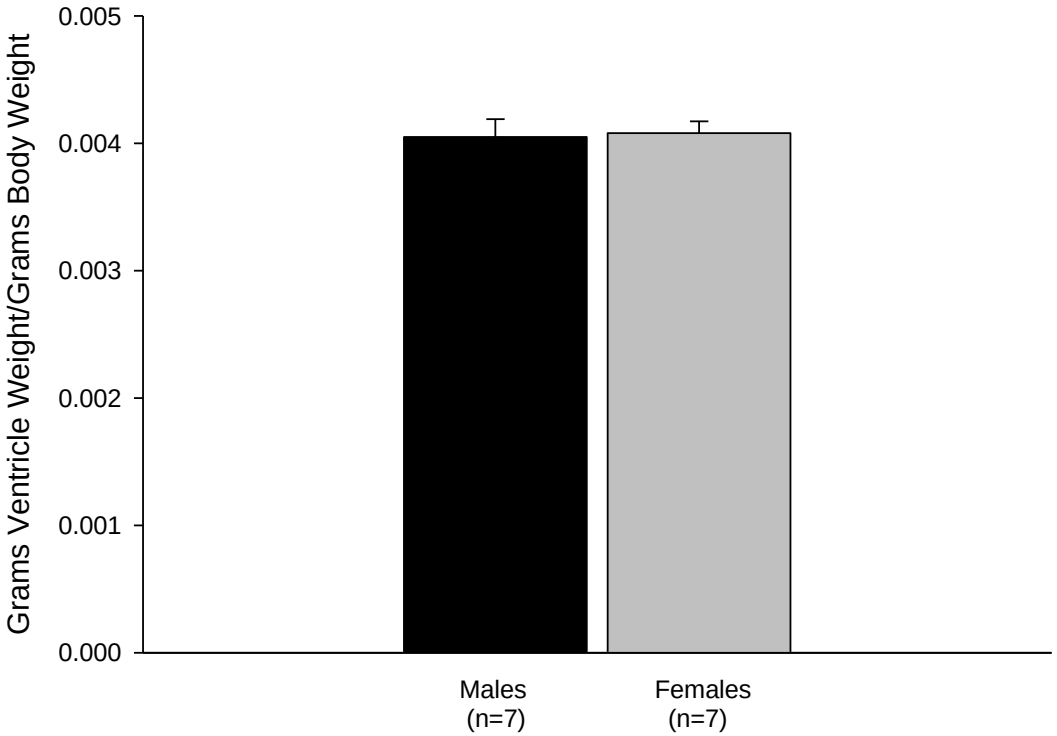


**Figure 3:** Comparison of the ventricle weight to body weight for male and female SHRs.

(Mean ± SEM, p = 0.859)

