A MITRAL VALVE PROLAPSE STUDY USING ELECTRICALLY INDUCED ARRHYTHMIA WITH NOREPINEPHRINE ADMINISTRATION TO PRODUCE PROLAPSING IN SHR AND WKY FEMALE RATS

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Mitral valve prolapse (MVP) affects 5-15% of the world’s population, it is the most common form of heart valve abnormality diagnosed in females, and shows a decrease in cardiac output and aortic distensibility. Hypertension is the number one most diagnosed cardiovascular disease in the United States, and has been observed to exacerbate and/or coincide with other existing diseases, also showing a decreasing cardiac output and aortic distensibility. The hypothesis for this project was: Hypertension increases the severity of mitral valve prolapse and its associated symptoms in female SHR rats compared to WKY female rats. WKY and SHR female rats that were older than 12 weeks were separated into 4 groups; the assigned groups were: WKY control (WKY CTL), WKY experimental (WKY EXP), SHR-control (SHR CTL), and SHR experimental (SHR EXP). Each rat was fitted with a Doppler probe to monitor flow velocity in the abdominal aorta to compare cardiac performance. Blood pressure, EKG and flow velocities were measured weekly for five weeks. Results showed that the SHR EXP had highest means where there was significance for treatment level (baseline vs. post treatment) for cardiac output, epinephrine and tissue damage. For cardiac output ($F=5.693$, $p=0.027$), for epinephrine ($F=43.985$, $p<0.001$), for tissue damage ($F=4.834$, $p=0.040$). For norepinephrine there was statistical difference among the different levels of treatment (baseline vs. post...
treatment) F=5.279, p=0.032 (SHR CTL had highest mean). These results suggest the catecholamine levels could be due to sympathetic dysfunction being more impaired in SHR EXP group, causing the decrease of norepinephrine and increase of epinephrine. This type of catecholamine response would drive up the cardiac output in the SHR EXP group. This data combined with the SHR EXP group having the most ventricle damage indicates heart failure initiating. In conclusion, hypertension is associated with the severity of symptoms for mitral valve prolapse.
DEDICATION

“When I dare to be powerful – to use my strength in the service of my vision, then it becomes less and less important whether I am afraid.” Audre Lorde

To my Abuelita Elvira d’Errico
1911-2006

Her life as collegiate educator and humanitarian set the standard of accomplishment I hope to obtain in my life.
Yo te amo.
It took a village to help me see my vision and research come to fruition. There have been so many people that played important roles in my obtaining this goal. I would like to thank the supportive and influential faculty of the Biology department at The University of Akron. Without their thought provoking inspiration and their ability to help students strive for answers, my analytical mind would not have been nurtured.

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CHAPTER I

INTRODUCTION

In a time where cardiovascular health has become more critical and population size increasing; there has been a call for research into various cardiovascular pathophysiology. Hypertension is the number one most diagnosed cardiovascular disease in the United States. In most recent accounts there has been an increase in the prevalence of hypertension in those aged 20-29 years in both males and females (NIH, 1996). The NIH Heart and Vascular center, announced in late 2004 that 1 in 3 adults is diagnosed with hypertension. Hypertension has been observed to exacerbate and/or coincide with other existing diseases and disease states, for example atherosclerosis, diabetes, and recently syndrome X. Understanding hypertension and the effect it has on the entire body and how it not only affects the physiology but also the anatomy is important to understanding this “silent killer”. How it affects other disease states and the normal function of the heart was the main focus of this project. More specifically, how it affects the function of the heart when there is a heart valve malfunction, like mitral valve prolapse.

Mitral valve prolapse (MVP) effects 5 -15% of the world’s population, and is the most common form of heart valve abnormality. MVP is more frequently diagnosed in females, but when diagnosed in males the symptoms are more severe. The higher
incidence of diagnosis in females has been discussed to be due to lower blood pressure, less dense muscle mass; which causes the mitral valve to set improperly in the annulus. The first mechanism that can lead to MVP is an autosomal dominant abnormality, known as primary MVP. The second mechanism that can cause MVP occurs later in life, known as secondary MVP. According to Boudoulas (2004), the pathophysiological consequences of MVP is the decrease in cardiac output due to the diminished blood flow into the left ventricle coupled with the backflow of blood through the annulus into the left atrium, known as regurgitation. Aortic function has been recognized as an important factor for measurement of left ventricular performance and myocardial perfusion (Boudoulas, 2004). Patients with MVP have shown evidence of decreased aortic distensibility, which also adds to the decreased cardiac output. Aortic distensibility is also decreased in patients with essential hypertension. Another similarity is both disorders show a decrease in cardiac output in patients along with ventricular hypertrophy. Taking into consideration these similarities between the two disorders, it could be postulated that combined the functional and structural symptoms displayed would be more severe.

**Hypothesis and Objectives**

For this project the hypothesis was: Hypertension increases the severity of mitral valve prolapse and its associated symptoms in female SHR rats compared to WKY female rats.
The objectives were:

- To implement new techniques to cause mitral valve prolapse in a rat model, without manually clipping the papillary muscles or chordea tendinea. Electrical stimulation of the vagus nerve was performed with norepinephrine IP (intraperitoneal) administration to cause an arrhythmia.

- To show that hypertension will increase the severity of mitral valve prolapse in female rats.

- That the rat can be used as an *in vivo* subject to observe MVP.

The following chapters will incur recent and archived literature reviews to add support for the rationale behind this project, the material and methods used and designed. Additionally, the last chapters will show the data obtained throughout the project, discuss the results statistically, and discuss future directions for subsequent projects.
CHAPTER II
LITERATURE REVIEW

In this chapter current and archived articles were used to give background and insight into this project, and to give the rationale for the methodology used. The objectives for this project were; to create new surgical techniques to produce mitral valve prolapse (MVP), to observe the effect of hypertension on MVP, and to introduce the rat as a potential model for use in MVP research. Therefore, throughout this chapter, topics will be discussed to support each of the objectives for this project. The normal heart and mitral valve anatomy and physiology will be discussed, along with the pathological state of MVP. Hypertension and some of its effects on the heart will be discussed. As well as the effects that MVP and hypertension have on cardiac output, blood pressure and norepinephrine. Norepinephrine will also be discussed for its therapeutic usage and its role in symptoms of both pathologies. Lastly, Doppler technology and the SHR and WKY strain will be discussed to support the rationale for their use in this project.

Normal Heart and Mitral Valve Anatomy and Physiology

To understand the abnormal structure and function that occurs in MVP, the normal anatomy and function must be understood. Healthy heart valve function and structure
allow the unidirectional flow of blood between the chambers and vessels of the heart. During healthy heart function the unidirectional flow of blood occurs without obstruction or regurgitation. With this decreased turbulent flow and regurgitation the red blood cells are not traumatized as they travel through the valve.

In the normal heart there are four valves, two atrioventricular and two semilunar valves. The atrioventricular valves are also referred to as the tricuspid and bicuspid valves. The bicuspid valve is also called the mitral valve. These two valves sit in between the atrium and the ventricles of the heart. The anatomy for the atrioventricular valves is comprised of the: annulus, commissures, the leaflets, the chordae tendinea, papillary muscles and the atrial and ventricular myocardium/wall (see figures 1 and 2).

Figure 1. Diagram of heart anatomy

Taken from web http://www.sxheart.com/heart_anatomy_labelled.jpg
Figure 2. Superior view of heart valves

Taken from web http://health.allrefer.com/health/heart-valve-surgery-heart-valves-superior-view.html

The mitral valve is the atrioventricular valve that sits in between the left atrium and the left ventricle. In normal heart anatomy the mitral valve is in close proximity to the aortic valve. This is unlike the tricuspid valve, which is separated by muscle from its right side counterpart the pulmonary valve. The mitral valve is formed by two leaflets, the aortic (anterior) and the mural (posterior), these leaflets sit inside a D shaped ring made up of a fibrous cord, called the annulus. The aortic leaflet has a semi circular shape and takes up 1/3 of the annulus’s dimensions. The mural leaflet takes up the other 2/3 and has a scalloped appearance, often divided into three segments called the lateral, middle and medial segments. The segments of the mural leaflet are not equal in size. Ho, (2002), noted that when the mural leaflet is deformed as in floppy valve the middle segment is likely to be prolapsed. During systole, the leaflets touch at the distal aspects
known as the commissure. The commissures are named according to their position in the heart, either anteriolateral or posteriomedial.

The mitral valve is open during the diastole stage of the heartbeat. The valve opens because of the relaxation of the ventricle myocardium, which causes a drop in the left ventricular pressure, known as ventricular diastole. During ventricular diastole, 70-80% of the blood flows through the mitral valve due to the pressure gradient caused by the relaxation of the ventricle but also due to the contraction of the atria. Once the ventricle fills and the ventricular pressure has reached the maximum, ventricular systole occurs, causing the ventricle to contract. As the ventricle contracts the papillary muscle are also contracted, pulling the chordae tendineae tight. This closes the mitral valve and seals it so no regurgitation of blood can pass back into the left atrium.

