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The Effects of Ovarian Hormones and Exercise on Gene Markers of Cardiac Dysfunction

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THE EFFECTS OF OVARIAN HORMONES AND EXERCISE ON GENE
MARKERS OF CARDIAC DYSFUNCTION

A Thesis Presented

by

ANISHA PATEL

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

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Department of Kinesiology

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ABSTRACT

THE EFFECTS OF OVARIAN HORMONES AND EXERCISE ON GENE MARKERS OF CARDIAC DYSFUNCTION

MAY 2015

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Heart disease is the leading cause of death in women in the United States. Premenopausal women appear to have better cardiac function and lower risk of heart disease compared to male postmenopausal female counterparts. Ovarian hormone loss influences blood pressure homeostasis and causes systemic inflammation, which may result in chronic stress on the heart. Two key physiological changes in cardiac dysfunction are reemergence of the fetal gene pattern and myocardial remodeling. Physical activity has been linked to improved cardiac function. The purpose of this study was to investigate the effects of ovariectomy on early markers of cardiac dysfunction and fibrosis and to determine if voluntary physical activity alters expression patterns in ovariectomized mice. We investigated the effects of ovariectomy and exercise on cardiac expression of fetal genes and markers and mediators of fibrosis in two cohorts of 8-10 week old female mice. Ovariectomized mice had greater expression of cardiac fetal genes and real time-PCR (RT-PCR) results indicated activation of the fibrosis pathway. Exercise was able to influence the expression of some markers of cardiac dysfunction. We concluded that ovarian hormone loss and associated physiological changes such as

increased adiposity and systemic inflammation trigger early changes in cardiac gene expression that precede overt cardiac dysfunction.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
CHAPTER	
1: INTRODUCTION	1
2: LITERATURE REVIEW	4
2.1 Introduction	4
2.2 Heart Disease & Women.....	4
2.2.1 Prevalence and Pathology	4
2.2.2 Ovarian Hormones	7
2.3 Evaluating Cardiac Pathology: The Fetal Gene Program	9
2.4 Fibrosis	13
2.5 Inflammation.....	17
2.6 Physical Activity.....	19
2.7 Overview.....	22
3: METHODS	24
3.1 Introduction	24
3.2 Animals.....	24
3.2.1 Cohort 1:	24
3.2.2 Cohort 2:	25
3.3 Tissue Analysis	26
3.3.1 Gene expression	26
3.3.2 Statistical Analysis	27
4: RESULTS	29
4.1 Cohort 1	29
4.1.1 Animal Characteristics	29
4.1.2 Gene Expression	29
4.1.3 Cohort 1: Fetal Genes	30

4.1.4 Cohort 1: Fibrotic Markers	31
4.1.5 Cohort 1: ECM fibers.....	31
4.2 Cohort 2.....	32
4.2.1 Animal Characteristics	32
4.2.2 Gene Expression	33
4.2.3 Cohort 2: Fetal Genes	33
4.2.4 Cohort 2: Fibrotic Markers	35
4.2.5 Cohort 2: ECM fibers.....	36
5: DISCUSSION.....	38
5.1 Ovariectomy Causes Activation of the Fetal Gene Program	38
5.2 Ovariectomy Activates Cardiac Fibrosis Associated Genes	42
5.3 Exercise inhibits a pathological gene expression pattern with ovariectomy	44
5.4 Exercise may play a role in limiting pathological remodeling of the myocardium	46
6: CONCLUSION.....	49
6.1 Strengths and Limitations	50
6.2 Future Directions	51
APPENDIX: RUNNING DATA	52
REFERENCES.....	54

LIST OF TABLES

Table	Page
1. Gene Expression Targets	28
2. Animal Characteristics for Cohort 1	29
3. Animal Characteristics for Cohort 2	33

LIST OF FIGURES

Figure	Page
1. CVD mortality trends for males and females	6
2. Prevalence of cardiovascular disease (CVD) in adults	6
3. Ventricle RNA expression of fetal genes in pressure overload model	12
4. Ventricle RNA expression of fetal genes in physiological hypertrophy model	20
5. Interleukin-1 α cardiac gene expression	21
6. Plasminogen activator inhibitor-1 (PAI-1) cardiac gene expression	21
7. Expression of fetal genes (Cohort 1)	30
8. Gene expression of fibrotic markers (Cohort 1)	31
9. Gene expression of ECM fibers (Cohort 1)	32
10. Expression of fetal genes (Cohort 2)	35
11. Gene expression of fibrotic markers (Cohort 2)	36
12. Gene expression of ECM fibers (Cohort 2)	37

CHAPTER 1

INTRODUCTION

Heart disease is the leading cause of death in women. Prior to menopause, women tend to have better cardiac outcomes compared to men, exhibiting preserved contractility with age and better responses to cardiac stressors such as hypertension and myocardial injury (19, 66). After menopause, mortality rates from heart disease increase and prognosis worsens. Most women who suffer from heart failure are postmenopausal and more women over the age of 45 who have a heart attack die within 1 year compared with men (29, 82).

The gender disparity associated with heart disease combined with the detrimental effects of menopause on female cardiac health provides evidence of a possible cardioprotective role of ovarian hormones. Ovarian hormone loss is linked to increased adiposity, insulin resistance, and systemic inflammation (85). Estrogen-mediated regulation of the renin-angiotensin-aldosterone system (RAAS) is lost after menopause, resulting in RAAS hyperactivity and consequential problems with natriuresis and diuresis (15, 34). Resulting blood pressure imbalance and increased hemodynamic load induce bouts of ischemia and increase wall stretch on the heart. This disease environment closely resembles the *in utero* environment of the growing fetal heart and is characterized by the re-expression of a fetal gene pattern (84, 106). While this regression in gene transcription has been well documented in models of acute cardiac injury or induced heart disease, early changes in expression patterns of cardiac dysfunction markers in a model of ovarian hormone loss have not yet been established (15, 84, 94).

In heart disease models, activation of the fetal gene pattern can exacerbate RAAS activity, resulting in increased levels of intracardiac angiotensin II (AngII) (23, 87). Acute injury or chronic stress can initiate an inflammatory response, cardiomyocyte hypertrophy, and myocardial remodeling (10). The myocardium is comprised of a variety of resident cells and AngII can trigger cardiac fibrosis through activation of cardiac fibroblasts (CFB) (48, 58). Activated cardiac fibroblasts differentiate to myofibroblasts (MFBs), cells that produce collagen and other ECM proteins, resulting in increased fibrosis (24).

Estrogen supplementation has been shown to reduce negative remodeling of myocardial tissue, indicating the hormone may have anti-fibrotic effects (70). Both cardiac myocytes and fibroblasts express functional estrogen receptors and 17β -estradiol has been shown to inhibit collagen synthesis in cardiac fibroblasts by downregulation of AngII receptors (32, 125). To date, severe cardiac fibrosis and tissue remodeling has been shown in models inducing acute injury or stress or have investigated the effects of estrogen related to disease pathology in an aged model or induced cardiac dysfunction; signs of cardiac tissue fibrosis at early stages of ovarian hormone loss without intentional ischemia or cardiac injury have not been established.

Physical activity has been shown to decrease the risk for heart disease. Exercise is linked to reduced cardiac dysfunction and attenuation for pathological remodeling (51). Recent studies have also shown exercise may decrease cardiac fibrosis attributed to low estrogen levels (70). Preliminary data from our lab has shown that physical activity influences a possible inflammatory and fibrotic response to low ovarian hormone levels in the heart. Understanding potential benefits of exercise on cardiac function in an

ovariectomy model may provide alternatives to hormone therapy, a controversial treatment for postmenopausal women.

The specific aims and hypotheses for this study have been outlined below:

Aim 1: To study the effects of ovariectomy on the markers of disease in mouse heart tissue.

Hypothesis 1: The hearts of ovariectomized (OVX) mice will exhibit evidence of dysfunction via a fetal gene expression pattern compared with sham surgery (SHAM) hearts.

Aim 2: To study the effects of ovariectomy on gene expression patterns that indicate fibrosis in mouse heart tissue.

Hypothesis 2: OVX mice will express more cardiac profibrotic markers and will exhibit more extracellular matrix (ECM) protein deposition compared with SHAM counterparts.

Aim 3: To determine whether exposure to voluntary wheel running physical activity (EX) influences markers of cardiac dysfunction and fibrosis associated with ovariectomy.

Hypothesis 3A: EX mice will exhibit lower expression of cardiac fetal gene markers compared with sedentary (SED) counterparts.

Hypothesis 3B: EX mice will exhibit lower cardiac ECM gene expression compared with SED counterparts.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review will highlight the prevalence of heart disease in women and the importance of studying the increase in risk that is associated with menopause. Changes in ovarian hormone levels will be assessed as key risk factors; and the relationship between ovariectomy, the cardiac fetal gene program, and fibrosis will be detailed. Lastly, the influence of exercise on heart disease pathology and dysfunction will also be explored.

2.2 Heart Disease & Women

2.2.1 Prevalence and Pathology

Heart disease is currently the leading cause of death in women. In 2010, Direct and indirect costs associated with heart disease exceeded \$300 billion (29). According to CDC data from 2009, one in four female deaths was caused by heart disease in the United States (56). This statistic increased to 1 in 3 female deaths by 2013 and will likely augment with the increasing prevalence of factors associated with heart disease such as hypertension, diabetes, obesity, and high cholesterol (73, 74). The increase in the aging population is also a significant risk factor as individuals over the age of 65 have a greater risk of stroke, myocardial infarction, coronary heart disease (CHD), hypertension, and heart failure (37).

Sex differences have been shown in a variety of heart and heart disease-related outcomes. While women preserve myocyte number and size with age, male myocardial mass decreases as a result of a reduction in the number of myocytes, and this loss of mass

leads to compensatory myocyte hypertrophy (78). This pathological hypertrophy results in decreased systolic function and increased left ventricle diameter over time (31). Chamber dilation and wall thinning result in poor cardiac contractility in males, while contractility is better preserved in females with age (14, 19). Premenopausal women fare better in response to hypertension, aortic stenosis, hypertrophic cardiomyopathy, and acute myocardial ischemia compared to their male counterparts (66, 82). Premenopausal women also appear to have better diastolic function than men, although diastolic function decreases in both sexes with age (31, 78). Gender disparities regarding heart health and function indicate that females have cardioprotection prior to menopause, potentially from ovarian hormones.

The contribution of ovarian hormones to cardiac pathology, function, and outcomes is evident when comparing pre- and postmenopausal women. Most women that suffer from heart failure are postmenopausal (82). After menopause, female mortality rates due to heart disease increase significantly and surpass those of men (Figure 1) (28, 97). Given the increases in the elderly population, women past menopause will have a direct impact on the prevalence of heart disease and heart disease associated costs. After 65, the incidence of heart failure nears 10 per 1000 people and American Heart Association projections show that by 2030, the prevalence of heart failure will increase by 25% (29). The prevalence of CVD, hypertension and heart failure increases drastically in women and surpasses prevalence in male counterparts with age (Figure 2) (17). The increase in risk associated with menopause combined with the dramatic increases in size of the elderly population pose a significant problem regarding the health burden and treatment costs in the United States.