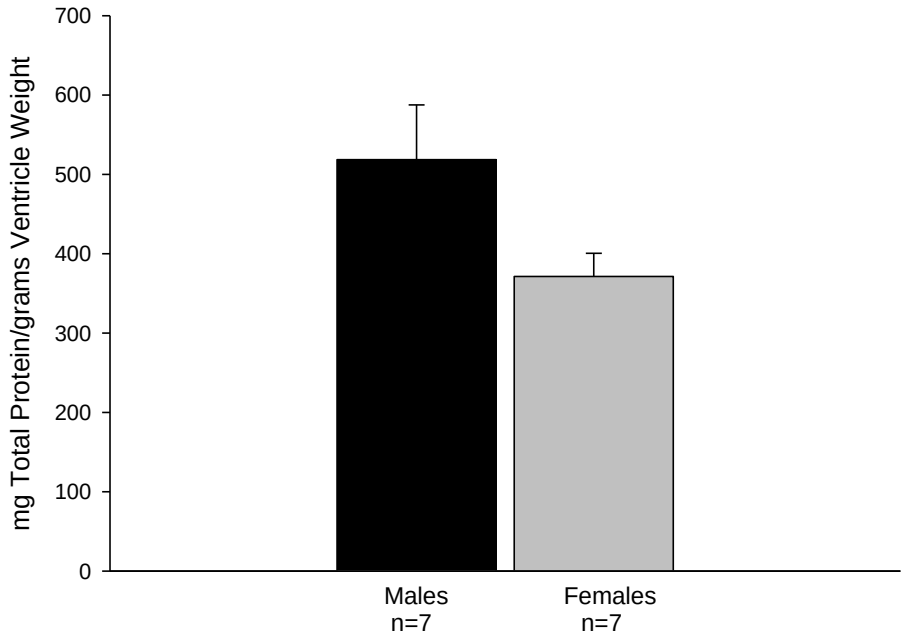


**Figure 4.** Comparison of the mg total protein/g ventricular weight for male and female SHRs. (Mean ± SEM, p = 0.073)

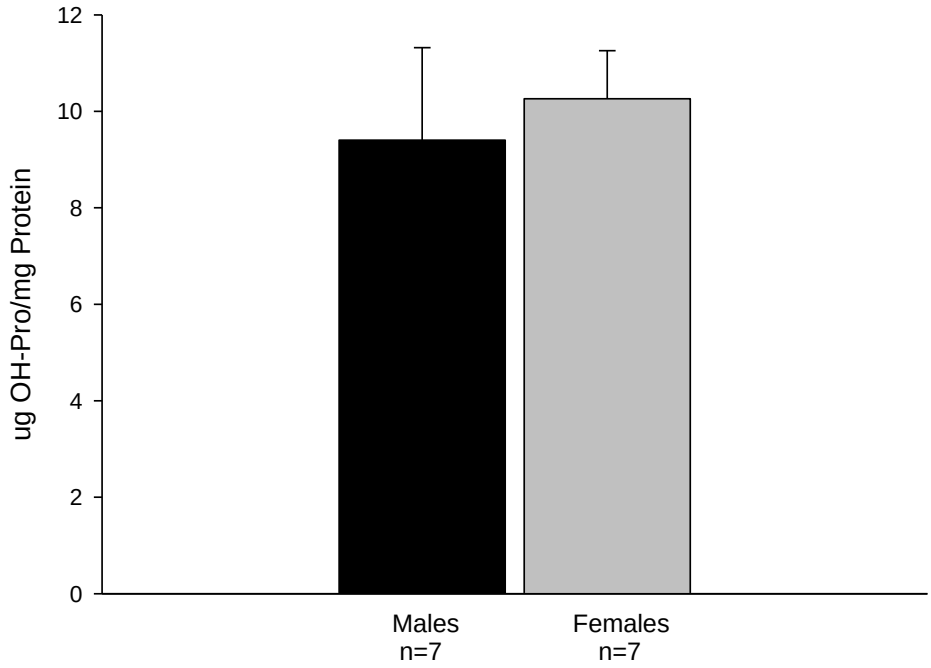


**Figure 5.** Comparison of the µg OH-Pro/mg protein for male and female SHRs. (Mean ± SEM, p = 0.699)

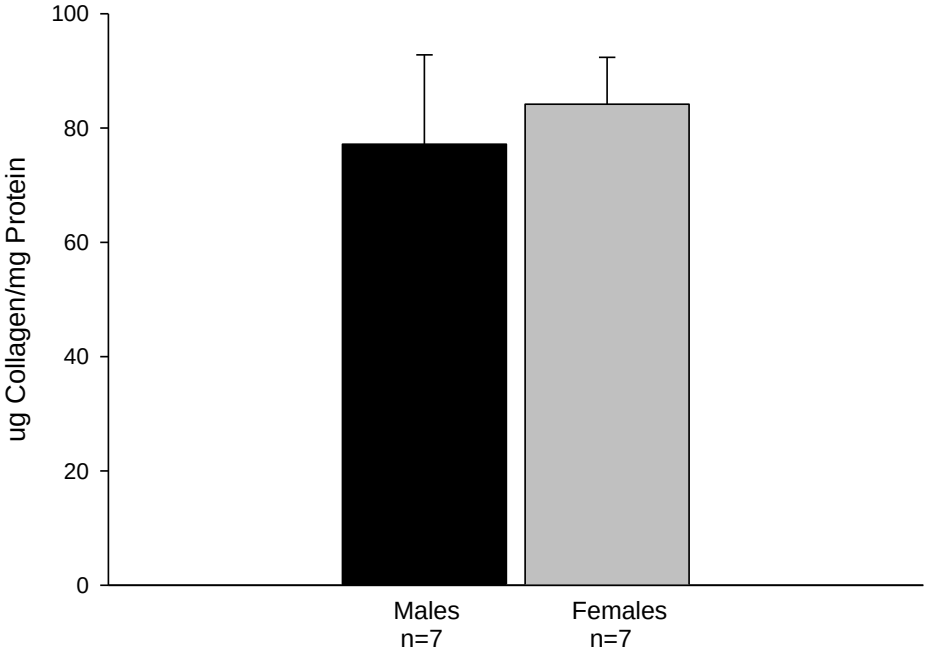


**Figure 6.** Comparison of the µg collagen/mg protein for male and female SHRs. (Mean ± SEM, p = 0.699)

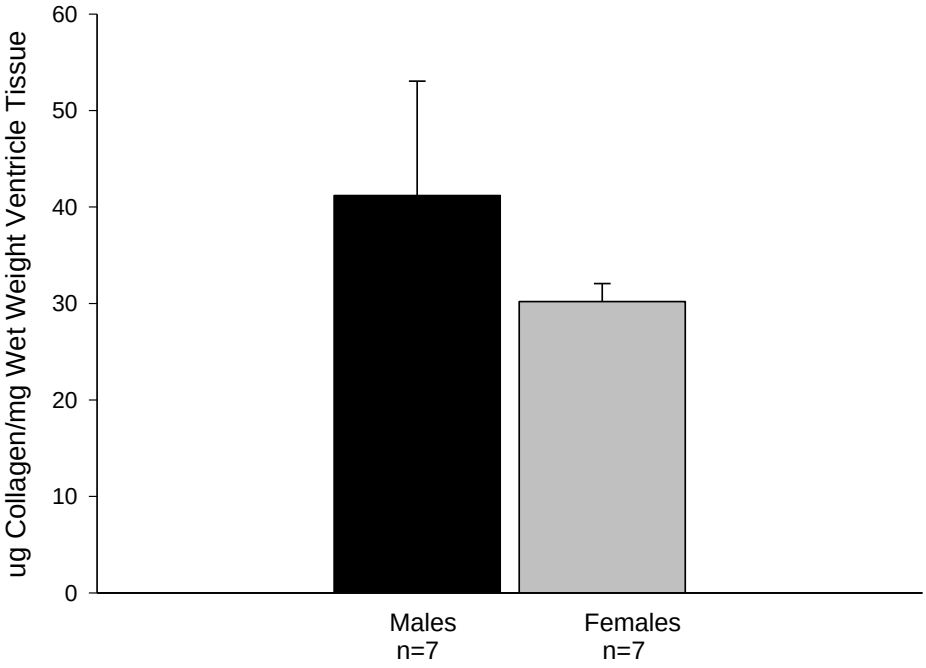


**Figure 7.** Comparison of the µg collagen/mg wet weight ventricle for male and female SHRs. (Mean ± SEM, p = 0.379)

## Discussion

Collagen is the main protein of connective tissue and plays an extensive role in connective tissue diseases in the human body, which are characterized by an increase or decrease of tissue collagen (Reddy and Enwemeka, 1996). The collagen matrix matrix of the heart consists of 85% type I collagen and 11% type III collagen (De Souza, 2002). During hypertension, an increase in collagen deposition accompanies cardiac hypertrophy, which can lead to altered cardiac function and heart failure (Yang et al., 1997).

In order to monitor the amount of collagen in various pathological conditions, such as hypertension, it is necessary to develop an assay that is reliable and can measure collagen quantitatively. Determination of hydroxyproline is the preferred method used when quantifying ventricular collagen, due to the specific location of hydroxyproline in collagen (Colgrave et al., 2008). This technique gives the most accurate results, is reliable, and inexpensive (Ignat'eva et al., 2007).