Figure 3. Anatomy of the mitral valve open and closed. “A” occurs during ventricular diastole (ventricle relaxed), “B” occurs during ventricular systole (ventricle contracting). During systole the papillary muscles and chordae tendineae are contracted and taunt to prevent the backflow of blood into the atrium. (Picture from Lippincott Williams & Wilkins Instructor’s resource CD-ROM to accompany Pershe’s pathophysiology concepts of altered health states 7th edition 2005)
Mitral Valve Prolapse

According to Boudoulas (2004), the pathophysiological consequences of MVP are lesions that occur in the left atrial space and the development of a third, but neutral, chamber (Figure 4). This third chamber is developed in the space between the annulus and the prolapsing leaflets. With the development of this third chamber cardiac output is decreased. The decrease in cardiac output is due to the diminished blood flow into the left ventricle coupled with the backflow of blood through the annulus into the left atrium, regurgitation. Aortic function has been recognized as an important factor for measurement of left ventricular performance and myocardial perfusion (Boudoulas, 2004). Patients with MVP have shown evidence of decreased aortic distensibility, which also adds to the decreased cardiac output. Interestingly, aortic distensibility is also decreased in patients with essential hypertension.

Figure 4. Diagram showing the 3rd chamber development between the left atrium (LA) and the left ventricle (LV) of prolapsing mitral valve. (Picture taken from Boudoulas, 2004 article)
Integrity and normal function of the mitral valve depends on several factors; most important are the shape and size of leaflets and the annulus. Mitral valve prolapse (MVP) can be caused by different mechanisms that affect the mitral leaflets, through either an inherited trait or by a physiological incident. The first mechanism that can lead to MVP is an inherited autosomal dominant abnormality, known as primary MVP. This autosomal abnormality affects the myxoma, (the embryonic tissue that gives rise to the cardiac tissue during development, i.e., the left atrium, the mitral valve and its supportive chordae tendinea). With any of the previous stated tissues and structures being malformed, the leaflet structure or length of tendons will not allow correct placement of leaflets within the annulus. Discussions about the genetic mutations in patients with MVP are in regards to the genes responsible for the production of the structural proteins, collagen and elastin. Primary MVP can also be associated with other disorders of the heart and syndromes, for instance Marfan and Ehlers-Danlos syndromes, in which there is a widespread defect in collagen. Also, MVP has been linked with atrial septal defect, aneurysm, and non-cardiac disorders such as Grave’s disease, sickle cell anemia, and muscular dystrophy. All of which have involvement of connective tissues abnormalities. In any event, each can affect the function and structure of the mitral valve.

The second mechanism that can cause MVP occurs later in life, is known as secondary MVP and is caused by several different possibilities. The following cardiovascular events can eventually lead to secondary MVP: papillary muscle rupture during myocardial infarction or an arrhythmia, ruptured chordae tendinea and leaflets from bacterial endocarditis. Mitral regurgitation (MR) can occur with either primary or
secondary MVP. Either route usually demonstrates a connective tissue malfunction involving mitral valve size, annulus and chordae tendinea. In conjunction with the previously stated mechanisms there are other cardiovascular disorders that have been associated with MVP. Hypotension, another cardiovascular pathology, is often associated with MVP, since this disorder affects the leaf placement in the annulus due to a decrease of pressure within the heart chambers. There has also been discussion that a patient’s weight can have significant impact on leaflet placement as well. Patients with eating disorders like anorexia nervosa have shown greater occurrence of MVP, this was observed by Johnson et al. (1986). Johnson, 1986, found strong evidence with the associations of body weight and the prevalence of MVP, by observing patients with anorexia. It has been speculated that the decrease in muscle mass allows for the chordae tendinea being elastic and loose, allowing the valve to prolapse. This suggests a reason for the prevalence in young women and a decrease in older women; increasing weight decreases MVP symptoms. This correlation with body mass and MVP is also supported by the Framingham study (Savage et al.,1983); which showed the highest diagnosis of MVP occurred in earlier stages of development i.e. adolescents, when body mass is lower.

Due to most patients being asymptomatic, MVP is difficult to diagnose. When diagnosing cardiac disorders and disease there are questions practitioners pose during assessment. The first question deals with the underlying etiology; is the disease congenital, infectious, hypertensive or ischemic in origin? The next consideration is in regards to the anatomic abnormalities that could be found in the heart; which chambers are involved, which valves are infected, has there been a myocardial infarction?
Additionally, what are the physiological disturbances; is there and arrhythmia present is there evidence of congestive heart failure or myocardial ischemia? Lastly, what is the extent of functional disability: how strenuous is the physical activity required to produce symptoms? Answering these questions aides the clinician with diagnosing cardiovascular disorders, like MVP.

The use of echocardiograms has been a useful diagnostic tool in diagnosing MVP. Echocardiograms allow clinicians the ability to asses mitral valve leaflet thickness, annulus size, and left ventricle and atria size. In conjunction with the echocardiogram, often auscultation can be used to detect apical non-injection systolic click with or without the presence of a mid to late systolic murmur, when used during valsalva maneuvers. Another useful tool is the transient external arterial occlusion technique. This is achieved through inflating bilateral arm cuffs to 20 mmHg above systolic blood pressure for 5 seconds. This is useful in identifying murmurs due to regurgitant lesions. Though each of the diagnostic tool described are useful in identifying MVP, often MVP is diagnosed during a routine physical that includes and echocardiogram.

There are associated symptoms that accompany MVP that are categorized under MVP syndrome, though the symptoms are not always indicative of MVP. MVP syndrome is considered to be a psychosomatic disorder. The type of symptoms can be: palpitations, chest pain, fatigue, exercise intolerance, dyspnea (shortness of breath), and syncope (fainting). Some other disorders that MVP syndrome has been linked to be: chronic pain sufferer syndrome, chronic fatigue syndrome, anxiety disorders, eating disorders and fibromyalgia (muscle pain due to connective tissue abnormalities). Fibromyalgia and anxiety disorders have been linked to sympathetic dysfunction, which
could correlate to the coincidence of MVP syndrome, since MVP patients have also shown sympathetic dysfunction evaluated by tilt table stress test.

**Hypertension**

There are interesting pathophysiology similarities between MVP and hypertension. Both disorders show a decrease in aortic distensibility. Another similarity is both disorders show a decrease in cardiac output on patients along with ventricular hypertrophy occurring. What makes these findings interesting are the large numbers of MVP patients are diagnosed with hypotension as well. As humans age, blood pressure naturally rises, due to age related hemodynamic changes i.e. structural changes in the heart and vessels. Therefore, an age related increase in blood pressure and the effects it has on hearts with MVP should be considered. With an increase in blood pressure the MVP associated symptoms have been shown to disappear in some patients. An increase in blood pressure is beneficial in alleviating the symptoms of MVP. This has been observed in patients diagnosed with MVP in adolescence, as young adults in their twenties symptoms and signs of MVP are alleviated. This disappearance of signs and symptoms has been linked to the increase in blood pressure and body mass. The extreme of this natural increase in blood pressure is the disease of hypertension. Therefore, what are the effects it has on a malformed and malfunctioning valve, since it is the most common form of heart disease diagnosed in the United States.

In the last 30 years there has been research focused on hypertension and its association with increased age, especially in the United States and the westernized world.
Hypertension is defined as continual elevated blood pressure (systolic blood pressure of 140 mm Hg or greater and/or diastolic blood pressure of 90 mm Hg or greater). Essential hypertension is a subtype of arterial hypertension in which no single specific etiology can be isolated as the cause of increased blood pressure. Over 95% of all cases of hypertension fall into the category of essential hypertension. It is thought that essential Hypertension is the result of a complex interaction between many physical, physiological, and environmental factors.

Arterial pressure is determined by the cardiac output (CO) and the systemic vascular resistance (SVR), i.e., BP = CO x SVR. (Figure 5. below). Therefore, Hypertension represents an imbalance between these two determinants, the following equation can be used to determine blood pressure. Hypertension can be caused by an increase in cardiac output and/or an increase in peripheral resistance.
With essential hypertension the proposed mechanism of hemodynamics are signs of decreased β-andrenergic responsiveness and decreased cardiac compliance (Hollenberg et al. 1997). Stroke volume is proportionate to the cardiac output of a person suffering from essential hypertension. Therefore, as stroke volume decreases, cardiac output decreases. With increased vascular resistance during essential hypertension, there is an increased incidence of arteriolar hypertrophy. As this resistance increases there is also increased incidence of ventricular hypertrophy. Over time, the hemodynamics of hypertension changes from a decrease in cardiac output to an increase in vascular...
resistance. Intengan et al. (2000) described this increase in vascular resistance by a combined restructuring process. Intengan et al. 2000, discussed that the changes were caused by eutrophic and hypertrophic remodeling. Both of these processes effect the media-lumen ration, where media is the vessel tissue (smooth muscle, endothelial tissue, adventia), and the lumen is the space or tube, which the blood travels through (Intengan et al., 2000). During eutrophic remodeling, both the lumen and the vessel diameter decrease. During hypertrophic remodeling both the media thickens/enlarges decreasing the lumen diameter. These two remodeling process explain the increased resistances of arteries during hypertension.