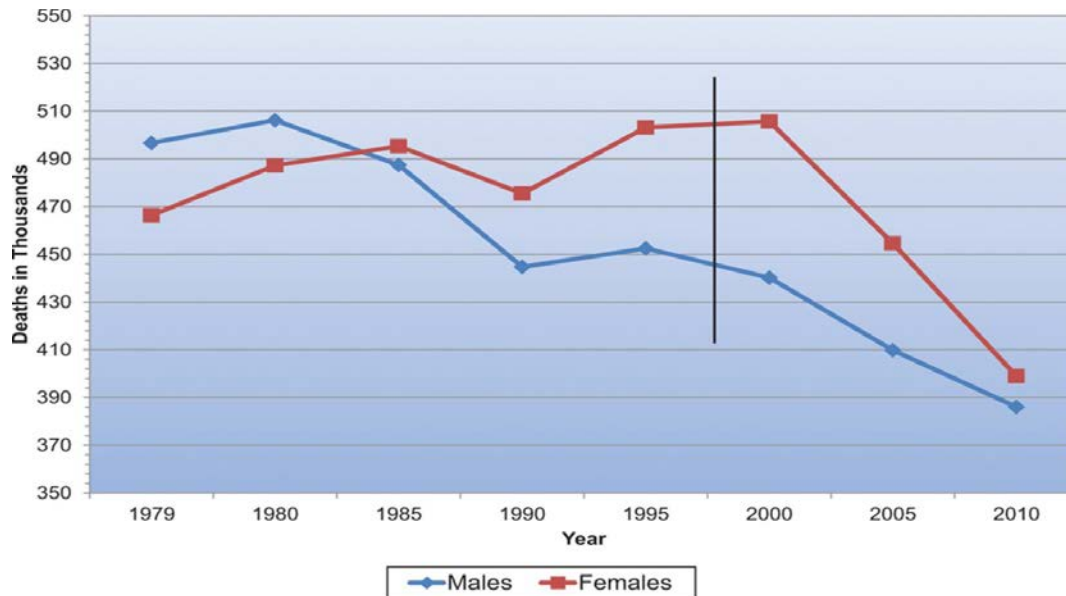
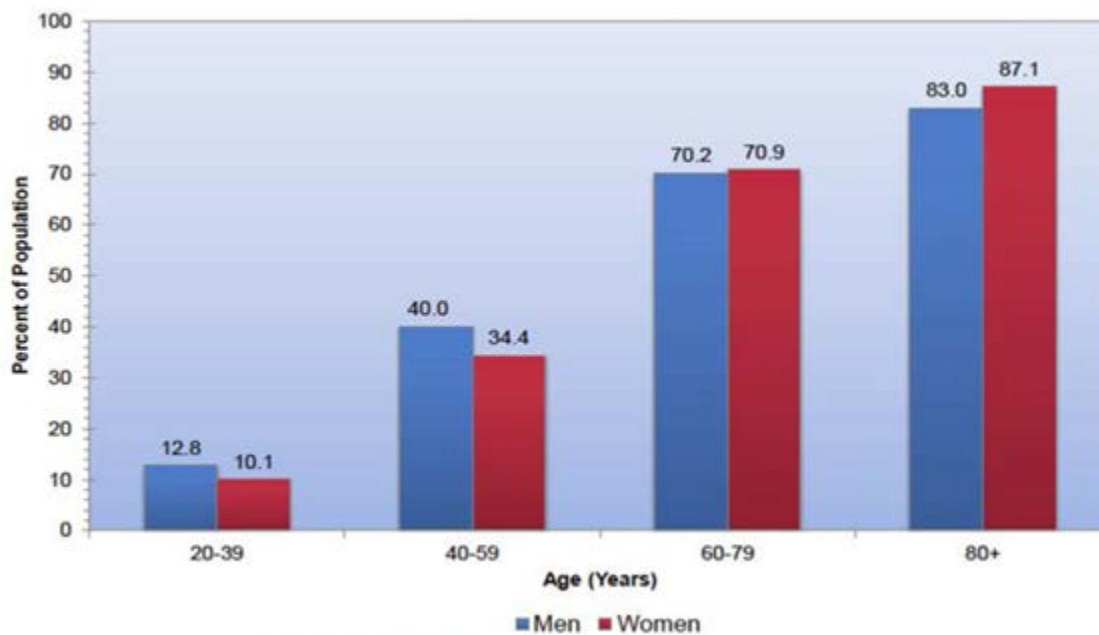


Figure 1: CVD mortality trends for males and females (United States:1979-2010) {{354 Go,A.S. 2014}}.



These data include CHD, HF, stroke, and hypertension.

Figure 2: Prevalence of cardiovascular disease (CVD) in adults ≥ 20 years of age (NHANES 2007-2010). Source National Heart, Lung, and Blood Institute {{355 Anonymous 2012}}.

The reduction in circulating ovarian hormones that accompanies menopause is linked to an increased risk for heart disease, indicating that ovarian hormones may be cardioprotective (66). The direct effects of ovarian hormone reduction on heart health

have not been thoroughly assessed although some literature suggests that cardioprotective effects of the ovarian hormone estrogen (E2) include decreased cardiac inflammation and pathological remodeling (55). For example, ovariectomization in mice, a model used to study postmenopausal physiology, results in greater left ventricular hypertrophy in response to pressure overload compared with ovariectomized mice supplemented with 17 β -estradiol, indicating that E2 may be involved in pathways regulating pathological hypertrophy (110).

To date, most investigations have initiated heart disease pathology through drastic interventions such as trans aortic constriction, ischemia, or other interventions that induce rapid hypertension and cardiac hypertrophy (24, 49, 122). These methods, while effective, accelerate heart failure and do not provide data on subtle changes in cardiac health and signaling that result solely from reduced ovarian hormones.

2.2.2 Ovarian Hormones

The two main hormones secreted by the ovaries are estradiol/estrogen and progesterone. Minor concentrations of E2 are also secreted by adipose tissue, skin, muscle and endometrial tissue. Carrier proteins bind to and transport about 99% of circulating E2s while the unbound 1% is considered physiologically active. E2s are steroid hormones that act on E2 receptors (ERs). Membrane E2 receptor activation can trigger signaling pathways in the cell or result in a conformational change in the protein that initiates downstream events in the nucleus via transcription factor activation or inhibition (26, 77). Additionally, E2 can induce genomic effects by binding to receptors in the cell nucleus (77). The two main ERs, ER- α and ER- β , act via the nongenomic pathway when activated by E2. These receptors exhibit different actions in the

cardiovascular system. In general, ER- α prevents vascular smooth muscle cell proliferation while ER- β regulates pathologic cardiac hypertrophy (54). Both receptor types are found in cardiac myocytes. An animal study showed that ovariectomized mice, aged mice, and aged ovariectomized mice had similar densities of ERs compared to sham surgery mice (104). Thus, prevalence of ER- α and ER- β do not appear to be influenced by not ovariectomy or aging.

Progesterone is another steroid hormone secreted by the ovaries and is well known for its function in pregnancy to prepare the endometrial lining for the fetus; however limited research has been conducted on the cardiovascular effects of progesterone independently of E2 (18). Progesterone binds to two main receptors, PR-A and PR-B, affecting gene expression and protein phosphorylation. Like E2, progesterone is also able to initiate signaling cascades or influence gene activity via cell membrane or nucleic PRs (26). Both of these receptors are found in cardiac tissue, indicating progesterone has potential influence on cardiac function; however evidence is equivocal regarding their effect. High serum levels of progesterone in men and women are associated with increased risk for congestive heart failure and *in vivo* animal studies have found progesterone causes inotropic dysfunction, or problems with contractility, although other studies have shown no effect of progesterone on contraction (76, 116).

While progesterone and E2 combination therapy is related to increased risk for non-cardiovascular events in women with prior heart disease, these hormones in combination appear to reduce risk for heart disease and benefit cardiac function in healthy postmenopausal women (40). A longitudinal study conducted on healthy postmenopausal women observed the effects of hormone replacement therapy (HRT)

with E2-only supplementation, E2 and progesterone in combination, and placebo. E2 alone or in combination with progesterone improved lipoprotein and reduced fibrinogen levels (116). Another study found that postmenopausal women with 4 months of combined hormone replacement therapy exhibited improved indicators of cardiac systolic and diastolic function including cardiac output, stroke volume, ejection fraction, and end diastolic volume (98).

The influence of HRT on coronary heart disease risk was investigated by the Heart and estrogen/progestin Replacement Study (HERS), the first large randomized, double-blind placebo controlled study to examine this relationship; Hulley et al., found that hormone replacement therapy was not cardioprotective as placebo and HRT participants developed similar numbers of heart problems over 4 years between controlled clinical time points. Additionally, more HRT participants experienced coronary events during the first year of the study compared to placebo group (41). A study by the Women's Health Initiative aimed to determine if HRT could prevent CHD and participants were to be followed over an 8-year period. After 5 years the study was halted as a disproportionate number of participants on HRT began to develop breast cancer. Data analysis at this point found a significant association ($p=0.05$) between HRT and CHD, stroke, breast cancer, and pulmonary embolism (16, 88). Due to the uncertainty regarding the influence of HRT on cardiac health and the high risks associated with HRT, other methods of treatment like exercise should be investigated.

2.3 Evaluating Cardiac Pathology: The Fetal Gene Program

In order to understand the increased risk for heart disease in postmenopausal women, the effects of ovarian hormone loss on the heart tissue must be studied. Initial

signs of menopause-induced stress in the heart tissue that precede outward cardiac dysfunction, such as poor contractility or hypertrophy, have not been established. The fetal gene program is a pattern of mRNA expression that is indicative of cardiac dysfunction in adults. An adult heart experiences bouts of ischemia, increasing hemodynamic load, and hypertrophy in a disease state, much like a fetal heart *in utero*. In response to chronic stress or acute injury, the adult heart begins to revert expression of cardiac genes associated with stretch and hypoxia from the healthy archetype to a disease pattern that resembles fetal expression (84).

In utero, the fetal heart exhibits gene expression patterns and a metabolic system that differ from the postnatal, healthy adult heart. In the low oxygen environment of the uterus, the heart metabolizes carbohydrates such as glucose and lactate (25). After the stress of birth and a 24-hour exposure to an environment rich in oxygen, the postnatal heart begins to shift the metabolic paradigm and relies primarily on fatty acid oxidation for energy (64). The fetal heart also experiences growth-induced stretch characterized by upregulation of cardiac hormones responding to stretch like atrial natriuretic peptide (ANP) and beta natriuretic peptide (BNP) (42, 84). Compared to fetal expression, these stretch-related hormones are downregulated in the healthy adult heart (44, 107). Under postnatal physiological conditions, atrial cardiomyocytes express ANP while BNP mRNA is found in both atrial and ventricular tissue (12, 65, 123). These hormones function to reduce blood pressure by regulating natriuresis and diuresis, and inhibiting activation of the renin-angiotensin-aldosterone system (RAAS) (3, 75).

Apart from stretch hormone activation, gradual increases in hemodynamic load are also accompanied by a transition in cardiac muscle fiber expression. Initially, the pre-

natal heart expresses the sarcomeric protein α -skeletal actin whereas the adult heart primarily expresses α -cardiac actin (93). Both isoforms are structurally similar, differing in only 4 four protein residues; however the transition from skeletal to cardiac actin is a characteristic difference between the fetal and adult heart (43). The murine fetal heart is also characterized by expression of β -myosin heavy chain (β -MHC) and this contractile element transitions to the α isoform (α -MHC) after birth. Humans, however, express β -MHC in cardiac tissue throughout fetal development as well as adulthood (91, 92). Additionally, the growing fetal heart slowly develops its inotropic pattern, gradually increasing expression of Calcium ATPase (SERCA-2a), a calcium handling protein. SERCA-2a allows for muscle relaxation by enabling the re-uptake of calcium into the sarcoplasmic reticulum after contraction. After birth, the established levels of SERCA-2a are maintained throughout adulthood (8, 84).

In a disease state, the adult heart is exposed to stressors that resemble the stimuli of the *in utero* environment. Bouts of ischemia induce periods of hypoxia and the heart experiences stretch in response to increased hemodynamic load. The heart reacts to these disease-related changes by reverting to a fetal heart pattern of metabolism (5, 84, 107). The failing adult heart has glycogen stores comparable to the fetal heart indicating that disease may activate a switch to a metabolic pattern that relies on glucose as a substrate (11). Chronic stress and the increased hemodynamic load exacerbates myocardial wall strain resulting in the upregulation of stretch hormones ANP and BNP; overall levels of BNP mRNA increase and ventricles are recruited to synthesize ANP (42, 90). Greater levels of cardiac ANP and BNP also function systemically to hormonally regulate RAAS activation in the kidneys and prevent increases in blood pressure (15).

As disease induced hypertrophy progresses, the heart reverts back to increased expression of α -skeletal actin and downregulation of the formerly predominant cardiac isoform (5, 42, 107). Expression of α -MHC is also downregulated in response to heart disease, while β -MHC levels are preserved, resulting in an isoform shift that resembles the fetal expression pattern in rodents. While human adult hearts only express β -MHC, these levels decrease with disease (84, 91, 92). Cardiac stress also affects calcium handling in the heart tissue, in models of induced pressure overload or severe hypoxia, SERCA-2a expression is reduced or diminished entirely (42, 84, 107). McMullen et al. used a mouse model of pathological pressure overload to characterize the expression pattern of fetal genes. The Northern blot in Figure 2 shows the difference in cardiac mRNA expression between an aortic banding model of pressure overload (BAND) and rats that underwent a sham surgery (SHAM). The BAND group, exhibited a pathological response to the pressure overload with greater expression of ANP, BNP, β -MHC, and α -ska, resembling the expression pattern established in developing fetal hearts (5, 71).