Hydroxyproline analysis consists of three main steps: hydrolysis, oxidation with chloramine-T, and the addition of Ehrlich's reagent to develop the chromophore, which is measured using a spectrophotometer (Reddy and Enwemeka, 1996). Hydrolysis is normally performed using 6N HCl at a temperature between 110-120$^0$C (Mukherjee and Sen, 1993). In our study we found that diluting the concentration of HCl to 3N after hydrolysis did not affect the assay. Reducing the concentration allowed us to eliminate the acid by boiling it off, as opposed to drying the samples using a flash evaporator or lyophilizer. When the oxidizing agent chloramine-T was added, it changed the pyrrolidine ring on hydroxyproline to a pyrrole ring. Ehrlich's reagent is used to detect certain phenols by producing a color reaction (Ignat'eva et al., 2007). This reagent specifically detects pyrroles, indoles, and nitrogen containing compounds

and is therefore widely used in hydroxyproline analysis (Ignat'eva et al., 2007). The only other molecules which react with p-dimethylamino benzaldehyde (chromophore of the Ehrlich's reagent) under conditions of acid hydrolysis of proteins are tyrosine and tryptophan. The colorometric reaction of pure tyrosine is 1.5% of hydroxyproline, while that of tryptophan is 0.7% of hydroxyproline (Neuman and Logan, 1950). Hence, these two molecules make minor contributions to the absorbance values in this study their contributions are incorporated into the collagen multiplication factor (8.2) used to convert hydroxyproline values into collagen content.