In most recent accounts there has been an increase in the prevalence of hypertension in those aged 20-29 years in both males and females (NIH, 1996). In 2004, the NIH heart and vascular center announced that hypertension affected one in three adults. Epidemiologic data suggest that elevated systolic pressures are linked to the occurrence of new cardiovascular events or disease like diabetes mellitus. With hypertension there is a decrease in ventricular function, increased aortic stiffness and ventricular mass, and decrease in cardiac output (Eren, 2005). According to Malmqvist et al., (2001), treated left ventricular hypertrophy due to hypertension with Ibersartan, by blocking the angiotensin II pathway and thus decreasing blood pressure. According to Zhang et al, (2003), the renin-angiotensin system (RAS) has a significant role controlling salt and water homeostasis and vascular tone regulation. Their research showed polymorphisms in the promoter region of the renin-angiotensin II receptor gene could be linked to essential hypertension in the Chinese population. The angiotensin II system is affected in symptomatic patients with MVP (Szombathy, 2000). In recent years, research has shown
that symptomatic patients with MVP also have autonomic or neuroendocrine dysfunction that can result in a number of related symptoms due to a polymorphism of the AT₁ gene (Szombathy, 2000). The AT₁ gene is responsible for vasomotion and autonomic nervous system control; which is a genetic predisposition for hypertension if a mutation occurs at this gene. Interestingly, the same phenotype for autonomic dysfunction occurs in patients with MVP.

With consideration of the genetic influence on MVP and autonomic nervous system control, this could explain the associated symptoms of MVP. For instance, the tilt table evaluation has been shown to demonstrate sympathetic activity impairment (dysautonomia, sympathetic nervous system dysfunction) in MVP patients. Some symptoms of MVP and dysautonomia are tachycardia, fainting, anxiety. Most patients with MVP have dysautonomia, though, dysautonomia does not cause MVP and vice versa. These findings indicate sensitivity to the neural hormone norepinephrine and a faulty autonomic response to postural changes.

**Norepinephrine Usage**

As previously discussed, β-adrenergic sensitivity and autonomic responsiveness is affected in both hypertensive and MVP patients, though this similar response seems to have opposite etiology. In hypertension, an increase in sensitivity to norepinephrine and increased autonomic responsiveness is etiologic for the pathology. In contrast, the increased sensitivity and autonomic responsiveness in MVP patients acts as a compensatory mechanism to adjust the cardiac output.
The use of agents such as norepinephrine is used to treat arrhythmias in the heart. In this paper Norepinephrine is referred to as a contractility agent; since when used to optimize cardiac function, it helps restore the natural rhythm of the heart, and EKG pattern. Norepinephrine is used during cardiac life support (Micromedex, 2005), to treat bradycardia and arrhythmias, such as ventricular fibrillation. Interestingly, though norepinephrine is used to optimize cardiac function, arrhythmias are listed as adverse reaction (Micromedex, 2004). These arrhythmias can occur due to sensitivity to the agents, or due to overdose. Ascertaining the overdose levels for a patient is difficult due to factors such as; norepinephrine dosages are not weight dependent, norepinephrine serum levels fluctuate frequently and are hard to monitor. Administration and dosing is accomplished by giving the lowest dose and to observe the results, if adequate results are not achieved over a prolonged period of time, concentration is increased as well as the rate it is administered. This reactive method in most cases is successful, but could lead to undetected complications with the cardiovascular system.

Norepinephrine has been used to observe vagal control of heart rate in dogfish, *Squalus acanthias*. According to Agnisola et al., 2003, that the use of norepinephrine and electrical stimulation increased the extent of recovery time of the dogfish heart after initiating cardiac arrest. Recovery time with norepinephrine administration and electrical stimulation occurring separately was less. Scheiber et al. (2002) examined the continuous administration of catecholamine and hospital mortality. Scheiber found that continuous administration of catecholamines, such as norepinephrine, increased in hospital morbidity. The absence of continuous administration of norepinephrine was associated with increased hospital survival (Schreiber et al., 2002).
Doppler Technology and Cardiac Output Measurements

The use of Doppler velocity has been proven useful in measuring flow velocity from the cardiac chambers and blood vessels. Flow determination of vessels can be determined by combining flow velocity and cross-sectional area and correlated through the use of the indication dilution technique (Hartley, 1974). One way to monitor the flow is to measure cardiac output (CO). Cardiac output is defined as the volume of blood pumped by the heart per unit of time, which is the rate of flow into the aorta from the left ventricle. The general equation is $CO = \text{heart rate} \times \text{stroke volume}$. The heart rate is measured as cycles/time and stroke volume is measured as volume/cycle, so CO is measured as vol/time. With the attention being paid to flow, it is important to remember Poiseuille’s law. According to Poiseuille, flow is proportional to pressure and the square of the radius of the blood vessel. For a vessel of outer radius $R$ and the length $L$, the flow velocity at radical position $r$ is given by $v(r) = \frac{P}{4L} (R^2 - r^2)$. Here, $P$ is the pressure difference between the ends of the vessel and $\mu$ is the viscosity. To gain precise measurements of CO consideration needs to be made that the velocity increases towards the center of the blood vessel. There is less resistance in the center of the vessel and less turbulent flow, which can slow down the flow of blood through the vessel. Since CO is denoted as a measure of vol/unit of time, it is beneficial to monitor stroke volume; which denotes the amount of blood pumped by each ventricle with each heartbeat. In a human adult heart the average amount of blood in ventricle is 70 ml during diastole (heart at rest). According to Delp et al. (1998), cardiac output in juvenile rats was 50-55 ml/min.
and was 110-112 ml/min in adult rats. Taking into consideration that a rat’s heart rate can range from 250 bpm to 450 bpm on average, stroke volume is estimated to be 70μL per beat in an adult rat (Ely, unpublished observation). Stroke volume represents the difference between end diastolic volume (EDV) and end systolic volume (ESV). By the time diastole ends, each ventricle has filled up with blood. This amount of blood is the end diastolic volume or EDV. The amount of blood ejected during the systole is the stroke volume. At the end of systole the volume of blood remaining in each ventricle is the end systolic volume or ESV. Each human ventricle normally contains about 120 ml of blood by the end of diastole this is the reason that normal blood pressure in humans is 120mmHg. In the rat the systolic blood pressure is the same as in the human; their blood pressure is 120 mm Hg systolic. This similarity in the blood pressures between the two species is the main reason the rat is used for hypertension studies. At the end of systole about 50 ml of blood are left in each ventricle. This means that 70 ml (or 70 μL in the rat) of blood were pumped out of each ventricle during systole. Regulating stroke volume measurement is the amount of stretching that occurs to ventricular cardiac muscle prior to ventricular contraction. The more cardiac muscle stretches, the more forcefully it contracts. These stronger contractions increase stroke volume. Rising blood pressure reduces sympathetic activity, decreasing heart rate. With high blood pressure there is also an increase in arterial pressure; which ventricles must overcome before semilunar valves open. This ventricular compensation increases ESV and decreases stroke volume. Reduced cardiac output helps bring blood pressure down to normal levels in acute conditions.
SHR and WKY Rat Strains

For the past 40 years the spontaneously hypertensive rat has been used as an animal model for hypertension studies in regards to humans. In 1963, Okamoto and Aoki published an article on their findings of a new strain of rat derived from the Wistar Kyoto (WKY) strain. The new strain of rat was called the spontaneously hypertensive rat (SHR). The development of the SHR strain was accomplished by selecting the WKY rats that had elevated blood pressures. Those WKY rats with elevated pressures were bred together, and each subsequent generation were selected for high blood pressure, and bred. Breeding the higher blood pressure rats lead to the divergence and genesis of the SHR strain from the WKY strain. During development the WKY and SHR rats have comparable blood pressures 120 mmHG or less, similar to humans, until they reach 150 gms. Once the rats reach 150 gms the difference in blood pressures begins. As the weight of the SHR rats increases their blood pressure rises proportionately. Contrastingly the blood pressure for the WKY rats remains consistent. To date, these two strains of rats have been used to study the genetic influence on blood pressure (Ely, 1993). According to Jeméniz-Atalyó (2003), the SHR rat shows greater potentiation to exogenous norepinephrine or stimulation of the intramural nerves. This suggests that there is a greater release of the neurotransmitter norepinephrine in SHR than WKY and support increased sensitivity to exogenous norepinephrine. This was discussed in earlier sections on hypertension and norepinephrine. The SHR rat showing a greater sensitivity to norepinephrine allowed this animal to become the primary model for use in this project. Since, MVP and hypertension both show evidence of increased norepinephrine sensitivity
and autonomic nervous system response. This allowed the rat to be the closest *in vivo* model to study the MVP phenomenon with minimal manipulation. The SHR rat already had half of the physiological responses of MVP in place, the increased sensitivity and autonomic response. The only thing left to produce was the MVP itself.
CHAPTER III
MATERIALS AND METHODS