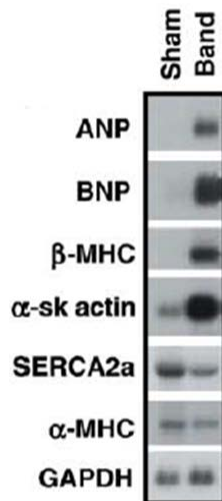


Figure 3: Ventricle RNA expression of fetal genes in pressure overload model (Band) exhibited an increase in markers responding to mechanical stretch and a decrease in contractile function markers compared to control (Sham) {{358 McMullen,J.R. 2003; 180 Bernardo,Bianca C. 2010}}.

2.4 Fibrosis

In response to chronic stress or acute injury, the myocardium begins to remodel. Increased peripheral resistance forces the heart tissue to stretch and as this pathological hypertrophy occurs, the distance between adjacent myocytes and other resident cells increases. The heart tissue compensates for this growth with fibrotic remodeling as cells increase secretion of ECM fibers and the matrix thickens. This process of malignant fibrosis is a combined effort of a variety of cardiac cell types.

Cardiac fibroblast (CFBs) cells make up 27% of the murine heart and 40%-60% of the adult human heart (96, 100). Forming a three-dimensional structure around cardiac myocytes, fibroblasts serve to maintain structural integrity of the heart tissue. More importantly, CFBs regulate extracellular matrix homeostasis by controlling ECM protein turnover rate (120). Acute injury or chronic stress conditions induce a fibroblast signaling response that serves to heal the injury or compensate for the increased load. In a stressed environment, often caused by increased peripheral resistance or systemic inflammation, cardiac fibroblasts differentiate into a new cell type with properties that resemble a combination of fibroblasts, smooth muscle cells, and vascular pericytes (10, 36). This cell type, known as the myofibroblast (MFB), is associated with pathology. Current research hypothesizes that in a disease state, quiescent (or inactive) CFBs along with other resident precursor cells, also differentiate into MFBs (10).

Initially the CFB becomes a proto-myofibroblast (proto-MFB), which is characterized by increased cytoplasmic actin stress fiber production and the formation of a dense matrix of collagen and fibronectin fibers (10, 108). Stage two of myofibroblast formation occurs as the protein transforming growth factor beta-1 (TGF β -1) binds to its

receptor, acting on a kinase enzyme to phosphorylate SMAD proteins (SMAD 2 and 3). These proteins form a complex and translocate to the nucleus of the proto-MFB in order to activate transcription of genes coding for profibrotic proteins. MFBs increase the production of fibronectin and collagen (I and III) and begin to produce and secrete the ECM protein vimentin, resulting in a denser, fiber rich matrix (81, 120). During this differentiation process, MFBs also begin to express endothelin-1 (ET-1) which participates in a positive feedback loop to promote MFB formation. ET-1 also stimulates the production of α -SMA (α -smooth muscle actin), a key indicator that the cell has reached the second stage of MFB formation (22, 81).

Acute injury and chronic stress are hypothesized to induce this MFB formation through different pathways. Acute injury, such as a myocardial infarct, initiates a wound healing response in the heart that promotes cardiac fibrosis. White blood cells and macrophages infiltrate the infarct area and initiate an inflammatory response. Secretion of TGF β -1 by the pro-inflammatory cells phosphorylates SMAD proteins in fibroblasts, resulting in MFB formation, migration, and ECM protein deposition (4, 68). TGF β -1 can also act through non-canonical mechanisms via the JNK and p38 kinase pathways (9, 58, 59). Chronic stress, like the systemic inflammation that accompanies increased postmenopausal adiposity, is characterized by the prevalence of pro-inflammatory cytokines in the blood and perturbations to blood pressure homeostasis. This stress is hypothesized to induce MFB formation in the heart via the renin-angiotensin system (10).

In a healthy heart the local natriuretic peptide system shares an inverse relationship with the systemic RAAS although this balance is lost in disease. In a healthy individual, the kidneys respond to low sodium levels, low blood volume and blood

pressure by releasing renin, a protease that leads to angiotensin II production, increased sodium and fluid retention and vasoconstriction to regain pressure homeostasis. On the other hand, with increased blood pressure and cardiac stretch, ANP and BNP are released and induce vasorelaxation, natriuresis and diuresis. In addition, ANP suppresses renin secretion producing an overall effect of reducing blood pressure and pressure on the myocardium (15, 95). On the other hand, in many pathological circumstances, increases in plasma levels of renin is accompanied by ANP and BNP upregulation in cardiac tissue in an attempt to prevent RAAS hyperactivity (15). *In vitro* research has also shown that ANP acts to protect the heart from pathological remodeling in that it inhibits TGF β -induced SMAD signaling and prevents MFB transformation in response to pressure-overload (62). Li et al. also found that ANP-null mice exhibited increased alpha-smooth muscle actin expression and colocalization with collagen deposits, indicating that low levels of natriuretic peptides are associated with greater pathological CFB differentiation, increased cardiac hypertrophy and pathological remodeling (62). Therefore, the activation of ANP occurs in an attempt to protect the heart from pressure overload and negative remodeling.

Recent studies have found that apart from the systemic role in RAAS, angiotensin II plays a direct role in hypertrophy and remodeling of the heart. Systemic renin accumulation in the heart increases intracardiac angiotensin II formation. High levels of cardiac angiotensin II exacerbate diastolic dysfunction, myocyte hypertrophy, and tissue fibrosis (39). Cardiac angiotensin II (AngII) is an upstream inducer of TGF β -1 signaling in fibrosis (6, 7, 35, 58). In response to higher levels of local AngII, fibroblasts increase TGF β -1 synthesis, promoting CFB differentiation to an MFB phenotype (7, 9, 10).

Myofibroblast proliferation and increased extracellular matrix fiber deposition causes pathological structural changes in the heart. As a result, heart contractile function is altered. Excess protein deposition in the ECM increases the distance between adjacent cardiac muscle cells which hinders proper contractility and increases wall stiffness (24, 124). Recent *in vitro* studies show that myofibroblasts exhibit increased intercellular signaling after differentiation, which may be a compensatory mechanism for the pathological effects of myocardial remodeling (124). Ovarian hormones such as E2 act directly on fibroblast differentiation pathways in response to cardiac hypertrophy. Mouse model studies show that ovariectomized mice, develop greater left ventricular hypertrophy as a response to pressure overload compared to ovariectomized mice supplemented with 17 β -estradiol, indicating that E2 may be involved in pathways regulating hypertrophy (110). Both cardiac myocytes and fibroblasts express functional E2 receptors and 17 β -estradiol has been shown to inhibit collagen synthesis in cardiac fibroblasts by downregulation of AngII receptors (32, 125). In response to ischemia/reperfusion, E2 improves cardiac recovery by downregulating inflammatory factors like tumor necrosis factor alpha (TNF- α) (122). Just as E2 supplementation allows for a better response to cardiac injury, E2 loss associated with menopause could have detrimental effects on heart health. Investigating the mechanisms underlying cardiac fibrosis may provide insight to the effects of menopause on female cardiac function.

As pathological cardiac fibrosis progresses, myocardial structure regulation is affected. Fibroblast proliferation and hyperactivity alters transcription of matrix metalloproteinases (MMP) and their tissue inhibitors (TIMPS), which normally maintain ECM homeostasis (48, 109). MMP activation has mostly been established as a response

to acute injury or in models of acutely induced cardiac hypertrophy (102, 103). MMP activity is also regulated by inflammatory biomarkers AngII and ET-1 (109). In vitro experiments of male and female Wistar rat cardiac fibroblasts observed the effects of E2 supplementation as well as E2 receptor (ER) antagonists on MMP-2 expression. 17 β -estradiol supplementation inhibited MMP-2 expression via activation of E2 receptor α (ER α). The study found that activated ER α acts via the ERK 1/2 pathway to phosphorylate Elk-1, which downregulates MMP-2 gene transcription (67). The influence of E2 on fibroblast expression of MMPs and overall activity suggests a mechanism linked to sex-related differences in cardiac fibrosis (67). However, the involvement of MMP activation at initial stages of disease is unclear.

2.5 Inflammation

Inflammation can be systemic or localized to specific tissues. Menopause is related to increases in systemic inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1), interleukin 1 beta (IL-1 β), IL-10, and TNF α (60, 113). Systemic inflammation after menopause may be in part a result of increased adiposity. Higher fat deposition and insulin resistance are associated with the reduction in circulating ovarian hormones that occurs after menopause (45). Ovariectomy in mice leads to significant increases in body weight and fat mass. Interestingly, studies measuring food intake found that ovariectomized mice did not consume more food than control (sham surgery) mice (45, 86). In one of these studies, despite similar food intake, the ovariectomized mice exhibited increased insulin resistance and gained 25% more weight than control mice after 8 weeks. This increase in mass was accompanied by increased hepatic fat deposition. Both visceral adipose tissue and lipid droplets in the liver exhibited increased

expression of pro-inflammatory markers and white blood cell infiltration the ovariectomized mice (86). Therefore, adipose tissue inflammation caused by ovarian hormone reduction results in a systemic reaction, initiating an inflammatory response in other tissues. A recent study reported that although ovariectomized mice became fatter at 12 weeks compared with control mice, by 20 weeks the groups were similar weight but ovariectomized mice had more adipose tissue inflammation. By week 26, ovariectomized mice had more insulin-resistance compared with controls (111). These studies demonstrate that ovarian hormone reduction expedites weight gain and fatty deposition initially; although with time ovariectomy does not result in more weight gain or fat deposition compared to control mice. The decrease in circulating ovarian hormones associated with the loss of the ovaries appears to upregulate adipose-related inflammatory markers and may result in an inflammatory response throughout the system.

Inflammation caused by risk factors for heart disease can also have direct influences on cell death and tissue damage in the myocardium. Cardiac tissue responds to low-grade stress with oxidative bursts, triggering the production of reactive oxygen species (ROS) by local neutrophils as well as mitochondria and various oxidase enzymes in the myocardium (69). Oxidative stress response causes platelet activation, endothelial swelling, and neutrophil infiltration, disrupting blood flow and resulting in dysfunction of cardiac endothelium (69, 112). This leads to reduced levels of nitric oxide, a common vasodilator, and increased production of proinflammatory cytokines and adhesion molecules (69).

2.6 Physical Activity

Physical activity has been shown to improve cardiac function and reduce the risk for heart disease. Exercise has been linked to reduced systemic inflammation, inflammatory biomarkers, and circulating white blood cells (13, 47). A recent study showed that exercise decreased negative remodeling in the hearts of spontaneously hypertensive rats (70). Therefore, it is possible that physical activity may be able to attenuate for the profibrotic outcomes associated with E2 loss and chronic inflammation. Exercise may have a direct effect on the heart or may act indirectly by reducing systemic inflammation.

Physical activity is also associated with physiological cardiac hypertrophy, though this increase in heart mass is generally benign (5, 114). Resistance training and endurance exercise induce concentric and eccentric myocyte hypertrophy, respectively, through a pathway regulated by insulin-like growth factor 1 (IGF-1) (72, 114). Unlike pathological remodeling, this physiological hypertrophy results in normal or enhanced cardiac function that is exhibited by proportional chamber enlargement and improved contractility (5). Additionally, physiological hypertrophy does not cause apoptosis or alter gene expression of pro-inflammatory/profibrotic markers such as AngII and ET-1 (5, 114). In a study comparing pathological and physiological cardiac hypertrophy, Kemi et al. found that 8-week old female mice, when aerobically trained (wheel running) for 6 weeks, exhibited cardiac myocytes that were approximately 20% longer and 30% wider than those of the sedentary group. Furthermore, the trained group exhibited cardiac hypertrophy via activation of the mTOR kinase pathway, with no increases in expression of fetal genes ANP, BNP, and skeletal muscle actin. The disease model, created using

transverse aortic constriction, also resulted in cardiac hypertrophy; however this remodeling was accompanied by increased mRNA expression of ANP, BNP, and skeletal muscle actin, indicating a pathological response (51). McMullen et al. also investigated the physiological expression pattern of fetal genes in swim-trained rats. Figure 3 shows difference between the pathological expression pattern (Band) and the physiological expression pattern (Exercise) of the fetal gene program. Both Band and Exercise groups exhibited cardiac hypertrophy although the hypertrophy in the Band group was accompanied by hypertension and cardiac dysfunction while the exercise group exhibited decreased resting heart rate and increased stroke volume, indicating enhanced cardiac function (5, 71).