Our results show that this method is highly sensitive and reproducible. Based on the data from the standard curve, an increase in hydroxyproline from 0.4-20 µg/ml led to a linear increase in absorbance at 550 nm. This demonstrates that there is greater chromophore development when more hydroxyproline is present in the tissues, resulting in higher absorbance values.

Cardiac collagen is an acid soluble protein and when prepared for analysis it is homogenized and centrifuged in phosphate buffered saline/protease inhibitor to maintain the proper pH, and with sodium dodecyl sulfate (SDS) to denature the proteins. Phosphate buffered saline is added to wash out the SDS, and acetone to dry the pellet. Formic acid is then used to dissolve the pellet protein. Acid soluble proteins are then treated with cyanogen bromide, which cleaves proteins at methionine residues and generates collagen peptide fragments that are readily identifiable in a 2-D gel electrophoresis profile (Mukherjee and Sen, 1993). At this point the tissue can be analyzed for collagen/hydroxyproline content, for 2-D gel peptide profiles or other Biochemical analyses. Pepsin degradation is a method that had been used for collagen isolation. However, there are several disadvantages associated with this method. One being that degradation of collagen is variable depending on the endogenous activity of the pepsin

preparation. The conditions of homogenization, as well as the amount of collagen protein substrate available are also potential problems.

Cyanogen bromide peptide fragments were resuspended in rehydration buffer for each hydroxyproline assay. Rehydration buffer was analyzed for interference in the hydroxyproline assay. The absorbance values for the rehydration buffer were lower than the absorbance values for the lowest standard. Thus, we can confirm that the reagents contained in this buffer do not interfere with the assay and assume that the absorbance values generated by the cardiac tissue are based upon hydroxyproline content and not rehydration buffer.

The amount of hydroxyproline present in the tissues was calculated using the standard curve and was then multiplied by a conversion factor, developed by Mukherjee and Sen, of 8.2 to estimate collagen content (1993). The absorbance values for cardiac tissue at 50 mg wet weight tissue/0.01 ml rehydration buffer fell towards the middle of the standard curve between 9-14 µg/ml.

When analyzing the ventricle and body weights for the seven 18 month male and female SHRs, the ventricle and body weights for the males were greater than the females. However, when comparing the ventricular weight/body weight ratio, the females had a slightly higher ratio. The mean values were nearly identical with only a 0.00003 g difference between the male and female SHRs.

A Bradford protein assay was performed to determine tissue protein content. Our data suggests that the protein/ventricle weights are greater in males than in females but does not show a significant difference. This data is consistent with that of previous studies that have shown higher total protein content in the ventricles of males, due to their larger ventricle size (Yang et al., 1997). Since 5 out of 7 male hearts had more total protein than all of the age-matched female

counterparts, one might predict that by increasing the sample size we might see a significant difference in protein content between males and females.

A comparison of collagen content (µg collagen/mg wet weight ventricle) in 18 month males and female SHRs has not been reported in the literature. Our data suggests the amount of µg collagen/mg wet weight ventricle was greater in males than in females, but does not show a significant difference. The significance of this data may have been influenced by two males whose hydroxyproline and collagen values were clearly different than the rest. Thus, while the amount of collagen between males and females was not significant, male SHRs typically expressed higher amounts of total protein and larger ventricular weights leading to a greater amount of collagen.

The goals of this research project were to develop a hydroxyproline assay to quantitate cardiac collagen and demonstrate that a gender-based difference in collagen content exists. Overall, this assay has been shown to be effective in quantitating the ventricular collagen content in the hypertrophied ventricles of male and female SHRs. Our data suggests that a difference in the amount of cardiac collagen exists between male and female SHRs, supporting the view that gender does influence hypertension (Douglas et al., 1998). We have also confirmed that the amount of cardiac collagen in the hypertrophied hearts of male SHRs is greater than in females. This may be a key factor contributing to the higher incidence of hypertension and transition to heart failure in males (Douglas et al., 1998).

## References


Badenhorst, D., Maseko, M., Oupa, J., Tsotetsi, O.J., Naidoo, A., Brooksbank, R., Norton, G.R., Woodiwiss, A.J. (2003). Cross-linking influences the impact of quantitative changes in myocardial collagen on cardiac stiffness and remodeling in hypertension in rats. *Cardiovascular Research*. 57: 632-641.