In this chapter the protocol used to obtain data will be outlined and described. First the rat strains used in this project will be described, how they were housed, and how they were identified. Second the two major surgeries will be detailed in two separate sections. The first surgery described will be the Doppler probe placement surgery; since all rats used in this project were fitted with Doppler velocity flow probes. The second surgery described will be the vagus nerve stimulation surgery, this was the treatment received by the experimental groups during the project. In addition to the two surgeries being described the in vivo measurement protocols will also be described. These measurements include; EKG tracings for heart rate and cardiac health monitoring, tail cuff blood pressures, cardiac output measurement from the Doppler probe and retro-orbital blood sampling for catecholamine analysis. Lastly, the tissue extraction protocol and the analysis methods used to quantify the in vivo measurements will be described. These methods will include: termination procedures, histological processing, tissue damage scoring, catecholamine analysis and statistical analysis.
Animals

For this project two strains of *Rattus norvegicus* were used; the spontaneous hypertensive rat (SHR) and the Wistar-Kyoto (WKY) which is the normotensive strain. The rats were divided into 4 main groups, each group containing 6 females (n=24). The age range for the rats was 12-16 weeks old. There were 2 groups per strain, 1 control and 1 experimental; WKY control (WKY CTL), SHR control (SHR CTL), WKY experimental (WKY EXP), and SHR experimental (SHR EXP). Each rat was identified by code written with permanent marker on their tails and matched with same code on the cage label. Code for ID issued was group number, female number, and treatment label and number (c-control, or e-experimental). An example of the code was group 1, female 2, control 3 was written as G1F2C3 on the tail and cage labels. This type of coding was used to reduce bias during blind review of data.

Retro-Orbital Blood Sampling and Catecholamine Analysis

One week prior to Doppler placement, surgery blood samples were collected for catecholamine (epinephrine and norepinephrine) baseline readings, from all rats used in this project. Blood samples were obtained using retro-orbital puncture under Pentothal anesthetic (0.2mg/100gm body weight of 2% Pentothal solution). During the fifth week after Doppler implantation, final measurements and recordings were completed; retro-orbital blood samples were taken for comparison with first blood sample obtained 6 weeks previously. Blood samples were assayed for catecholamine measurements using a
Waters HPLC system (Waters 1515 Isocratic HPLC Pump, 717 autosampler and 2465 electrochemical detector). Plasma epinephrine and norepinephrine samples were needed to compare difference in levels between strains and treatments. Once pretreatment/baseline catecholamines were recorded and the general health of the rats was confirmed, probe placement surgery was scheduled.

Flow Probe Placement Surgery

The first surgery performed in this project was placement of a Doppler velocity flow probe around the abdominal aorta. Placement of a flow probe allowed for the direct measurement of stroke volume; which was used to calculate cardiac output. All rats used in this project were fitted with a 0.5 mm Doppler velocity flow probe (Crystal BioTech). On the day of probe placement surgery, each rat was weighed and then anesthetized with 0.4mg/100g body weight with a 2-2.5% Pentothal solution given i.p. (intraperitoneal). After anesthesia was established, noted by diminished response to foot reflex, the rat’s abdominal and dorsal cranial regions were shaved and cleaned with betadine and alcohol. After prepping, rats were given 0.1mg/100g body weight of 1% Atropine solution (intramuscular) in the hind limb to reduce saliva secretion production that occurs with the use of Pentothal. The rat was then placed on a surgery stage and appendages were restrained using gauze tape looped around restraint hooks on the stage. The use of restraints held the appendages out of the field and the abdominal region open. After restraints were secured the abdomen was locally anesthetized with 2% lidocaine solution, the injection path was the mid-saggital line, which was also the incision site. After 60
seconds had passed since the lidocaine was injected, a 1-1 ½ inches (2.54cm - 3.81cm) incision via scalpel was made running down the mid-saggital line. The incision ran from the inferior aspect of the ribs (below the sternum and diaphragm muscle) to the umbilicus region. After the incision was made and the skin was separated from the underlying muscle, a tunnel was made using hemostats from the incision site, under armpit, to the area on the back of the head between the ears. One side of rat was unrestrained to allow rotation of the body, sterile gauze was placed over abdominal incision when the rat was ventrally positioned while the exit site (dorsal) was made. By opening the hemostats the skin was stretched and made a reference point to create the exit point of the tunnel. Opening the hemostats allowed easy insertion of the scalpel tip creating an opening 2-3mm wide, large enough for closed hemostat tips to be exposed. With tips of hemostats exposed a piece of sterile gauze tape, moistened with sterile saline, was grabbed with hemostats and pulled back through the tunnel to the abdominal incision. The exposed gauze tape would later be used to tie around probe wires and pulled back through the tunnel. This would guide the probe wires to opening between the rats’ ears. Sterile gauze wet with sterile saline was placed over exit site and rat was place on back and restrained again.

Returning to the abdominal incision, blunt dissection with the hemostats was used to penetrate the muscle layers and enter into the abdominal cavity. Once the abdominal cavity was penetrated the initial hole was widened to match the skin incision length. After lengthening, the index fingers were used to check the width of the opening to ensure there was enough space to place rib spreaders in. If there was inadequate space both the skin and muscle incisions were lengthened. Once the incision was appropriate
length, two pieces of sterile gauze wet with sterile saline were placed on the abdomen side by side meeting at the point of incision, and secured in place with hemostats. Then intestines and stomach were removed from the cavity, taking care not to pinch, perforate or twist the organs and placed on the gauze on the right side of the body. The exposed organs were then covered with a second piece of gauze wet with sterile saline to prevent the organs from drying out. By removing the intestines and stomach the left kidney was easier to access. Remaining organs inside the cavity were surrounded with wet sterile gauze and pushed gently to the perimeter of cavity and secured in place with rib spreaders. By palpating the remaining adipose tissue on the medial aspect of the left kidney, the pulse from the abdominal aorta was felt. Doing this allowed a reference point for dissecting the aorta, avoiding overlying and underlying arterial and venous vessels. To place the probe around the aorta, vessel was blunt dissected with cotton swabs. Once vessel was blunt dissected, a pair of small forceps was used to further expose and lift vessel slightly, holding vessel in place. With forceps in place a 3 inch piece of suture thread was looped and fed under vessel between the two prongs of the forceps. With the loop through, the center of the loop was cut, leaving two strings. The two separate strings were moved to opposite ends of the vessel, between the two prongs of the forceps. The silicone cuff of the probe was trimmed down from original length to fit the length of the exposed vessel. The probe was pinched close to the transducer, but not on the transducer, to cause the ends of the probe to protrude out. The probe was then lowered down close to the vessel, and a second person with a set of microstats, placed microstats under the vessel in between the open prongs of the forceps. Once under the vessel the microstats were used to grab lower end of the probe cuff and guided under the vessel.
The person holding microstats, pinching the cuff, maneuvered the top portion of probe around the upper portion of the vessel. This resulted in the cuff surrounding the vessel. With the probe surrounding the vessel, the two pieces of suture were crisscrossed under the probe. Next, still allowing for the crisscrossed pattern under the vessel the opposing ends of the strings were tied around the transducer on the probe. Doing this allowed for required contact between the vessel and probes, which ensured the suture, would not slip off the probe onto the vessel. Once the probe was secured, a baseline auscultatory measurement was taken; using a 20 MHz Pulsed Doppler amplifier to ensure there was adequate contact between probe and vessel. After the probe was secured and tested: the intestines and stomach were guided back into the cavity, again taking care to not pinch perforate or pinch the organs, the cavity was sutured closed. The Doppler leads were then guided outside of the cavity subcutaneously, and exposed between the ears on the dorsal cranial side of rat, via the previous placed gauze tape. This placement of the leads reduced the risk of the rat chewing on the leads.

Before suturing the abdominal skin closed, an additional reading of the flow probe was taken; to ensure the probe still had proper contact with the vessel. This time the reading was done with the PowerLab equipment, which was the time the pretreatment/baseline stroke volume measurement, was obtained. After the closing incision, the area was cleaned with betadine, and the rat was administered a 500 unit/100 gm weight of rat (0.1cc/100gm) intramuscular dose of 5000 units Penicillin G, to reduce risk of infections. Subcutaneous sterile saline injection or lactated ringers was given (3-5 cc) to reduce dehydration during recovery. Upon recovery from anesthesia the rat was
given a .2 cc of Metacam orally, in order to reduce pain from surgery. Metacam was given twice a day for 72 hours post surgery.

**Electrical Stimulation of the Vagus Nerve (one week post Doppler implantation)**

The same anesthesia protocol was used as described in the previous section. Limb restraints were required to ensure proper EKG lead placement and connection, and to keep limbs out of the field. EKG leads/pins were placed as follows; negative lead to left armpit region, positive lead to right armpit region, and grounding lead to left hind limb. Armpit region placement of leads reduced the amount of noise that occurs during normal breathing of rat during EKG monitoring. After leads were in place baseline EKG and heart rate measurement were observed using Powerlab® receiver (AdInstruments) equipment and attached IBM® laptop with Chart 5® software (AdInstruments). Baseline EKG and heart rate were taken for 60 to 90 seconds allowing adequate equipment calibration at initiation of EKG monitoring. After baseline EKG and heart rate were obtained, program was paused and animal was prepped for the next surgical segment.