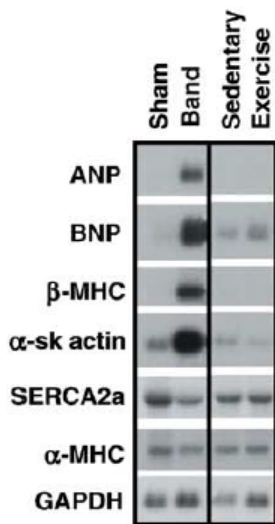


Figure 4: Ventricle RNA expression of fetal genes in physiological hypertrophy model (Exercise) exhibited a diminished expression of markers responding to mechanical stretch and an expression pattern of contractile function markers similar to control (Sedentary) {{180 Bernardo,Bianca C. 2010; 358 McMullen,J.R. 2003}}.

Exercise-mediated hypertrophy is also reversible and does not involve myocardial remodeling, while hypertrophy associated with heart disease is irreversible and results in WBC infiltration, ECM protein deposition, fibrosis, and eventual heart failure (114). These principal differences between physiological and pathological hypertrophy

distinguish disease-mediated mechanisms and exercise-induced changes in myocardial structure.

Physical activity may influence key inflammatory markers in an ovariectomy model. In a preliminary study from our lab, hearts from female C57/BL6 mice were analyzed for gene expression of inflammatory markers. Mice were divided into four groups: OVX, OVX+EX, SHAM

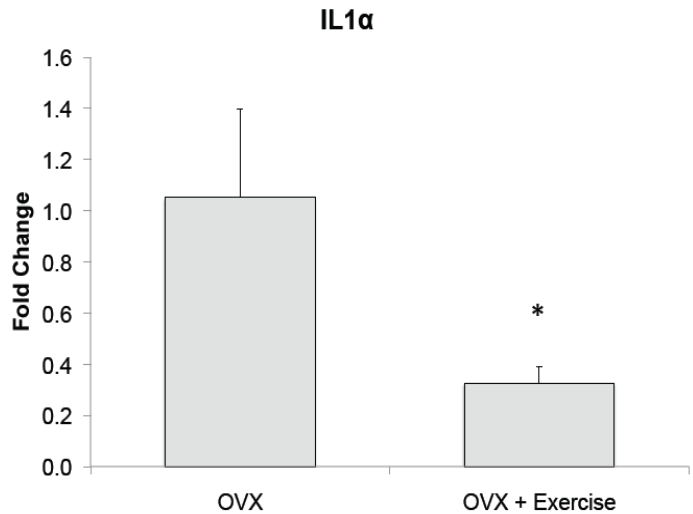


Figure 5: Interleukin-1 α cardiac gene expression. * Significantly different from ovariectomized (OVX) female mice.

and SHAM+EX. Both OVX and OVX+EX mice underwent a bilateral ovariectomy. For 8 weeks, these groups were housed in cages and the +EX mice were provided voluntary

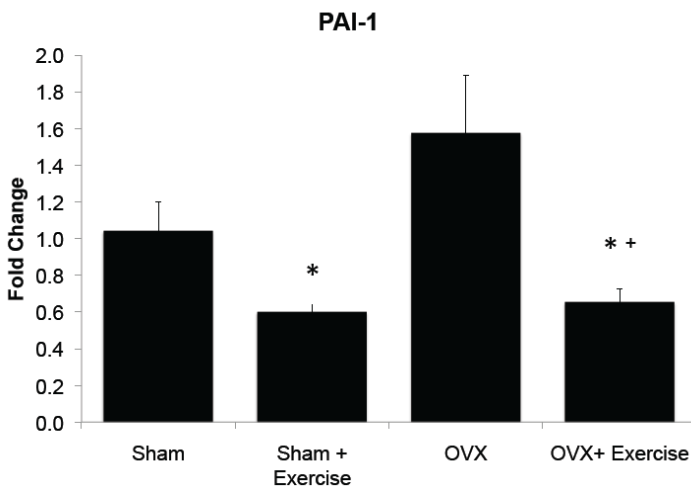


Figure 6: Plasminogen activator inhibitor-1 (PAI-1) cardiac gene expression. * Significantly different from sham surgery (SHAM) female mice, + Significantly different from ovariectomized (OVX) female mice.

exercise on a running wheel. Preliminary data showed exercise-exposed mice exhibited significantly lower cardiac gene expression of IL1- α , a pro-inflammatory marker, compared to OVX (Figure 4). IL1- α is secreted by activated macrophages, neutrophils, and

endothelial cells and causes the proliferation of fibroblasts.

The cardioprotective effects of exercise may play a role in other inflammatory marker levels. Plasminogen activator inhibitor-1 (PAI-1) functions to prevent fibrinolysis, or the degradation of blood clots, and is associated with CVD development (30). Plasma levels of PAI-1 have been shown to be significantly decreased after short-term aerobic training in postmenopausal women (46). In addition to an exercise effect on IL1- α levels in our preliminary study, mice exposed to physical activity had lower PAI-1 cardiac gene expression compared to their sedentary counterparts (Figure 5). More research must be conducted in order to see the effects of exercise on inflammation and cardiac fibrosis.

2.7 Overview

Common risk factors associated with heart disease induce RAAS activation and myocyte hypertrophy during the initial stages of pathological remodeling of the heart. RAAS activation and chronic cardiac stress may effect fetal gene expression patterns in the heart. Over time, the heart is unable to compensate for continuous overload and fibrotic pathways are activated. Ovarian hormones interact with multiple factors involved in the RAAS system as well as pathological remodeling pathways in the heart. The eventual increase in ECM protein deposition and fibrosis that result from this remodeling cause decreased contractility and cardiac dysfunction, which results in heart failure.

The following study evaluates pathways implicated in heart disease pathology as they relate to menopause. The murine ovariectomy model is used to illustrate the proposed pathways of heart disease related to ovarian hormone loss in postmenopausal women. Further, it is used to evaluate the effectiveness of countermeasures, such as physical activity to reduce the negative consequences of ovarian hormone loss on the

cardiovascular system. In this study, the mice received surgery at 8-10 weeks of age (young adult) and were sacrificed 8 weeks post-surgery therefore this study evaluates the early effects of menopause without the added effects of aging. Due to the early time period investigated, we chose to use gene expression analyses as gene expression is a sensitive technique that can identify early markers of dysfunction prior to translation and protein expression. Classic markers of cardiac dysfunction, the fetal gene program, were evaluated in addition to markers of RAAS activation and fibrosis. Importantly, this study aimed to evaluate the effects of ovarian hormone loss without induction of hypertension or acute pathological stress on the cardiovascular system. Therefore, our results will be relevant to the independent effects of ovarian hormones on heart disease.

CHAPTER 3

METHODS

3.1 Introduction

The primary aims of this study are to investigate the effects of ovariectomy and exercise on cardiac dysfunction in mice. This chapter will outline the methodologies planned to achieve these aims.

3.2 Animals

Mouse models exhibit similar responses to surgical procedures as humans (61, 66). Bilateral ovariectomy is the most common intervention in animals to simulate physiology related to ovarian hormone loss (50). In mice, ovariectomy has been shown to reduce circulating estradiol by 67% and does not cause hyperphagia, eliminating the effect of overweight and obesity due to consumption (99, 115). Ovariectomy leads to loss of all ovarian-derived hormones and therefore precludes analysis of the effects of any one hormone; therefore, this model may be more representative of changes that occur with menopause. As we propose to study *early* markers of cardiac dysfunction we will use two cohorts of 8 week old (early adult) mice and investigate the effect of exercise at this stage.

3.2.1 Cohort 1:

Hearts from twenty female C57/BL6 (Harlan) mice were obtained from a study conducted at the University of Maryland (118). At 8-10 weeks of age, mice were divided into two groups: a sham surgery group (SHAM; n=10) and a bilaterally ovariectomized group (OVX; n=10). The mice in the SHAM group were anesthetized but the ovaries were left intact. All mice were given *ad libitum* access to water and standard rodent chow

(Purina Laboratory Rodent Diet 5001:23% protein, 4.5%fat, 6% fiber) and were housed in a temperature controlled room on a 12h light/dark cycle for eight weeks after surgery. Food was removed from all groups 24 hours before mice were sacrificed and weighed. Hearts and other tissues were harvested, snap frozen in liquid nitrogen, and stored at -80°C until processing.

3.2.2 Cohort 2:

Hearts were obtained from a study conducted at the University of Maryland (45). Similar to cohort 1, twenty 8-week old C57/BL6 (Harlan) cohort 2 mice were divided into two groups: a sham operated group (SHAM) and a bilaterally ovariectomized group (OVX). All mice were housed in cages for eight weeks and half of each group (n=5) was provided a running wheel for voluntary exercise (EX). Exercise animals were individually housed to measure each animal's activity. Wheel revolutions were recorded by a computer attached to a photocell on each wheel. Data was populated through customized software (Lafayette Instruments, Lafayette, IN). The other half of the two groups were housed in a standard cage with no wheel and were considered the sedentary (SED).

All mice were given *ad libitum* access to water and standard rodent chow (Purina Laboratory Rodent Diet 5001:23% protein, 4.5%fat, 6% fiber) and were housed in a temperature controlled room on a 12h light/dark cycle. Mice were removed from the wheel cages 24 hours prior to sacrifice and food was removed from all groups at this time as well. All mice were then sacrificed, weighed, and tissues were harvested, snap frozen in liquid nitrogen, and stored at -80°C until processing. The study was approved by the University of Maryland institutional Animal Care & Use Committee (IACUC).

3.3 Tissue Analysis

3.3.1 Gene expression

RNA was isolated from the heart tissue using the Trizol method (63). Briefly, 50-100mg of tissue was homogenized in 1ml Trizol and incubated at room temperature for 5 minutes. Then, 0.2ml chloroform was added to each sample, mixed, and incubated for 2-3 minutes. Samples were centrifuged to separate the aqueous from solid phases. The aqueous phase was removed and RNA was precipitated through the addition 0.5ml of isopropanol. Samples were incubated then centrifuged. The resultant RNA was washed with a 75% ethanol solution then dried. RNA was then rehydrated and stored at -80°C. RNA content was determined via spectrophotometry (NanoDrop).

To synthesize complementary DNA (cDNA), 0.2-1 µg of total RNA (depending on the target gene) was reverse transcribed and the reaction was inactivated by incubation at 70°C for 15 minutes. Primers were designed for each target (Table 1) and optimized for annealing temperature, dilution, and greater than 95% efficiency. Dissociation melt curves were analyzed to determine the specificity of the PCR products. cDNA produced from the reverse transcription reaction was mixed with SsoFast EvaGreen (BioRad), and forward and reverse primers for each rtPCR reaction (Table 1). PCR was employed using GAPDH as a reference for each reaction. GAPDH was evaluated as a reference gene by statistical analysis of mean threshold (C(t)) values for each sample. For cohort 1, the average C(t) values for each SHAM sample across all genes were compared with those for each OVX sample using a two-tailed T-test (p=0.05). For cohort 2, intergroup differences between the average C(t) values across all genes were determined with a two-

way ANOVA. There was a significant effect of group and condition ($p=0.013, 0.001$), but no significant group by condition interactions.

Quantification of gene expression in each treatment group was analyzed according to the $2^{-\Delta\Delta C_T}$ method where the $\Delta C_T = \text{GAPDH CT} - \text{target gene CT}$. Data was expressed normalized to the SHAM SED condition, which was set to 1.

3.3.2 Statistical Analysis

Data is expressed as means \pm standard error. Both cohorts were tested for normality and equal variance. Differences between groups in cohort 1 were determined using two-tailed T-tests with Microsoft Excel. Due to limited sample size, cohort 2 did not pass assumption tests. Cohort 2 served as a preliminary analysis, and SigmaPlot Software was used to conduct an Analysis of Variance (ANOVA) to determine the effect of group (OVX, SHAM) and the effect of condition (SED, EX). Significant interactions were analyzed using Tukey's post hoc testing. Significance was set at an alpha level of $p < 0.05$.