Brodsky, B., Persikov, A.V. (2005). Molecular structure of the collagen triple helix. *Advances in Protein Chemistry*. 70: 301-339.

Brodsky, B., Ramshaw, J.A.M. (1997). The collagen triple-helix structure. *Matrix Biology*. 15: 545-554.

Burjanadze, T.V. (1982). Evidence for the role of 4-hydroxyproline localized in the third position of the triplet (Gly-X-Y) in adaptational changes of thermostability of a collagen molecule and collagen fibrils. *Biopolymers*. 21: 1489-1501.

Campbell, J.E., Janicki, J.S., Weber, K.T. (1995). Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone. *J. Mol. Cell Cardiol.* 27: 1545-1560.

Colgrave, M.L., Allingham, P.G., Jones, A. (2008). Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. *J. of Chromatography A*. 1212: 150-153.

Conrad, C.H. (1995). Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. *Circulation*. 91: 161-170.

Conrad, C.H., Brooks, W.W., Robinson, K.G., Bing, O.H.L. (1991). Impaired myocardial function in spontaneously hypertensive rats with heart failure. *Am. J. Physiol.* 260: H136-H145.

De Souza, R.R. (2002). Aging of myocardial collagen. *Biogerontology*. 3: 325-335.

Debessa, C.R.G., Maifrino, L.B.M., De Souza, R.R. (2001). Age related changes of the collagen network of the human heart. *Mechanisms of Ageing and Development*. 122: 1049-1058.

Douglas, P.S., Katz, S.E., Weinberg, E.O., Chen, M.H., Bishop, S.P., Lorell, B.H. (1998). Hypertrophic remodeling: gender differences in the early response to left ventricular pressure overload. *J. of the Am. College of Cardiology.* 32(4): 1118-1125.

Edwards, C.A., O'Brien, W.D. (1980). Modified assay for determination of hydroxyproline in a tissue hydrolyzate. *Clinica Chimica Acta*. 104: 161-167.

Eghbali, M. (1990). Collagen gene expression and molecular basis of fibrosis in the myocardium. *Heart Failure*. 6: 125-128.

Eghbali, M., Weber, K.T. (1990). Collagen and the myocardium: fibrillar structure, biosynthesis and degradation in relation to hypertrophy and its regression. *Molecular and Cellular Biochemistry*. 96: 1-14.

Eghbali, M.E., Eghbali, M., Robinson, T.F. (1989). Collagen accumulation in the heart ventricles as a function of growth and aging. *Cardiovasc. Res.* 23: 723-780.

Fleg, J.L. (1986). Alterations in cardiovascular structure and function with advancing age. *Am. J. Cardiol.* 57:33c-44c.

Forman, D.E., Cittadini, A., Azhar, G., Douglas, P.S., Wei, J.Y. (1997). Cardiac morphology and function in senescent rats: gender-related differences. *J. Am. Coll. Cardiol.* 30(7): 1872-1877.

Ignat'eva, N.Yu., Danilov, N.A., Averkiev, S.V., Obrezkova, M.V., Lunin, V.V., Sobol, E.N. (2007). Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *J. of Analytical Chemistry*. 62(1): 55-57.

Inouye, K., Kobayashi, Y., Kyogoku, Y., Kishida, Y., Sakakibara, S., Prockop, D.J. (1982). Synthesis and physical properties of (Hydroxyproline-Proline-Glycine)$_{10}$: Hydroxyproline in the X-position decreases the melting temperature of the collagen triple helix. *Arch. Biochem. Biophys.* 219: 198-203.

Jenkins, C.L., Bretscher, L.E., Guzei, I.A., Raines, R.T. (2003). Effects of 3-hydroxyproline residues on collagen stability. *J. Am. Chem. Soc.* 125: 6422-6427.

Klabunde, R.E., (2005). Hypertrophy. *Cardiovascular Physiology Concepts.* www.cvphysiology.com/Heart%20Failure/HF009.tm.

Lakhan, S.E., Harle, L. (2008). Cardiac fibrosis in the elderly, normotensive athlete: case report and review of the literature. *Diagnostic Pathology*. 3: 12.

McBride, S.M., Flynn, F.W., Ren, J. (2005). Cardiovascular alteration and treatment of hypertension: do men and women differ? *Endocrine*. 28(2): 199-207.

McCormick, R.J., Thomas, P.D. (1998). Collagen crosslinking in the heart: relationship to development and function. *Basic Appl. Myol.* 8(2): 143-150.