Norepinephrine solution was made by taking hospital grade 1mg/1ml (1:1) norepinephrine solution and diluting by half. The norepinephrine vial contains 4mg/4ml total. Since a small amount was used for this experiment, 0.5 ml of the 1:1 solution was drawn into a 1 ml syringe. Then 0.5 ml of sterile water was drawn into same syringe to fill the 1 ml capacity of the syringe. The syringe was recapped, inverted and swirled to mix the solutions. The resulting solution concentration in the syringe was 0.5 mg/1ml. For rats receiving electrical stimulation and norepinephrine, the same syringe was used.
tipped with a 30 gauge short needle, containing 0.5mg/ 1 cc for later injection into the peritoneal cavity. Fresh dilution of norepinephrine was made each day this procedure was done since the half life of norepinephrine dilution is < 24 hours.

After a baseline EKG was obtained, the leads were removed until time to obtain the treatment EKG. The neck and chest regions were numbed with 2% lidocaine solution. To isolate the vagus nerve an incision was made on the left lateral ventral cervical region about 1 inch to 1 ½ inches long, starting at about the fourth vertebrae and extending to the superior aspect of the left clavicle. Subdermal connective tissue was bluntly separated exposing underlying muscle groups via hemostats. Identification of the sternocleidomastoid group was the landmark desired. By gently manipulating the muscle encasing the trachea, with hemostats, going with the grain of the muscle, the muscle was separated. Doing this exposed the sheath containing the vagus nerve and carotid artery. Separating the sheath was unnecessary. Insertion of hemostats into the site where the muscle was separated, the hemostat was maneuvered to gently bring up the vagus nerve and carotid artery along with surrounding muscle tissue. Once the tissue was brought up out of original placement, precautions were taken with the placement of the hemostat to ensure that the trachea was not crushed by the weight of the hemostat (so hemostats are not on top of, or going across trachea). Next the stimulator leads were placed on the muscle with the vagus nerve. There was no need to place the leads directly on the vagus nerve, which can damage the nerve; the electrical impulse is transferred from the muscle to the vagus nerve. The rat was administered 0.2 cc of the norepinephrine solution in the intraperitoneal cavity as electrical stimulation commenced. The Grass SD9 Stimulator settings were: 4 volts, duration of 2 millisecond intervals, at a frequency of 2/sec.
Stimulation commenced for a total of 60 seconds, EKG was recorded for two minutes following electrical stimulation. After a 2 minute reading, stimulation with norepinephrine administration was repeated with an additional 2 minute EKG recording. The incision site was then closed with sutures and the area was cleaned with betadine. Post–surgery recovery protocol was the same as stated in the previous section.

**Unit Conversion of Power Lab**

The PowerLab was automatically set up to read measurements in voltage. Since the measurements of interest were to measure volume/unit of time the units needed to be changed. The first step in unit conversion was to pull up the input amplifier screen. The input amplifier icon was located in the right upper corner of the chart screen. Double clicking the input amplifier icon pulled up the input amplifier screen. At the bottom left hand corner of the input amplifier screen was the unit button, clicking on that icon button pulled up the unit conversion dialog box. In the upper right corner of the unit conversion window was the unit icon, that when clicked upon a pop-up menu appears listing the different units you can use. There was the option to make your own unit for use. For this project the unit used was Liters (L), which was on the menu of options for units. After the unit is selected and confirmed by clicking ok, the value parameters were set up. Further adjustments were made accordingly, by setting the numeric values to represent microliters by using scientific notation for liters.

Working with Dr. Kevin Kreider of the Mathematics Department at the University of Akron, unit conversion from mV to μL was accomplished. By taking previous
Doppler readings from a male WKY rat which was recorded using the mV units (figure 6), highest points in mV were obtained by tracing the recorded measurements with cursor. Doing this showed individual point measurements.

![Figure 6. Doppler tracing from male WKY without unit conversion](image)

By obtaining maximum points and minimum points from this tracing, these measurements were used to set the range of values for the microliter conversions. By initiating the unit conversion window as described in materials and methods or by using the analysis mode with Chart5 software without the powerlab running the unit conversions were made. With the unit conversion box up and the units set to Liters (L) and decimal places set to six; which allows for the microliter log exponential notation. Point 1 was set to correspond to the lowest microliter point. Lowest measured mV point is entered here, this point was -0.7047mV and was converted to 0 L. Point 2 was set to correspond to the highest mcL point, so the highest mV point was entered into point 2 box. The number conversion was 0.2912mV converted to 7e-005L. The number 7e-005L is derived as follows: 7e represents 70 µL the max stroke volume that would be recorded, -005 represents the decimal places moved to the left to decrease liters to microliters. The
units were converted and the tracing was adjusted in the program according to new unit parameters, (see figure 7). For subsequent readings, even if the lowest point dropped below the designated “0” point, the important factor was the difference between the max and min points. This difference was how stroke volume was determined.

![Doppler tracing from male WKY with unit conversion](image)

Figure 7. Doppler tracing from male WKY with unit conversion

Once unit conversion was set, the next step was to quantify the data and ultimately determine the stroke volume. This was ascertained by using the Chart5 software and the x= icon at the top of the chart. The steps to set up “x=” to represent the calculations of choice, was done by clicking on the tabulation icon, which then brings up the tabulation pad. By clicking on the fist column heading a window opened to show the mathematical choices used to interpret the data recorded. Selecting “statistical” brought up a second column within the window, where what type of statistical measurement needed can be selected. In that column, only one measurement was selected, i.e. “mean”. Selecting “OK” applied that measurement. By selecting the next columns heading, the same
protocol was repeated to obtain max point, min point, and max-min. Only one measurement/column is allowed in this program. By measuring the highest and lowest peaks, which were interpreted as the volume/unit of time at which the blood is passing through the vessel past the transducer on the probe. When reviewing the tracing form the velocity probe, the peaks represented higher flow past the transducer on the probe. Conversely, the troughs represented decreased or slow flow past the transducer on the probe. By using the max-min calculation stroke volume could be determined. This was accomplished by highlighting a section of the tracing, 1 minute time frame was used, and then selecting the max-min calculation would calculate the difference; which was determined to be the stroke volume. The measurement obtained from the max-min calculation was then multiplied with the heart rate obtained during time of stroke volume measurement to calculate cardiac output (figure 8).

Figure 8. Doppler tracing used to approximate Stroke Volume from adult male WKY rat
Weekly Blood Pressure Measurement, Heart Rate, and Cardiac Output Measurement

Pretreatment/baseline blood pressures and heart rate were taken prior to flow probe placement surgery. The baseline cardiac output was measured from stroke volume readings taken during the flow probe surgery. Starting one-week post surgery bi-weekly blood pressures, heart rate and aortic flow readings were taken. Blood pressure was taken using the tail cuff procedure.

Using the Doppler amplifier linked to the powerlab monitored heart rate and cardiac output. Light anesthesia was needed for this procedure. A 2mg/100 gm of weight of 2% Pentothal was administered i.p. to anesthetize the rat. The Doppler leads were hooked up to the Doppler amplifier, which was linked to the Powerlab. Upon running the chart 5 program, visible tracing of the Doppler was taken in 1 to 2 minutes. Using the software as described in the previous section stroke volume was measured. After stroke volume measurement, the rat was placed on the stage dorsal side down, and appendages restrained to allow for good EKG contact. Leads were placed at the base of the two upper in the appendages armpit region, and ground lead was placed at a lower appendage. After the EKG was obtained, the heart rate and stoke volume mean, measured that day, were used to calculate cardiac output (Heart rate*Stroke volume=Cardiac output).

Cardiac output, blood pressure, and heart rate data for each animal was placed into data table using Excel® from MicroSoft®, where mean scores for each animal were calculated for later use in statistical analysis.
Termination and Tissue Extraction

At the conclusion of the 5-week monitoring period, after the last EKG and stroke volume measurement was obtained, leads were removed and additional anesthesia was given (4mg/100g body weight of rat of 2-2.5% Pentothal solution) for euthanasia. After the anesthesia was administered, an incision was made running from the top of the sternum to the umbilicus, separating the pectoral muscles and exposing the sternum and ribs. Using hemostats, a puncture hole was made into the thoracic cavity and expanded by opening the hemostats. Widening the incision allowed for the viewing of the lungs and heart, this site acts as point of reference for placement of the scissors. Scissors were placed at the top of the hole and used to cut across the sternum. Once the heart was removed, it was gently blotted with gauze soaked with sterile water to remove excess blood and placed into a container filled with 10% formalin solution. After the heart was extracted, the probe was removed from the abdominal aorta, was cleaned and rinsed in sterile saline.