Table 1: Gene Expression Targets

Primer	Function		Primer Sequence (5'-3')
GAPDH	Reference Gene	F:	CTCATGACCACAGTCCATGC
		R:	CACATTGGGGGTAGGAACAC
Atrial Natriuretic Peptide (ANP)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	TGCCGGTAGAAGATGAGGTC
		R:	AGCCCTCAGTTTGCTTTTCA
B-type Natriuretic Peptide (BNP)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	CTGAAGGTGCTGTCCCAGAT
		R:	CCTTGGTCCTTCAAGAGCTG
α-Myosin Heavy Chain (α-MHC)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	CTGGGCAAATCCAACAACCTT
		R:	TCTTGCCTCCTTTGCCTTTA
β-Myosin Heavy Chain (β-MHC)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	TGCAGCAGTTCTTCAACCAC
		R:	TCGAGGCTTCTGGAAGTTGT
α-Skeletal Actin (α-sk)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	CGATATCCGCAAAGACCTGT
		R:	GCTGGAAGGTGGACAGAGAG
Ca²⁺ ATPase (SERCA2a)	Fetal Gene Program & Cardiac Dysfunction Marker	F:	CTGTGGAGACCCTTGGTTGT
		R:	CAGAGCACAGATGGTGGCTA
Angiotensin II (AngII)	Induces TGF β secretion	F:	AGCAGCCGTCCTTTTGATAA
		R:	TGCTGGACACCTTTTAGGG
Endothelin-1 (ET-1)	Induces TGF β secretion and Stimulates CFB Differentiation to MFB	F:	GTGTCTACTTCTGCCACCTGGACAT
		R:	GGGCTCGCACTATATAAGGGATGAC
Transforming Growth Factor β1 (TGFβ-1)	Stimulates CFB Differentiation to MFB	F:	ATACGCCTGAGTGGCTGTCT
		R:	GGTTCATGTCATGGATGGTG
α-Smooth Muscle Actin (α-sma)	Marker of MFB formation	F:	ACTGGGACGACATGGAAAAG
		R:	AGAGGCATAGAGGGACAGCA
Collagen 1 (Col1)	ECM Fiber Involved in Myocardial Remodeling	F:	TGACTGGAAGAGCGGAGAGT
		R:	ATCCATCGGTCATGCTCTCT
Collagen 3 (Col3)	ECM Fiber Involved in Myocardial Remodeling	F:	GTCCACGAGGTGACAAAGGT
		R:	GATGCCCACTTGTTCCATCT
Vimentin (Vim)	ECM Fiber Involved in Myocardial Remodeling	F:	AAGGAAGAGATGGCTCGTCA
		R:	TTGAGTGGGTGTCAACCAGA
Fibronectin (FN)	ECM Fiber Involved in Myocardial Remodeling	F:	CAAGACCATACCTGCCGAAT
		R:	CAACTGGTTGGCATGAAATG

CHAPTER 4

RESULTS

4.1 Cohort 1

4.1.1 Animal Characteristics

Cohort 1 heart weight and body weight information was provided by the University of Maryland where the animals were sacrificed (117). The OVX group had significantly greater body weight and significantly lower heart weight to body weight ratio compared to SHAM ($p < 0.001$; 0.001 , respectively). There was no difference in heart weight between groups (Table 2).

Table 2: Animal Characteristics for Cohort 1

	HW (g)	SEM	BW (g)	SEM	HW/BW	SEM
SHAM (n=10)	0.1295	0.003	22.18	0.270	0.0058	0.0001
OVX (n=10)	0.1250	0.004	25.33*	0.484	0.0049*	0.0001

* indicates significant difference from sham surgery (SHAM) female mice ($p < 0.005$).

4.1.2 Gene Expression

Gene expression data for cohort 1 is depicted in fold change relative to the SHAM group (set to 1.0) and the genes investigated have been grouped by function for clarity. The first group of genes is the signature fetal gene program: ANP, BNP, α -MHC, β -MHC, α -sma, and SERCA-2a. The second group of genes, angiotensin II, TGF β -1, endothelin-1, and α -sma are implicated in MFB activation and pathological fibrosis in the heart. ECM fibers collagen 1 & 3, vimentin, and fibronectin comprise the third group and are indicators of myocardial remodeling and fibrosis.

4.1.3 Cohort 1: Fetal Genes

Cohort 1 expression of many of the fetal genes resembled the expression pattern of the pathological model of heart disease (See Figure 3). ANP and BNP, cardiac hormones that respond to stretch, showed greater expression (3.43-fold, $p=0.063$ and 2.52-fold, $p=0.026$, respectively) in the OVX group. Expression of contractile filaments α -MHC and β -MHC was significantly lower in OVX (0.79-fold, $p=0.048$ and 0.64-fold, $p=0.007$, respectively) compared to SHAM. The skeletal muscle component α -ska showed 1.62-fold greater gene expression in the OVX group ($p=0.007$) while the calcium handling protein Serca-2a showed a 0.735-fold downregulation compared to SHAM ($p=0.444$)(Figure 5).

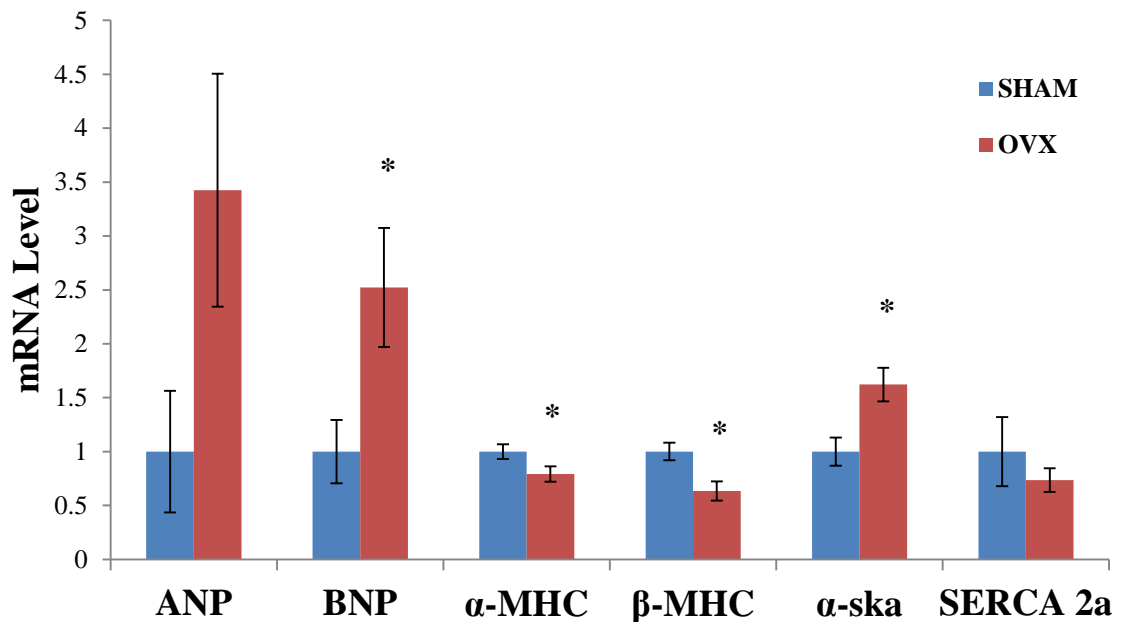


Figure 7: Expression of fetal genes (Cohort 1) Atrial Natriuretic Peptide (ANP), B-type Natriuretic Peptide (BNP), α -Myosin Heavy Chain (α -MHC), β -Myosin Heavy Chain (β -MHC), α -Skeletal Actin (α -ska), and Calcium ATPase (SERCA 2a) depicted as fold change of ovariectomized (OVX) female mice ($n=10$) compared to sham surgery (SHAM) female mice ($n=10$). * indicates that OVX expression was significantly different from SHAM ($p<0.05$).

4.1.4 Cohort 1: Fibrotic Markers

Mediators of pathological fibrosis showed greater expression in ovariectomized mice compared to the sham group. Angiotensin II was 5.28-fold higher in OVX ($p=0.021$) and expression of TGF β -1 in OVX was 2.97-fold greater than SHAM expression ($p = 0.017$). Expression of endothelin-1, a mediator of fibrosis involved in stimulating MFB formation, was 1.7-fold greater in OVX, but this was not statistically significant ($p=0.255$). The MFB marker α -sma mRNA levels were 1.89-fold higher in OVX compared to SHAM ($p=0.057$) (Figure 6).

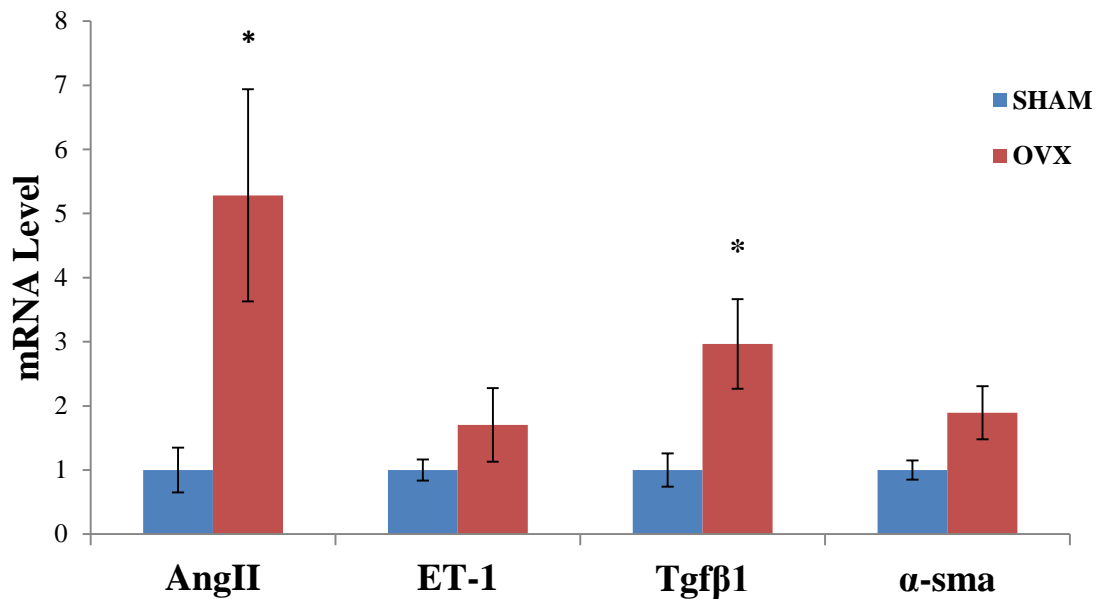


Figure 8: Gene expression of fibrotic markers (Cohort 1) Angiotensin II (AngII), Endothelin-1 (ET-1), Transforming Growth Factor β 1 (Tgf β 1), and α -Smooth Muscle Actin (α -sma) depicted as fold change of ovariectomized (OVX) female mice ($n=10$) compared to sham surgery (SHAM) female mice ($n=10$). * indicates that OVX expression was significantly different from SHAM ($p<0.05$).

4.1.5 Cohort 1: ECM fibers

Expression of extracellular matrix fibers was higher in OVX mice compared with SHAM, although not statistically significant. Collagen 1 and collagen 3 showed 2.16- and

2.47-fold higher expression in OVX compared to SHAM ($p=0.099$; 0.065 , respectively). OVX also exhibited 2.16-fold greater expression of vimentin and fibronectin was upregulated 3.23-fold compared to SHAM ($p=0.99$; 0.062 , respectively) (Figure 7).