Mukherjee, D., Sen, D. (1993). Alteration of cardiac collagen phenotypes in hypertensive hypertrophy: role of blood pressure. *J. Mol. Cell Cardiol.* 25: 185-196.

Neuman, R.E., Logan, M.A. (1950). The determination of hydroxyproline. *J. of Biological Chemistry*. 184: 299-306.

Okamoto, A.K. (1963). Development of a strain of spontaneously hypertensive rat. *Jap. Circ. J.* 27: 282-293.

Orshal, J.M., Khalil, R.A. (2004). Gender, sex hormones, and vascular tone. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 286(2): R233-R249.

Perret, S., Merle, C., Bernocco, S., Berland, P., Garrone, R., Hulmes, D.J., Theisen, M., Ruggiero, F. (2001). Unhydroxylated triple helical collagen I produces in transgenic plants provides new clues on the role of hydroxyproline in collagen folding and fibril formation. *J. Biol. Chem.* 276: 43693-43698.

Radin, M.J., Holycross, B.J., Sharkey, L.C., Shiry, L., McCune, S.A. (2002). Gender modulates activation of renin-angiotensin and endothelin systems in hypertension and heart failure. *J. Appl. Physiol.* 92(3): 935-940.

Reddy, G.K., Enwemeka, C.S. (1996). A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical Biochemistry*. 29(3): 225-229.

Safar, M. (1990). Ageing and its effects on the cardiovascular system. *Drugs*. 39 (Suppl. 1): 1-8.

Sakikabara, S., Inouye, K., Shudo. K., Kishida, Y., Kobayashi, Y., Prockop, D.J. (1973). Synthesis of $(Pro\text{-}Hyp\text{-}Gly)_n$ of defined molecular weights. Evidence for stabilization of collagen triple-helix by hydroxyproline. *Biochem. Biophys. Acta*. 303: 198-202.

Sellers, A., Reynolds, J.J. (1977). Identification and partial characterization of an inhibitor of collagenase from rabbit bone. *Biochem. J.* 139: 359-368.

Shapiro, L.M., McKenna, W.J. (1984). Left ventricular hypertrophy. Relation of structure to diastolic function in hypertension. *Br. Heart J.* 51: 637-642.

Shimamoto, N., Goto, N., Tanabe, M., Imamoto, T., Fujiwara, S., Hirata, M. (1982). Myocardial energy metabolism in the hypertrophied hearts of spontaneously hypertensive rats. *Basic Res. Cardiol.* 77: 359-367.

Thrainsdottir, I.S., Hardarson, T., Thorgeirsson, G., Sigvaldason, H., Sigfusson, N. (2003). Survival and trends of occurrence of left ventricular hypertrophy, gender differences, 1967-92: The Reykjavik Study. *J. Int. Med.* 253(4): 418-424.

Trippodo, N.C., Frohlich, E.D. (1981). Similarities of genetic (spontaneous) hypertension: man and rat. *Circ. Res.* 48: 309-319.

Tsotetsi, O.J., Woodiwiss, A.J., Netjhardt M., Qubu, D., Brooksbank, R., Norton, G.R. (2001). Attentuation of cardiac failure, dilation, damage, and detrimental interstitial remodeling without regression of hypertrophy in hypertensive rats. *Hypertension*. 38: 846-851.

Wallen, W.J., Cserti, C., Belanger, M.P., Wittnich, C. (2000). Gender-differences in myocardial adaptation to afterload in normotensive and hypertensive rats. *Hypertension*. *36*(5): 774-779.

Weber, K.T. (2001). Cardioreparation in hypertensive heart disease. *Hypertension*. 38: 588-591.

Weber, K.T., Clark, W.A., Janicki, J.S., Shroff, S.G. (1987). Physiologic versus pathologic hypertrophy and the pressure-overloaded myocardium. *J. of Cardiovascular Pharmacology*. 10 (Suppl. 6): S37-S49.

Welgus, H.G., Jeffrey, J.J., Eisen, A.Z. (1985). Human skin fibroblast collagenase: Interaction with substrate and inhibitor. *Coll. Relat. Res.* 5: 167-175.

Yang, C.M.Y., Kandaswamy, V., Young, D., Sen, S. (1997). Changes in collagen phenotypes during progression and regression of cardiac hypertrophy. *Cardiovascular Research*. 36: 236-245.

Zak, R. (1973). Cell proliferation during cardiac growth. *Am. J. Cardiol.* 31: 211-219.

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:dka

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