Histological Analysis and Tissue Damage Scoring

Hearts from containers of 10% formalin were cross-sectioned into three sections. The superior and inferior sections were placed back into the original container filled with 10% formalin. The middle section (containing largest section of the ventricles with top section containing atria removed) was placed into small weaved pattern basket, labeled identically to the original container, for later identification. The tissue processing used to dehydrate the tissue for later paraffin sectioning and
staining, was done automatically by the Tissue-Tek VIP equipment (Miles Scientific). After tissue processing, sectioned hearts were embedded into paraffin blocks. After blocks had hardened, they were sectioned on the microtome (Surgipath); sections were cut at a width of 7 microns. Microsections were placed onto slides and allowed to dry for later picrosirius red stain or hemotoxylin & eosin stain. Staining occurred automatically (Fisher Histomatic slide stainer model 172). After staining, the slides were viewed and analyzed under a microscope to examine and quantify extent of tissue damage between the groups. Pictures of slides were taken using an Olympus BX60 microscope with an attached DAGE-MTI DC-330 camera integrated with a Dell PC using a Scion image program by Scion Corp, for easier viewing and quantifying the tissue damage.

Tissue damage scoring was done via the principle investigator and 3 individuals blind to the experiment. Slides for scoring were selected based on clarity of picture; one from each rat was selected (total of 24 slides). Using Powerpoint® from MicroSoft®, selected pictures were grouped 4 per slide. Pictures were paired by similarity of region pictured, strain and treatment level, see Figure 8. The columns were separated by strain and the rows were by treatment level. The numerical value scale used to score the different levels of damage was 0-4. The value of “0” represented no damage, “1” little damage, “2” little to moderate damage, “3” moderate to severe damage, “4” considerable/severe damage (Seachrist et al. 2000). The blind reviewers were asked to use this scale, to indicate the level of damage that they saw for each slide, and were allowed to use half values. Each slide’s score, from each individual review, was then added together, the average for that slide was then used for statistical analysis.
Figure 9 is representative of a slide used during blind review of tissue damage. Actual arrangement of pictures on the slides was only known by the principle investigator. Examples of tissue damage in picrosirius red stains and hemotoxilyn and eosin stains were obtained from Histology text and lab books, and were given to blind reviewers for referencing during scoring. Once scoring was completed answer sheets were returned to the principle investigator, all scores for each animal were then placed into data table using Excel® from MicroSoft®, where mean scores for each animal were calculated for later use in statistical analysis.
Statistical Analysis

All data from cardiac output, heart weight, relative heart (heart weight/body weight of rat/100gms of body weight), blood pressure, epinephrine levels, norepinephrine levels, and tissue damage scoring were tested for statistical significance between WKY CTL (control), WKY EXP (experimental), SHR CTL and SHR EXP. The program used to run statistical analysis was Sigma Stat® from SysStat. The factors used for statistical analysis were strain (control vs. experimental) and treatment (baseline vs. post treatment). Tables were created in SigmaStat; empty cells were filled with the mean from the cells available from that group to keep cell count even. This was for done for 12 cells total in epinephrine (6) and norepinephrine (6), post level catecholamines. The six empty cells were from rats that died before scheduled termination day when post treatment retro-orbital blood draws were performed to obtain catecholamine levels (2 WKY CTL, 2 WKY EXP, 1 SHR CTL and 1 SHR EXP).

Statistical tests run were Paired T-Test, One-Way ANOVA, Two Way ANOVA, and Two-Way Repeated Measures ANOVA. Paired T-Tests were run for cardiac output, heart weight, relative heart weight and blood pressure comparing strains with similar treatment (ex. SHR-c vs. WKY-c). Two-way Repeated Measures ANOVA tests were run between strains and treatment levels for cardiac output, blood pressure, epinephrine, norepinephrine. A Two Way ANOVA test was run for tissue damage. One Way ANOVA analysis was performed for relative heart weights. Graphs were created using Sigma Plot from Systat using the mean and standard error means from the ANOVA results.
CHAPTER IV
RESULTS

Cardiac Output

Figure 10 is a graph showing the comparison of cardiac output between strain (control vs. experimental) and treatment levels (baseline vs. post treatment), means, s.e.m. (standard error mean), 2-way Repeated Measures ANOVA were used to create the graph. The following abbreviations were used to denote the different groups: WKY CTL (WKY Control), WKY EXP (WKY experimental), SHR CTL (SHR control), and SHR EXP (SHR experimental). The baseline means for the groups were respectively: WKY CTL (24.58 ml/min), WKY EXP (16.83 ml/min), SHR CTL (12.58 ml/min), SHR EXP (13.17 ml/min). The post treatment means for the groups were respectively: WKY CTL (18.56 ml/min), WKY EXP (22.07 ml/min), SHR CTL (17.17 ml/min), SHR EXP (32.50 ml/min). See Table 1. There was significance for different levels of treatment (baseline vs. post treatment) F= 5.279, p=0.032. There was a significant interaction between strain (control vs. experimental) and treatment (baseline vs. post treatment), F=5.693, p=0.027.
**Epinephrine**

Figure 11 is a graph representing the baseline and post treatment plasma epinephrine levels between strain and treatment levels (means, s.e.m., 2-way ANOVA). The baseline means for the groups were respectively: WKY CTL (267.20 pgs/ml), WKY EXP (195.50 pgs/ml), SHR CTL (300.83 pgs/ml), SHR EXP (328.71 pgs/ml). The post treatment means for the groups were respectively: WKY CTL (920.50 pgs/ml), WKY EXP (570.00 pgs/ml), SHR CTL (748.00 pgs/ml), SHR EXP (1989.00 pgs/ml). See Table 2. There was significance between the different treatments (baseline vs. post treatment) $F=43.985$, $p<0.001$.

**Norepinephrine**

Figure 12. is a graph showing the comparison between treatment levels and strains for plasma norepinephrine levels (means, s.e.m., 2-way ANOVA). The baseline means for the groups were respectively: WKY CTL (218.4 pg/ml), WKY EXP (279.0pg/ml), SHR CTL (666.33 pg/ml), SHR EXP (761.71 pg/ml). The post treatment means for the groups were respectively: WKY CTL (1011.0 pg/ml), WKY EXP (1200.0 pg/ml), SHR CTL (1698.00 pg/ml). SHR EXP (1435.00 pg/ml). See Table 3. There was significance among the different levels of treatment (baseline vs. post treatment) $F=5.279$, $p=0.032$. There was a significant interaction between strain and treatment, $F=5.693$, $p=0.027$. 
Blood pressure

Figure 13 is a graph representing a two way ANOVA comparison of blood pressure between strain and treatment levels (mean, s.e.m., 2-way ANOVA, 2-way repeated measure ANOVA). The baseline means for each group were respectively: WKY CTL (130.65 mmHg), WKY EXP (129.9 mmHg), SHR CTL (127.8 mmHg), and SHR EXP (142.75 mmHg). The post treatment means for each group were respectively: WKY CTL (131.5 mmHg), WKY EXP (130.25 mmHg), SHR CTL (128.25 mmHg), SHR EXP (143.00 mmHg). See Table 4. There was no statistical difference between the different strains (F=0.125, p=1.727) or between treatment levels (F=1.472, p=0.238).

Relative Heart Weight

Figure 14 is a graph representing the relative heart weights for strain and treatment level (mean, s.e.m, 1-way ANOVA). The means for the groups were respectively: WKY CTL (0.728 gm/100gms body weight), WKY EXP (0.647 gm/100gms body weight, SHR CTL (0.57 gm/100 gms body weight), SHR EXP (0.675 gm/100 gms body weight). See Table 5. There was no statistical difference between the groups, F=2.084, p=0.143.

EKG Analysis

Figure 15a-c are of EKG tracings of an SHR female. Figure 15a was taken prior to norepinephrine and electrical stimulation administration heart rate was 240 bpm. Figure
15b is an EKG tracing of same female during treatment of norepinephrine and electrical stimulation heart rate was 225 bpm. Note irregular pattern of EKG when compared to 15a, QRS complex has narrowed and elongated which is indicative of ventricular tachycardia. Figure 15c., heart rate is 210 bpm, was taken post treatment, notice the continuation of QRS elongation when compared to 15a, pattern is not irregular as noticed in 15b.

Figure 16a-c are EKG tracings of a WKY female. The Figure 16a tracing was taken prior to norepinephrine administration and electrical stimulation, the heart rate was 348 bpm. Figure16b was taken during electrical stimulation and norepinephrine administration, notice the elongated and narrowed QRS complex, this is indicative of ventricular tachycardia, heart rate was not able to be measured due the EKG moving past the range. Figure 16c. is the EKG tracing for the WKY rat taken after electrical stimulation and norepinephrine administration. There is no evidence of the ventricular tachycardia that was seen in Figure 16b, heart rate was 372.