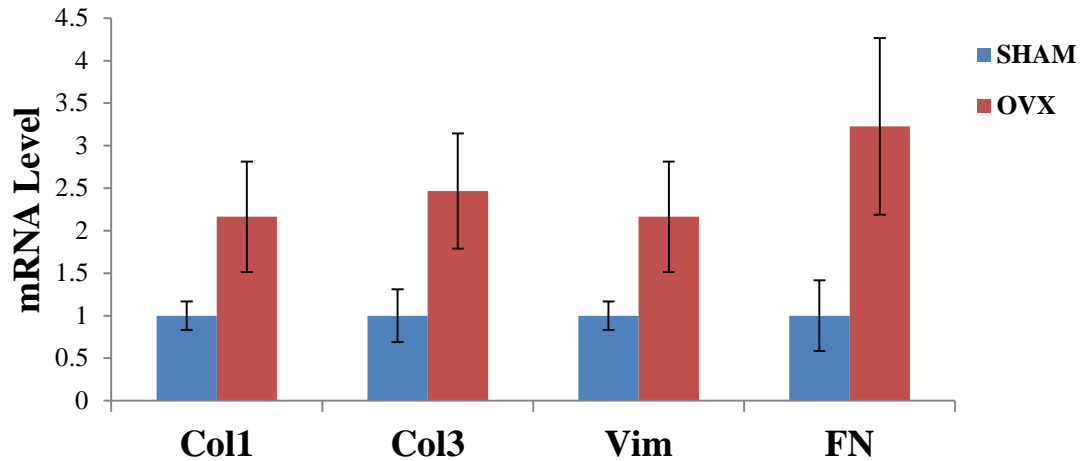


Figure 9: Gene expression of ECM fibers (Cohort 1) Collagen 1 (Col1), Collagen 3 (Col3), Vimentin (Vim), and Fibronectin (FN) depicted as fold change of ovariectomized (OVX) female mice ($n=10$) compared to sham surgery (SHAM) female mice ($n=10$).

4.2 Cohort 2

4.2.1 Animal Characteristics

Body weight, heart weight, and food consumption data for cohort 2 animals was provided by the University of Maryland, where the animals were sacrificed (45, 117). There was no effect of ovariectomy or exercise on heart weight between groups. Ovariectomy had a significant effect on body weight and food consumption ($p=0.039$ and $p=0.027$, respectively). Exercise had a significant effect on food consumption as well ($p=0.037$). Running data was not acquired on this cohort. Data that was collected previously on a similar cohort of animals reveals reduced running distance in OVX compared with SHAM (Appendix A) (118).

Table 3: Animal Characteristics for Cohort 2

	HW (g)	BW (g)	HW/BW	Average Food Consumption (g)
SHAM SED (n=5)	0.1380	24.19	0.0057	4.943
OVX SED (n=5)	0.1282	26.73	0.0048	4.229
SHAM EX (n=5) [†]	0.1492	24.15	0.0062	5.189
OVX EX (n=5) [†]	0.1555	25.81	0.0060	4.912

There were no significant interactions between group and condition. [†]**Note:** This data represents the full cohort. A sample from both SHAM EX and OVX EX groups was lost during shipping and is not included in the gene expression data below.

4.2.2 Gene Expression

Gene expression for cohort 2 is depicted in fold change relative to the SHAM group (set to 1.0). The gene targets have been grouped by function for clarity with the first group consisting of the signature fetal gene program: ANP, BNP, α -MHC, β -MHC, α -ska, and SERCA-2a. The second group consists of angiotensin II, TGF β -1, endothelin-1, and α -sma; genes implicated in MFB activation and pathological fibrosis in the heart. ECM fibers collagen 1 & 3, vimentin, and fibronectin comprise the third group and are associated with cardiac remodeling and fibrosis.

4.2.3 Cohort 2: Fetal Genes

Expression levels are depicted in fold change relative to the sham sedentary group (set to 1.0) and the genes have been organized in the same grouping as that of cohort 1. Cohort 2 fetal gene expression showed the influence of exercise on ovariectomized mice. There was no effect of ovariectomy or exercise on cardiac gene expression of ANP and

BNP. Both group and condition had significant effects on cardiac mRNA expression of myosin isoform α -MHC ($p=0.008$, 0.001) as well as a significant group by condition interaction ($p=0.008$). α -MHC expression was significantly upregulated in OVX SED and SHAM EX compared to sham sedentary (2.07-fold, $p=0.001$ and 2.34-fold, $p=0.001$, respectively). There was a significant group effect ($p=0.006$) and group by condition interaction ($p=0.001$) on gene expression of β -MHC. OVX SED and SHAM EX had greater mRNA expression of β -MHC compared to SHAM SED (2.11-fold, $p=0.001$ and 1.49-fold, $p=0.039$, respectively). OVX EX expression of β -MHC was significantly lower than OVX SED (1.37-fold, $p=0.04$). Gene expression of skeletal muscle isoform α -ska showed a significant overall effect of exercise ($p=0.013$). There was a significant effect of group and condition on gene expression of contractile protein SERCA 2a ($p=0.001$, 0.001).

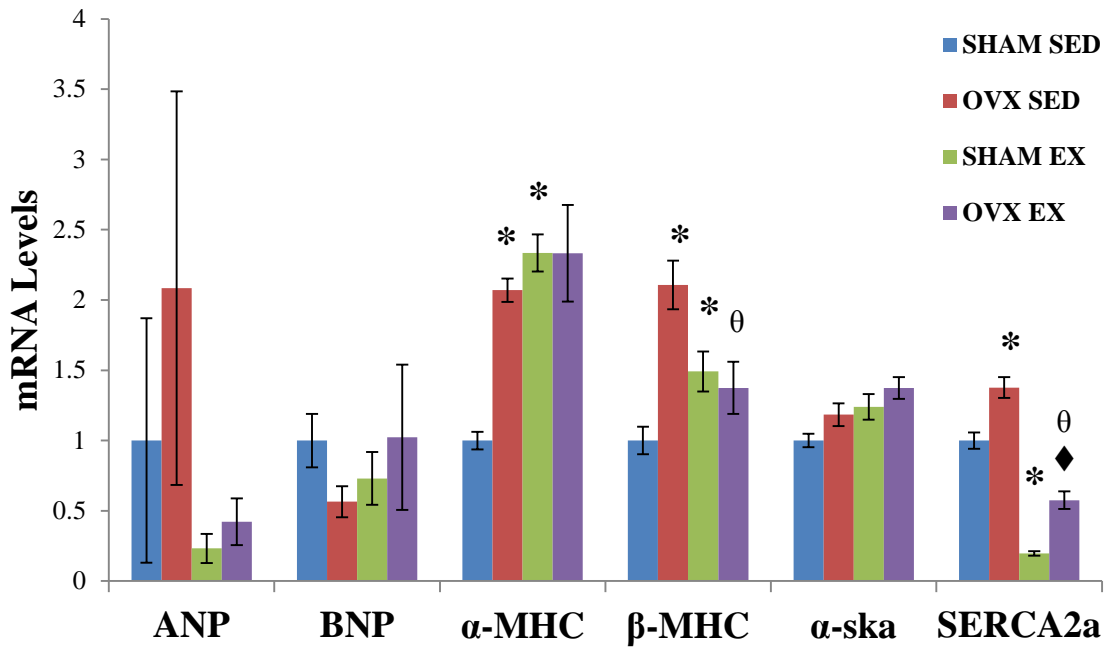


Figure 10: Expression of fetal genes (Cohort 2) Atrial Natriuretic Peptide (ANP), B-type Natriuretic Peptide (BNP), α -Myosin Heavy Chain (α -MHC), β -Myosin Heavy Chain (β -MHC), α -Skeletal Actin (α -ska), and Calcium ATPase (SERCA 2a) depicted as fold change of ovariectomized sedentary (OVX SED) female mice (n=5), ovariectomized exercise (OVX EX) female mice (n=4), and sham surgery exercise (SHAM EX) female mice (n=4) compared to sham surgery sedentary (SHAM SED) female mice (n=10). * indicates fold change was significantly different from SHAM SED, θ indicates significant difference from OVX SED, and \blacklozenge indicates significant difference from SHAM EX ($p < 0.05$).

4.2.4 Cohort 2: Fibrotic Markers

There was a significant effect of ovariectomy and exercise on gene expression of Angiotensin II, a mediator of cardiac fibrosis ($p=0.016, 0.015$). Angiotensin II expression also had a group by condition interaction and OVX EX hearts had significantly greater expression of AngII compared to OVX SED and SHAM EX (4.15-fold, $p=0.001, 0.001$). Gene expression of endothelin-1, another fibrotic mediator, showed a significant effect of ovariectomy ($p=0.043$). TGF β -1, involved in cardiac growth and remodeling, and α -sma, a marker of MFB formation, both showed no significant effects of group or condition (Figure 10).

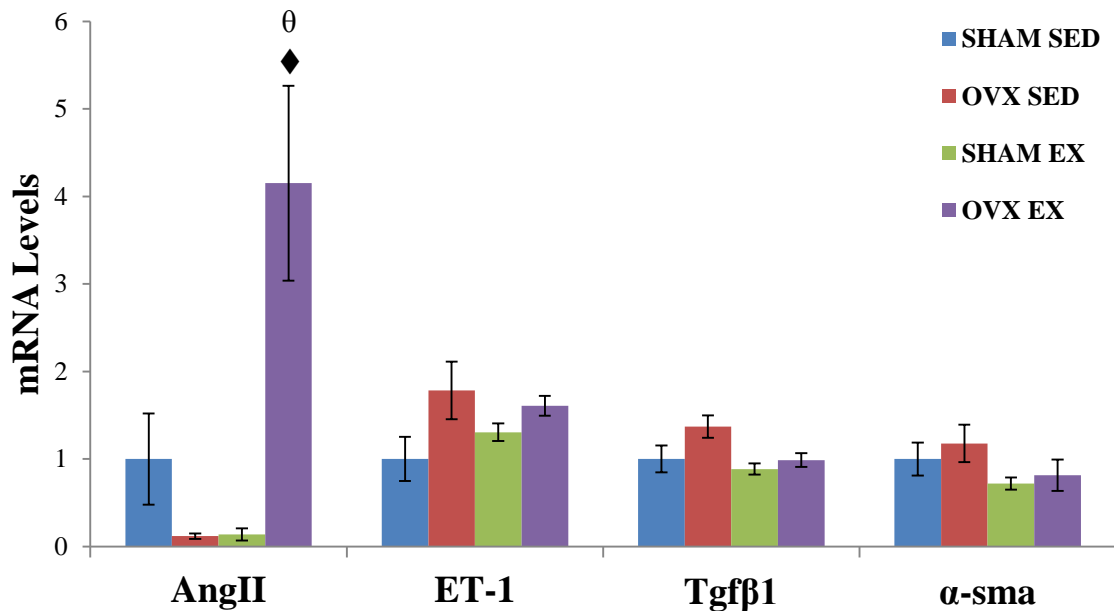


Figure 11: Gene expression of fibrotic markers (Cohort 2) Angiotensin II (AngII), Endothelin-1 (ET-1), Transforming Growth Factor β 1 (Tgf β 1), and α -Smooth Muscle Actin (α -sma) depicted as fold change of ovariectomized sedentary (OVX SED) female mice (n=5), ovariectomized exercise (OVX EX) female mice (n=4), and sham surgery exercise (SHAM EX) female mice (n=4) compared to sham surgery sedentary (SHAM SED) female mice (n=10). * indicates fold change was significantly different from SHAM SED, θ indicates significant difference from OVX SED, and \blacklozenge indicates significant difference from SHAM EX (p<0.05).

4.2.5 Cohort 2: ECM fibers

ECM fibers, indicators of cardiac fibrosis and tissue remodeling, followed a relatively consistent pattern of expression. Collagen 3 gene expression had a significant overall effect of exercise (p=0.048). There was no effect of group or condition on cardiac gene expression of collagen 1, vimentin, or fibronectin (Figure 12).