**Ventricle and Mitral Valve Damage**

Figure 17 is a graph showing the comparison of tissue damage between strain and treatment level (mean, s.e.m, 2-way ANOVA). The means for each group were respectively: WKY CTL (0.167), WKY EXP (0.313), SHR CTL (0.333), and SHR EXP (1.183). See Table 6. There was statistical significance between strains F=4.834, p=0.040. There was statistical significance between treatment levels F=4.454, p=0.048.
Figure 18 is a representative slide of the mitral valve comparison between the strain and treatment level. Slides were taken at 400x. These slides are from SHR and WKY female rats from the project, these slides were made using a picrosirius red stain and magnified 400x. The darker red color indicates higher collagen concentration/damage. The SHR control female the papillary muscle (PM) found in the left ventricle with its chordae tendinae (CT) not attached. The WKY control female slide shows the PM without its CT; note the lighter color of the PM compared to the SHR control. The SHR experimental slide shows the left ventricle wall (LVW) and the mitral valve (MV). The WKY experimental slide also shows the LVW and the MV; note the darker red and increased amount of red in the SHR experimental LVW and MV.
Figure 10. Cardiac Output. Comparison of baseline and post treatment cardiac output between strains and treatment levels (means, s.e.m., 2-way Repeated Measures ANOVA), significance between treatment (baseline vs. post treatment) $F=5.279$, $p=0.032$.
Table of 2-Way Repeated Measure ANOVA Data for Cardiac Output

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
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<td>1.126</td>
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<td>432.160</td>
<td>432.160</td>
<td>5.279</td>
<td>0.032*</td>
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<td>Strain x Treatment</td>
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<td>466.096</td>
<td>466.096</td>
<td>5.693</td>
<td>0.027**</td>
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<td>1719.277</td>
<td>81.870</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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<td>5653.159</td>
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Table 1. Table of 2-Way Repeated Measure ANOVA For Cardiac Output data. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P=probability. * and ** indicate significances
Figure 11. Epinephrine. Comparison of plasma epinephrine levels baseline and post treatment between strains (means, s.e.m., 2-way Repetaed Measure ANOVA). There was significance for the different treatment levels (baseline vs. post treatment) $F=43.985$, $p<0.001$ (*). There was significance between strain (control vs. experimental) vs. post treatment $p=0.01$ (**).
Table of 2-Way Repeated Measure ANOVA for Epinephrine Data

<table>
<thead>
<tr>
<th>Source of Variation</th>
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<th>P</th>
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<td>43.985</td>
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Table 2. Table of 2-Way Repeated Measure ANOVA for Epinephrine Data. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P= probability. * indicates significance.
Figure 12. Norepinephrine. Comparison of norprinephrine levels between strains (control vs. experimental) and treatments (means, s.e.m., 2-way Repeated Measures ANOVA). There was significance among the levels of treatment $F=111.214$, $p<0.001$ (*).
### Table of 2-Way Repeated Measures ANOVA For Norepinephrine Data

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<td>Treatment</td>
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<td>8384354.319</td>
<td>8384354.319</td>
<td>111.214</td>
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<td>53597.101</td>
<td>0.711</td>
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<td>13939550.67</td>
<td>296586.184</td>
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Table 3. Table of 2-Way Repeated Measures ANOVA For Norepinephrine Data. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P= probability. * indicates significance.
Blood Pressure

Figure 13. Blood Pressure. This is a graph representing a two way ANOVA comparison of blood pressure between strain and treatment levels (mean, s.e.m., 2-way Repeated Measures ANOVA). There was no significance comparing strain (control vs. experimental) vs. treatment (baseline vs. post treatment).
### Table of 2-Way Repeated Measures ANOVA for Blood Pressure Data

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<td>2.750</td>
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Table 4. Table of 2-Way Repeated Measures ANOVA for Blood Pressure Data. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P= probability. There was no significance.
Relative Heart Weight

Figure 14. Relative Heart Weights. This is a graph representing the relative heart weights for strain and treatment level (mean, s.e.m, 1-Way ANOVA). There was no significant difference between the strain (control vs. experimental) $F=2.084$, $p=0.143$. 
Table of 1-Way ANOVA Data for Relative Heart Weights

<table>
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<tr>
<th>Source of Variation</th>
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<th>SS</th>
<th>MS</th>
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<th>P</th>
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</thead>
<tbody>
<tr>
<td>Between Groups</td>
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<td>0.0652</td>
<td>0.0217</td>
<td>2.084</td>
<td>0.143</td>
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<tr>
<td>Total</td>
<td>19</td>
<td>0.232</td>
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Table 5. Table of 1-Way ANOVA Data for Relative Heart Weights. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P= probability. There was no significance.
Figure 15a. SHR female– Pre Norepinephrine and Electrical Stimulation

Figure 15b. SHR female During Norepinephrine Delivery and Electrical Stimulation

Figure 15c. SHR female- Post Norepinephrine Delivery and Electrical Stimulation

Figure 15a is an EKG tracing of an SHR female taken prior to norepinephrine and electrical stimulation administration heart rate was 240 bpm. Figure 15b is an EKG tracing of same female during treatment of norepinephrine and electrical stimulation heart rate was 225 bpm. Note irregular pattern of EKG when compared to 15a, QRS complex has narrowed and elongated which is indicative of ventricular tachycardia. Figure 15c., heart rate is 210bpm, was taken post treatment, notice the continuation of QRS elongation when compared to 15a, pattern is not irregular as noticed in 15b.
EKG Tracing for WKY Female

Figure 16a. EKG from WKY EXP female baseline EKG, HR 348 bpm.

Figure 16b. EKG from WKY Female during electrical stimulation with norepinephrine administration, note QRS elongation, some so severe tip of peak went beyond range of graph unable to calculate HR.

Figure 16c. EKG from WKY Female after electrical stimulation with norepinephrine. EKG returned to normal rhythm HR 372.
Figure 17. Tissue Damage. This is a comparison of tissue damage between strain (control vs. experimental) and treatment (baseline vs. post treatment). (means ± S.E.M., 2 way ANOVA). There was statistical significance between strains $F=4.834$, $p=0.040$ (*). There was statistical significance between treatment levels $F=4.454$, $p=0.048$ (**).
Table of 2-Way ANOVA Data for Tissue Damage

<table>
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<tr>
<th>Source of Variation</th>
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<td>Strain</td>
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<td>1.615</td>
<td>1.615</td>
<td>4.843</td>
<td>0.040*</td>
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<tr>
<td>Treatment</td>
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<td>1.488</td>
<td>1.488</td>
<td>4.454</td>
<td>0.048**</td>
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<tr>
<td>Strain * Treatment</td>
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<td>0.744</td>
<td>0.744</td>
<td>2.227</td>
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<td>Residual</td>
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</table>

Table 6. Table of 2-Way ANOVA Data For Tissue Damage. DF=Degrees of Freedom, SS = Sum of Squares, MS=Mean of Squares, F= F variance, P= probability. * and ** indicate significance.
Figure 18. These slides are Form SHR and WKY female rats from the project, these slides were made using a picrosirius red stain and magnified 400x. The darker red color indicates higher collagen concentration/damage. The SHR control female the papillary muscle (PM) found in the left ventricle with its chordae tendinae (CT) not attached. The WKY control female slide sows the PM without its CT; note the lighter color of the PM compared to the SHR control. The SHR experimental slide shows the left ventricle wall (LVW) and the mitral valve (MV). The WKY experimental slide also shows the LVW and the MV; note the darker red and increased amount of red in the SHR experimental LVW and MV.
CHAPTER V
DISCUSSION

The hypothesis was: hypertension increases the severity of progression of mitral valve prolapse in females and that the functional and structural symptoms would be more severe in the SHR EXP group. It was expected that the SHR EXP group would have reduced cardiac output, elevated catecholamine levels, elevated tissue damage, and reduced blood pressure after receiving the stimulation and mitral valve prolapse treatment.

In support of the hypothesis the plasma catecholamine levels in the SHR EXP were elevated compared to the WKY EXP. Post treatment epinephrine analysis showed the SHR EXP had the highest plasma epinephrine levels. One explanation for this result was that the compensatory mechanism for cardiac output was functioning in response to the trauma induced to the heart. One compensatory mechanism is the sympathetic nervous system and its control of cardiac output and blood pressure via norepinephrine and epinephrine.

Most of the plasma norepinephrine is produced through spillover from the adrenergic neurons. In contrast, the adrenal medulla, contributes a small amount of plasma norepinephrine, but produces most of the plasma epinephrine content. Both of these catecholamines are released by the sympathetic nervous system via the
neurotransmitter acetylcholine. The combined effect of these catecholamines is increased CO, heart rate, blood pressure and respiration. A chronic release in norepinephrine and epinephrine can cause symptoms of anxiety, which is often a symptom of mitral valve prolapse.

The SHR rat strain has been shown to have increased sympathetic activity and sensitivity to norepinephrine (Ely et al. 1997). This increase in the catecholamines would indicate the compensatory mechanism for decreasing cardiac output was working. Therefore an increase in the catecholamine levels would also indicate increased sympathetic activity to increase the cardiac output to sustain life, by compensating for the reduced cardiac function. Marks (2003) stated; “It has been known for over 40 years that heart failure patients live in a continuous hyperadrenergic state with high levels of circulating catecholamines.”