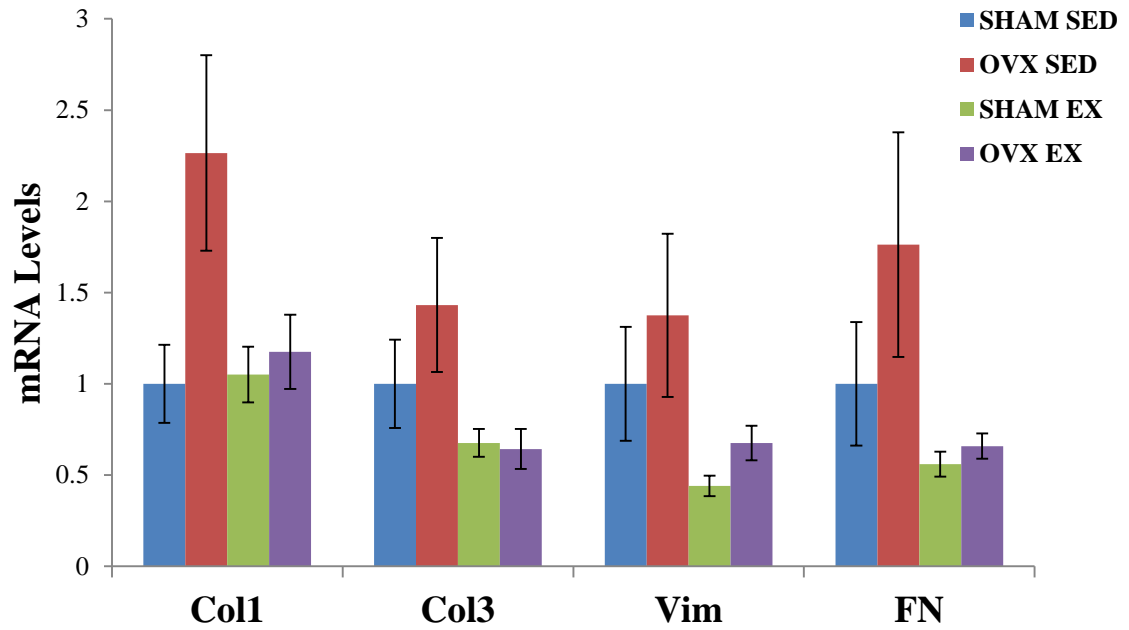


Figure 12: Gene expression of ECM fibers (Cohort 2) Collagen 1 (Col1), Collagen 3 (Col3), Vimentin (Vim), and Fibronectin (FN) depicted as fold change of ovariectomized (OVX) female mice (n=10) compared to sham surgery (SHAM) female mice (n=10).

CHAPTER 5

DISCUSSION

Ovarian hormone loss after menopause is associated with an increased risk for heart disease and coronary events. In this study, we were interested in cardiac gene expression patterns at early stages of ovarian hormone loss in a postmenopausal animal model. We also explored exercise as a possible modality for attenuating ovariectomy induced changes in cardiac expression. We hypothesized that ovariectomy would cause a pathological cardiac gene expression pattern and fibrosis and that voluntary wheel running would be related to lower expression markers of dysfunction. Our major findings were 1) compared to sham surgery mice, ovariectomized mice expressed a disease phenotype of the cardiac fetal gene program, 2) ovariectomized mice upregulated cardiac expression of ECM genes and other genes associated with fibrosis, which may be related to increased angiotensin II expression and 3) exercise reduced the expression of many fibrosis-associated genes.

5.1 Ovariectomy Causes Activation of the Fetal Gene Program

Adult re-expression of the fetal gene program in cardiac tissue is a well-characterized key indicator of heart disease (79, 84, 107). Acute injury or chronic systemic stressors produce an environment of hypoxia and increased hemodynamic load resulting in compensatory cell hypertrophy in the heart. These pathological changes mimic the low oxygen environment and growth-induced wall strain of the fetal heart, resulting in the reemergence of fetal expression signature in the diseased heart (84, 107). Activation of the fetal gene program has been characterized in various heart disease models, including hypertension, myocardial infarction, and heart failure, but it is

unknown whether the expression pattern is an early indicator of cardiac dysfunction in postmenopausal women (8, 20, 79, 107).

In this study, intergroup differences in mRNA levels of fetal genes show that only 8 weeks of ovarian hormone loss caused changes in cardiac gene expression. Ovariectomized mice had greater mRNA levels of cardiac stretch hormones ANP and BNP compared to sham mice, suggesting that ovarian hormone loss is associated with changes indicative of increased cardiac stretch. Though we cannot confirm this with mechanical data on the degree of chamber stretch or wall strain or blood pressure in these animals, the data suggest that OVX was associated with altered natriuretic peptide maintenance of blood pressure homeostasis.

In normal physiological conditions, cardiac expression of ANP and BNP functions to counterbalance the renin angiotensin aldosterone system (RAAS), primarily by activating guanylyl cyclase-A (GC-A), which promotes vasorelaxation, natriuresis, and diuresis when active (15, 21, 38, 52, 83). ANP and BNP also suppress the RAAS by reducing plasma renin activity and ANP is able to directly inhibit renin production in the kidneys, providing a direct hormonal link between the heart and renal system (15, 57, 75). In pathological cases of hypertension and heart failure, this balance is lost and natriuretic peptide expression increases as RAAS activity escalates (15).

This pathological failure of homeostasis may also be a product of postmenopausal ovarian hormone loss as an increasing amount of evidence suggests that estrogen is a critical regulator of the RAAS (119). Estrogen controls synthesis of angiotensinogen, an upstream precursor of AngI and AngII that is activated by renin (53). Studies have found that compared to E2-replete counterparts, E2 deficient rats as well as postmenopausal

women have higher levels of circulating plasma renin and have greater activity of angiotensinogen-converting enzyme (ACE), the enzyme that regulates AngII production. Additionally, E2-deficient hypertensive rats have greater levels of circulating AngII compared to E2-replete counterparts (27, 119). These studies show that estrogen regulates production of angiotensinogen and conversion to angiotensin II by regulating enzyme activity. As a result, estrogen is able to modulate RAAS activity and prevent homeostatic imbalance and pathological upregulation of natriuretic peptides in the heart.

Our data suggests that ovarian hormone deficiency led to increased RAAS activity as cohort 1 OVX mice had 5.28-fold greater levels of cardiac AngII mRNA compared to SHAM mice and greater cardiac natriuretic peptide expression pattern in the hearts of ovariectomized mice. Surprisingly, the negative effects of ovarian hormone loss on the heart occurred within only 8 weeks of ovariectomy. Increased adiposity, expression of systemic inflammatory markers, and other the systemic changes associated with ovariectomy may also have increased cardiac strain and resulted in ANP and BNP upregulation (60). The degree of wall strain and the mechanics of cardiac chamber dilation in a model of ovarian hormone loss should be investigated to further understand the effects of postmenopausal physiology on cardiac pathology.

Our data revealed additional indicators that OVX animals experienced cardiac dysfunction compared with SHAM animals. OVX mice expressed lower levels of cardiac contractile protein isoforms α -MHC and β -MHC compared to SHAM mice. Downregulation of α -MHC and greater prevalence of β -MHC is the disease-associated re-expression of a fetal pattern in mice hearts. Changes in myosin heavy chain expression have been established in models of acute stress or extreme cardiac dysfunction. Studies of

pressure/volume overload and spontaneous hypertension in rat models showed a significant transition in cardiac gene expression from predominantly α -MHC to the disease-associated β -MHC (42, 107). These models involved drastic perturbations to cardiac homeostasis; the myosin heavy chain expression pattern in cohort 1 ovariectomized mice may have not been affected as severely after only 8 weeks of ovarian hormone loss; and therefore, only an upregulation of β -MHC was observed without a downregulation of α -MHC. Potentially, the upregulation of β -MHC precedes the downregulation of α -MHC during the earliest stages of cardiac dysfunction.

McMullen et al. reported that the calcium handler SERCA 2a mRNA expression was downregulated in a model of cardiac pressure overload indicating that calcium reuptake from the sarcoplasmic reticulum suffers in a model of cardiac dysfunction (71). Other animal models of induced hypertrophy have shown radical upregulation of SERCA-2a, suggesting that calcium handling exhibits a bimodal response to cardiac stress. Initial increases in SERCA-2a levels are hypothesized to be part of an energy-conserving mechanism in environments of mild cardiac stress; though some studies have shown discrepancies in SERCA-2a regulation with mild hypertrophy (1). In cohort 1 of our study, there were no significant differences in SERCA-2a gene expression between OVX and SHAM mice indicating that calcium handling was not affected by 8 weeks of ovarian hormone loss. Future studies should examine whether contractile dysfunction and SERCA-2a upregulation is initiated in a model of ovarian hormone loss at later time points.

The expression of muscle filament α -ska was significantly greater in OVX mice compared to sham, suggesting a transition from cardiac actin to the skeletal muscle

isoform. Skeletal actin is predominantly expressed during fetal heart growth periods, and the re-emergence of this expression pattern has been documented throughout the progression of cardiac hypertrophy and heart disease (5, 8, 42, 107). Upregulation of α -ska may show an effect of short term ovarian hormone loss on heart muscle filament expression that resembles the stress-inducing effects of fetal growth and heart disease.

5.2 Ovariectomy Activates Cardiac Fibrosis Associated Genes

Cardiac tissue fibrosis is associated with pathological remodeling of the myocardium in response to increased total peripheral resistance, cardiac hypertrophy, and acute cardiac injury such as a myocardial infarction (24, 96). These risk factors increase the volume load on the heart and in order to achieve the necessary contractile strength, cardiac myocytes begin to hypertrophy. The accompanied infiltration of inflammatory cells as well as the muscle growth increases intercellular space (124). Resident cells, predominantly cardiac fibroblasts (CFBs), upregulate protein expression of ECM fibers in order to maintain wall structure and proper contractility (24, 48). While the heart is able to compensate this way for some time, increased cardiac hypertrophy and fibrosis can lead to maladaptive chamber dilation and wall stiffening, eventually resulting in contractile dysfunction. We aimed to explore if ovarian hormone loss and the associated systemic physiological changes induced cardiac fibrosis or initiated mRNA expression of profibrotic markers in the heart. Assessing transcription of profibrotic genes in ovariectomized mice could provide a strong link between the effects of ovarian hormone loss on heart tissue and the increased risk of heart disease after menopause. The majority of the literature has explored increases in ECM fiber deposition and myofibroblast (MFB) formation using models of acute cardiac injury or induced spontaneous hypertension,

showing that ovarian hormones such as estrogen can reduce pathological remodeling of the heart (23, 80, 81, 110). Our mouse model can help elucidate fibrosis-associated changes caused by ovarian hormone loss alone; without the effects of acute injuries, hypertension, or transgenically induced diseases.

Analysis of gene expression data revealed that ECM fiber expression was affected by ovarian hormone loss. Cardiac expression of collagen 1 and collagen 3 was 2.16- and 2.47-fold greater in ovariectomized mice compared to mice with sham surgery. Both collagen isoforms are present in the ECM of a healthy myocardium, but prolonged or drastic upregulation of collagen fibers is indicative of pathological fibrosis. An integral part of maladaptive fibrosis is the differentiation of CFB cells into the MFB phenotype and the initial stage of this differentiation process is characterized by the formation of an intercellular matrix that is dense with collagen. As differentiation progresses, these proto-myofibroblasts also begin to secrete fibronectin and vimentin fibers that infiltrate the ECM (9, 10). Cardiac gene expression of fibronectin and vimentin was 3.23- and 2.16-fold greater in OVX mice compared to mice that underwent sham surgery. This upregulation may be a product of preliminary shifts in the CFB phenotype resulting from ovarian hormone loss and associated systemic changes.

The estrogen-controlled RAAS is part of a possible mechanism that provides a relationship between ovarian hormone loss and changes in ECM fiber gene expression; this pathway may also explain the effects of postmenopausal physiology on cardiac dysfunction in women (15, 119). Apart from balancing natriuretic peptide activity, AngII (circulating and local) is an upstream activator of TGF β -1 in cardiac fibroblasts, which promotes fibrosis and CFB differentiation (87). Higher levels of cardiac AngII mRNA in

ovariectomized mice may have stimulated TGF β -1 expression, which was 2.97-fold greater in OVX compared to SHAM. In pathological cardiac fibrosis, TGF β -1 acts via a SMAD pathway to initiate CFB differentiation to an MFB cell type (58, 59, 62, 81). The activation of this pathway by RAAS hyperactivity in ovariectomized mice could provide a mechanism for the ECM fiber expression results found in cohort 1.

There were no intergroup significant differences in gene expression of endothelin-1 and α -smooth muscle actin, two markers that characterize MFB differentiation and cardiac tissue fibrosis. The hearts of the ovariectomized mice may be expressing early signs of cardiac dysfunction that precede myocardial remodeling. The mice were sacrificed 8 weeks post surgery and this early time point highlights the influential role of ovarian hormones on cardioprotection. More importantly, the reduction in circulating ovarian hormones may impact cardiac function in women at early stages post menopause. Differences in expression levels of genes associated with fibrosis and ECM fiber deposition provide preliminary information regarding the effects of ovarian hormone loss on cardiac health. Future studies with analysis of protein expression and ECM thickness can provide more information regarding physical changes in the myocardium.