Another explanation for this result could be due to the interaction of epinephrine levels upon norepinephrine. Goldstein et al. (2003) reported that administration of physiologically active amounts of epinephrine enhances neurogenic vasoconstrictor responses or enhances release of norepinephrine during sympathetic stimulation. The epinephrine hypothesis explains this as follows: Sympathetic nerve terminals take up circulating epinephrine; sympathetic stimulation coreleases the removal of epinephrine with norepinephrine. The coreleased epinephrine binds to β-adrenoceptors on sympathetic terminals and augments further norepinephrine release, enhancing neurogenic vasoconstriction during sympathetic stimulation (Goldstein et al., 1999). Therefore, the increase in epinephrine could be another compensatory mechanism to increase the norepinephrine plasma concentration, and alleviate the impending decrease
in cardiac output. This can also explain why the SHR EXP group had elevated cardiac output, epinephrine levels but not norepinephrine levels post treatment compared to SHR CTL, WKY CTL and WKY EXP groups.

Cardiac output is used to monitor cardiovascular performance and function. Severely decreased cardiac output is indicative of heart failure. The anticipated decrease in cardiac output in MVP was thought to be due to diminished blood flow into the left ventricle coupled with the back flow of blood through the annulus into the left atrium (regurgitation) (Boudoulas, 2004). This coupled with increased aortic stiffness and ventricular hypertrophy usually account for the decrease in cardiac output found in subjects presenting with MVP. Symptoms of essential hypertension are similar to those found in MVP: increased aortic stiffness and ventricular mass, and a decrease in cardiac output. Therefore, it was postulated that having both disorders would exacerbate the symptoms of each. Results confirming this hypothesis would have shown a greater decrease in cardiac output in the SHR EXP group since this group already had hypertension and was receiving treatment to promote mitral valve prolapse.

Additionally, Nakata et al. (2005) showed that impairment at the sympathetic presynaptic neurons reduces myocardial uptake of norepinephrine. This reduction in norepinephrine uptake causes accelerated leakage and spillover of the catcholamine at the nerve endings; which contributes to the increase in post synaptic drive during heart failure (Nakata, et al. 2005). Therefore, impairment of the cardiac sympathetic innervation (sympathetic dysfunction or dysautonomia) contributes to arrhythmias i.e ventricular tachycardia and an increased incidence of sudden cardiac death from heart failure.
Cardiac output has been shown to affect other cardiovascular functions, such as, blood pressure. With regards to blood pressure, the anticipated decrease may not have been observed after MVP treatment, because the compensatory mechanism, the catecholamines, were still effective in preventing reduced heart function. The blood pressure would remain elevated since cardiac output was elevated, and cardiac output is a component of blood pressure (blood pressure = cardiac output * vascular resistance). Therefore, since cardiac output remained elevated in the SHR EXP group, the blood pressure did not decrease as anticipated. Julius, (1988), stated that during the early phase of hypertension; a hyperkinetic circulation characterizes this stage. Excess in sympathetic and decreased parasympathetic control in the heart causes the hyperkinetic circulation. During this early phase of hypertension, CO is increased via neurogenic mechanisms, but later begins to decrease and normalize despite the hypertensive state continuing. During this phase, Julius, 1988, indicated that there is an increase in plasma norepinephrine levels. There is also an increase in epinephrine levels; which in combination with norepinephrine, increases cardiac output, and ultimately blood pressure. A longer sequence of data collection for 8 to 10 weeks would have potentially allowed heart failure to develop. The structural and functional changes postulated would have followed the decrease in norepinephrine, since neurohumoral changes are the first to occur after cardiac trauma. Following the decrease in the catecholamines, the cardiac output would drop as well as blood pressure, which was the anticipated result.

Heart failure from arrhythmias and/or hypertrophy is due to the left ventricle not pumping adequate blood volume to the body. There are no EKG changes that would indicate heart failure; rather, the EKG changes that occur are in response to structural
changes that occur with heart failure, such as, hypertrophy. With regards to EKG changes, hypertension can produce hypertrophy. On an EKG tracing hypertrophy is indicated by ST segment elevations and T-wave changes. The lack of this finding could be explained by two possibilities; not enough damage occurred to promote electrical conductivity changes, or not enough time had elapsed from the treatment to allow for electrical conductivity changes to occur. Possibly, more than 6 weeks needs to elapse to observe physiological and anatomical changes to occur after trauma.

Dickout (1997) stated that elevated catecholamines have been shown to cause ventricular hypertrophy. Breist et al. (2004) used male and female Sprague-Dawley rats, which were injected with norepinephrine and caused increased fibrosis (thickening) of the ventricle myocardium. This type of fibrosis and hypertrophy occurs in both hypertension and mitral valve prolapse, and is often caused by ventricular tachycardia (La Vecchia et. al. 1998). It was postulated that the SHR EXP group would have the greatest relative heart weight. Having an increased heart weight in this group would support increased ventricular mass and hypertrophy in SHR rats with treatment. Indeed, both hypertensive and MVP hearts showed increased ventricular mass and hypertrophy.

Norepinephrine has been suggested to be the myocardial hypertrophic hormone, responsible for hypertrophy and cardiac tissue damage, Schlaich et al (2003), Patel et al. (1991). With regards to tissue damage, the treatment of electrical stimulation with norepinephrine administration did promote damage, and was greater in the SHR EXP group, as hypothesized. The increased tissue damage could be due to the combined effects of the treatment and the effects of hypertension on the myocardium. Schlaich et al. (2003) concluded that increased cardiac norepinephrine release is related to the
development of hypertensive left ventricular hypertrophy. Patel et al. (1991), adds, that chronic infusion of subpressor dose of norepinephrine in mongrel dogs was shown to increase mass and thickness of the left ventricle wall. Despite the absence of increased relative heart weight, there was structural damage. Structural damage (cardiac fibroplasia) and remodeling (cardiac fibrogenesis) are the first steps involved in hypertrophic restructuring. Therefore, the treatment, used in this study, was enough to cause damage in the experimental groups, and was more severe in the SHR EXP group, most likely due to the additional effects of hypertension.
CHAPTER VI
CONCLUSION

The hypothesis for this project was that hypertension increases the severity of mitral valve prolapse (MVP) and its associated symptoms in female SHR rats compared to WKY female rats.

The SHR EXP rat showed greater cardiac damage compared to WKY EXP and controls, which supports the idea that the experimental treatment was sufficient to produce ventricular tachycardia to cause papillary muscle and ventricle wall damage shown to promote MVP. The catecholamine levels were increased to possibly maintain cardiac function. This may be due to enhanced sympathetic activity which controls the compensatory mechanism against heart failure, by increasing blood pressure and cardiac output. Therefore, the combined effects of the treatment and hypertension on the heart may have caused greater damage. In summary, this research supports the use of the rat model for MVP studies, since the SHR strain already has the sympathetic dysfunction and ventricular structural changes which has been shown to occur in both MVP and hypertension.
Future Directions

One future direction would be to extend the length of time of the study to observe the postulated decrease in cardiac output. Also it would be important to allow several end points to perform histological analysis on the hearts to compare damage between the time, strains, and treatment. Weekly blood pressure and cardiac output changes should be monitored to isolate the time the functional changes occur, while extending the time for structural changes. Weekly EKG’s should be taken for heart rate, but would also be used to monitor any electrophysiological changes that could occur with the continued restructuring occurring after damage. This could show evidence of ST elevation, or ventricular tachycardia.

Another option for the surgical procedure would be to increase the interval of stimulation and norepinephrine administration. Instead of one cycle the use of, multiple cycles may promote greater functional changes. This could potentially exacerbate the damage that occurs allowing the functional and structural changes to be more prominent.

With regards to molecular biology, measurement of the norepinephrine transporter protein in the left ventricle myocardium could be analyzed to quantify sympathetic dysfunction and activity. This type of analysis would help to validate the decrease in cardiac output in the SHR EXP. Finally, the use of a genetically engineered rat with a connective tissue disorder, such as, Marfan’s syndrome, could be used as a model for primary mitral valve prolapse. In contrast of using
the surgically induced secondary MVP model proposed with this completed project.
REFERENCES


Boudoulas H. The floppy mitral valve, mitral valve prolapse, mitral valve regurgitation, and the floppy mitral valve/mitral valve prolapse syndrome current concepts. Hellenic J Cardiol 2004;45


Marks A. Calcium and the heart: a question of life and death. *J of Clin Invest.* 2003 111:5

MICROMEDEX Healthcare Series, located at [http://www.agmc.org/mdxcgi/mdxhtml.exe?&tmpl=mdxhome.tm1&SCNAME=mdxhome&ctl=d:\mdx\mdxcgi\megat.sys](http://www.agmc.org/mdxcgi/mdxhtml.exe?&tmpl=mdxhome.tm1&SCNAME=mdxhome&ctl=d:\mdx\mdxcgi\megat.sys) used through Akron General Medical Center pharmacy. All micromedex information on Norepinephrine and calcium gluconate.


October 19, 2004

Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Ely, Ms. Annissa Langworthy

On October 12, 2004 the Institutional Animal Care and Use Committee reviewed your protocol titled:

Dr. Ely, Ms. Annissa Langworthy, "Hypertension's Effects on Mitral Valve Prolapse"
IACUC # 04-10-F
48 rats

Your project has received unanimous approval pending modifications. Investigators need to include how the abdominal surgery will be performed. Also, investigators names need to be removed from protocol.

You must provide the committee with documentation of updated risk information, or serious adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Sincerely,

James Holda
IACUC Chair