5.3 Exercise inhibits a pathological gene expression pattern with ovariectomy

Physical activity has been shown to improve cardiac function, diminish pathological myocardial remodeling, and lower the risk for heart disease (39). Using cohort 2, we investigated the effects of physical activity on the pathological cardiac expression pattern. With swim training, McMullen et al. found that mouse cardiac gene expression of ANP and BNP were lower compared with sedentary animals, exhibiting a healthy adult pattern, thus demonstrating the potential benefits of exercise on lowering

markers of cardiac dysfunction (71). In cohort 2, we found no effect of group or condition on cardiac ANP and BNP gene expression; despite high standard error, ANP expression is 2.08-fold greater in hearts of OVX SED mice compared to SHAM SED. Between the two natriuretic hormones, ANP plays a greater role in RAAS regulation than BNP. ANP directly inhibits renin secretion from the juxtaglomerular cells of the kidneys and functions to maintain natriuresis and diuresis by inhibiting RAAS hyperactivity (15). Research shows that exercise improves renal function and is effectively able to prevent, reduce, and delay chronic kidney disease. ANP expression may have been downregulated as exercise was able to regulate RAAS activity.

On the other hand, in a study investigating fetal gene reactivation in 8 week old treadmill trained rats, Kemi et al. found that after 6 weeks, cardiac expression of ANP was not different between trained and sedentary groups (51). In this study, animals were not diseased. The re-expression of fetal genes has been established as a sign of pathological changes in cardiac tissue, while exercise associated physiological changes in cardiac expression follow a distinctly separate pathway (42, 51, 107).

There was a significant group by condition interaction in expression of α -MHC and β -MHC though contrary to cohort 2 results, cohort 1 OVX SED mice expressed greater levels of cardiac α -MHC and β -MHC compared to SHAM SED. The increase in expression of β -MHC with ovariectomy may illustrate the pathological effects of ovarian hormone loss on myosin heavy chain isoform expression in cardiac tissue. With exercise, ovariectomized mice showed significantly lower mRNA expression of β -MHC compared to sedentary counterparts, suggesting a cardioprotective role of exercise that influences myosin heavy chain isoform expression in the heart. The greater β -MHC expression in

the SHAM EX group compared to SHAM SED may be a result of low sample size and high variability between individual mice. These limitations may also explain the expression pattern of α -ska, which showed no significant difference with ovariectomy, unlike the results of cohort 1 samples. Gene expression of α -ska was not different between sedentary and exercise conditions, which mirrors findings by Kemi et al. wherein cardiac mRNA expression of α -ska showed no significant differences between sedentary and treadmill trained rats.

Calcium handling in the heart becomes compromised with cardiac injury and the associated pathological response. McMullen et al showed that SERCA 2a, a calcium handler in cardiac tissue, is downregulated in the disease model compared to sham surgery and that exercise showed mRNA expression similar to sham (5, 71). The fetal gene program and calcium handling in particular has not been characterized in a model of ovarian hormone loss. Cohort 2 showed greater SERCA 2a expression with ovariectomy and lower expression with exercise. The expression pattern found with cohort 2 may be a compensatory mechanism for the systemic stress associated with postmenopausal physiology.

5.4 Exercise may play a role in limiting pathological remodeling of the myocardium

Exercise induced physiological cardiac hypertrophy and remodeling occurs through molecular pathways involving growth factors and other signaling molecules distinct from the inflammatory pathways activated in pathological myocardial fibrosis (51). This beneficial adaptation results from sustained exercise training, is associated with normal or improved heart function, and is a reversible process (5). We hypothesized that mice exposed to voluntary wheel running would express lower levels of cardiac

markers associated with fibrosis and genes coding for ECM proteins compared to the sedentary counterparts within OVX and SHAM groups. There was a significant group by condition interaction in cardiac gene expression of Angiotensin II and OVX EX mice hearts had 4.15-fold greater AngII expression compared to OVX SED and SHAM EX ($p=0.001$). Physical activity is associated with decreased plasma renin activity and lowered levels of circulating AngII in models of heart failure (126). Circulating angiotensin and activation of RAAS balances natriuretic peptide release from the heart to regulate blood pressure in normal physiology (89, 121). The AngII expression pattern in cohort 2 may differ from our initial hypothesis because of lower levels of ANP and BNP in the exercise conditions. ET-1 and Tgf β -1, mediators of MFB formation, had similar expression patterns as α -sma, a characteristic marker of MFB cells, that may indicate activation of the differentiation pathway. There was a significant group effect ($p=0.043$) on ET-1 expression in cohort 2. There was a general pattern of ET-1, Tgf β -1, and α -sma upregulation with ovariectomy and slight downregulation with exercise, indicating that exercise may influence the pathological differentiation of cardiac fibroblasts at early stages of ovarian hormone loss.

Exercise has been shown to reduce myocardial remodeling and fibrosis in ovariectomized spontaneously hypertensive rats. The expression pattern of ECM fibers in cohort 2 suggests that physical activity may also influence myocardial remodeling in an ovariectomized rodent model that is not subject to a major insult to the heart. Gene expression of ECM proteins followed a similar pattern of expression as ET-1, Tgf β -1, and α -sma. There was a significant effect of condition on collagen 3 gene expression as this ECM fiber was downregulated in the exercise condition ($p=0.048$). Though collagen

1, vimentin, and fibronectin mRNA levels did not have significant effects of group or condition, the overall expression pattern of all four ECM fibers was consistent. Cardiac gene expression of Col1, Col3, Vim, and FN was greater in OVX SED mice compared to SHAM SED and was lower in the exercise conditions. This expression pattern suggests that the early pathological effects of ovarian hormone loss on cardiac gene expression can be attenuated for with exercise within a short period of time, highlighting the importance of physical activity in terms of disease prevention.

CHAPTER 6

CONCLUSION

The American Heart Association reports that as of 2013, 26% of women age 45 and older who have an initial recognized MI die within a year compared to 19% of men, mainly because women are more likely to suffer a heart attack later in life compared to men (28). This disparity between males and females illustrates the cardioprotective role of ovarian hormones and the importance of establishing the effects of menopause. We hypothesized that the hearts of ovariectomized mice would upregulate gene expression of markers of cardiac dysfunction and pathological fibrosis and that exercise would be able to lower the expression of these markers. Based on our results we found that OVX hearts did manifest early signs of cardiac dysfunction and expressed greater mRNA levels of profibrotic markers.

As of 2015, approximately 20% of the United States population is between 50-64 years of age as the country undergoes a demographic transition to an aging society. Seventy percent of this population enters their 60s previously diagnosed with at least one chronic condition (28). As a result, the disease burden of the aging population and associated healthcare costs are due to increase over the next few years and in order to diminish this amount of waste and improve quality of life for a large portion of the population, effective early interventions need to be assessed and established. Physical activity is not only associated with improved cardiac function, but is able to diminish insult-induced cardiac dysfunction and fibrosis in animal models of ovarian hormone loss. Despite limitations, cohort 2 data supported the finding that ovariectomy increased

expression of cardiac dysfunction markers and that exercise was able to attenuate for some of these gene expression differences.

The mice in this study were sacrificed 8 weeks post surgery, at a young adult age (16 weeks old), which shows that even a limited period of ovarian hormone loss affected cardiac expression pattern and activated a fetal expression program, indicating heart disease pathology. Cardiac fibrosis and myocardial remodeling has been well established in models of acute cardiac injury or advanced stages of hypertension and heart disease (33, 96, 105). Our novel findings showed that mRNA levels of profibrotic markers and ECM fibers were upregulated in ovariectomized mice compared to sham. The effect of short term ovarian hormone loss on cardiac gene expression highlights the relationship between postmenopausal physiology and the increased risk for heart disease and necessitates the early timing of healthy preventative measures such as exercise. The CDC has reported that 1 in 3 female deaths is caused by heart disease and as this risk increases drastically after menopause, it is important to understand the immediate effects of ovarian hormone loss when determining treatment options.

6.1 Strengths and Limitations

Most studies assessing expression patterns of fetal genes and cardiac fibrosis use models of acute or direct injury to the heart. Strengths of this study include the use of ovariectomy to replicate post menopausal physiology in order to investigate changes in expression of cardiac dysfunction markers due to the systemic changes associated with ovarian hormone loss. More importantly, this model was able to assess pathological effects of ovarian hormone loss on the heart at **early time points**, highlighting the degree of cardioprotection of ovarian hormones as well as the need for early intervention

methods to reduce the risk of heart disease in postmenopausal women. By investigating differences in gene expression, we were able to observe early changes in the heart caused by postmenopausal physiology.

This study is not without limitations. Cohort 2 consisted of a limited number of animals, with five mice in each of the sedentary conditions (OVX and SHAM) and only four mice in each of the exercise conditions. The low sample size as well as other methodological differences between cohorts likely resulted in high individual variability and as a result some of the OVX versus SHAM comparisons in cohort 2 contradict findings with cohort 1. Primarily, cohort 1 animals were housed in groups together while cohort 2 animals were housed individually. Studies have shown that rodents prefer a group living dynamic and exhibit increased heart rate when housed individually (2, 101). Additionally food consumption was monitored in cohort 2 and animals may have had more human contact as food was removed for weighing daily.

6.2 Future Directions

Future research should use Western Blot or immunohistochemistry to investigate ECM protein expression as well as the differences in cardiac fibrosis in the hearts of ovariectomized mice compared to sham. Mouse echocardiography can also be utilized in order to determine the timing of symptoms indicating cardiac dysfunction in models of ovarian hormone loss. Establishing early effects of ovarian hormone loss on cardiac dysfunction markers can help develop possible preventative interventions, such as exercise, to reduce the risk of heart disease that is correlated with ovarian hormone loss.

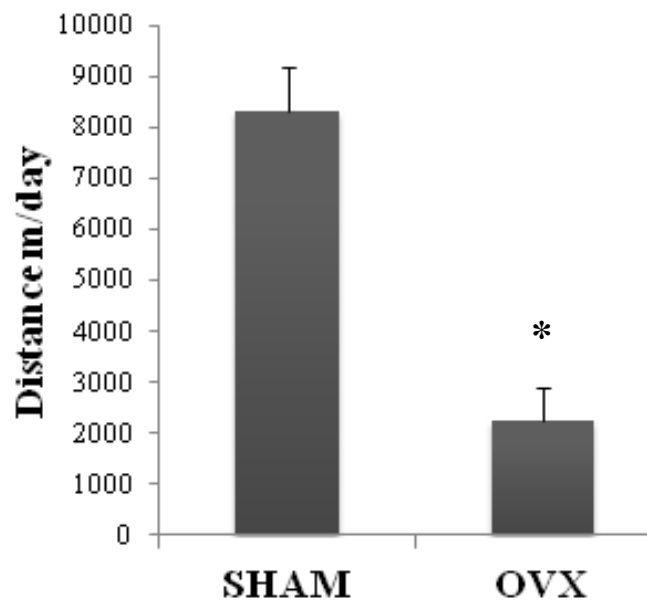
APPENDIX

RUNNING DATA

Wheel running had been recorded for the cohort 2 subgroups in the EX condition; however the recorded data was lost and unable to be recovered during the original study. Running data below was collected from a study with a similar animal protocol at University of Maryland with previously published results (118). This study was conducted on C57/BL6 mice that were divided into sedentary and exercise cohorts. At 8-weeks old, eleven mice were divided into two groups: a sham surgery group (SHAM, n=5) and a bilaterally ovariectomized (OVX, n=6). For 8 weeks, all mice were housed in individual cages that contained a voluntary wheel for exercise. Wheel revolutions were recorded by a computer attached to a photocell on each wheel. Data was populated through customized software (Lafayette Instruments, Lafayette, IN).

All mice were given *ad libitum* access to water and standard rodent chow (Purina Laboratory Rodent Diet 5001:23% protein, 4.5%fat, 6% fiber) and were housed in a temperature controlled room on a 12h light/dark cycle. Mice were removed from the wheel cages 24 hours prior to sacrifice and food was removed from all groups at this time as well. Tissues were harvested, snap frozen in liquid nitrogen, and stored at -80°C until processing. The study was approved by the University of Maryland institutional Animal Care & Use Committee (IACUC).

Running Distance



Recorded running data for two groups; * indicates significant difference from sham surgery group ($p < 0.05$).

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