***Cardiac Hypertrophy and Regression during Postpartum in C57Bl/6 Mice***

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ABSTRACT

 Cardiac hypertrophy is an increase in heart size due to either physiological or pathological reasons. Physiological inducers include exercise or pregnancy. For instance, athletes experience cardiac hypertrophy during their seasons of workouts. However, when they stop working out consistently, their hearts undergo cardiac regression, the decrease of heart size back to normal. This regression also happens after a woman delivers a baby. However, hypertrophy may still continue throughout breastfeeding; in such cases, regression would occur after lactation has ended.

 Although cardiac hypertrophy has been extensively researched, hypertrophy during lactation and the regression of hypertrophy has been significantly less investigated. Thus, the objective of this study was to evaluate changes in the heart mass after pregnancy-induced cardiac hypertrophy, and identify the signaling pathways responsible for these changes. Mice were divided into the following groups: control (non-pregnant diestrus cycle), 17 days of gestation (late stage of pregnancy), 7 days of postpartum, 21 days of postpartum (time of weaning), 7 days after weaning, and 21 days after weaning. We hypothesized that the heart size would increase throughout pregnancy and even more so during lactation by modifying pro-hypertrophic signaling pathways, and then it would decrease in size after weaning by altering pro-atrophic signaling.

 We found that lactation further increases pregnancy-induced cardiac hypertrophy. In addition, signaling pathways including phosphorylated FoxO and ERK were significantly increased during lactation. Cardiac regression begins to occur after the lactation period has ended, but hearts did not regress completely back to normal after 3 weeks of weaning. Our results suggest that lactation further increases pregnancy-induced cardiac hypertrophy and this is mediated by inactivation of FoxO and ERK signaling pathways.

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CHAPTER I

INTRODUCTION

Cardiac hypertrophy is defined as an increase in heart muscle mass with changes in cardiac geometry (Frey & Olson, 2003; Razeghi & Taegtmeyer, 2006; Shiojima & Walsh, 2006; Dorn, 2007; McMullen & Jennings, 2007; Hill & Olson, 2008; Chung & Leinward, 2014). Cardiac hypertrophy can be induced by either pathological or physiological factors. Pathological hypertrophy occurs with sustained pressure overload and leads to an increase in mass in order to compensate for the increase in workload (Frey & Olson, 2003). Overloads in both pressure and volume change the geometry of the heart chambers; these changes can be either concentric or eccentric (Dorn, 2007). Pathological cardiac hypertrophy is not reversible and ultimately leads to decreased cardiac function and heart failure (Chung & Leinwand, 2014; Dorn, 2007). However, in contrast to pathological cardiac hypertrophy, exercise training and pregnancy induce physiological cardiac hypertrophy (Kametas, McAuliffe, Hancock, Chambers, & Nicolaides, 2001; Chung, Heimiller, & Leinwand, 2012; Chung, Yeung, & Leinwand, 2012; Chung & Leinwand, 2014). In this case, the function of the heart either remains the same or is enhanced, a trend that is often seen in an athlete’s heart compared to a sedentary person’s heart (Dorn, 2007). Thus, unlike in pathological situations, physiological hypertrophy is reversible (Sen, 1983; Friddle, Koga, Rubin, & Bristow, 2000; Razeghi & Taegtmeyer, 2006; Hill & Olson, 2008). This reversibility is known as cardiac regression, or when the heart reverses back to its normal size after cardiac hypertrophy (Sen, 1983; Friddle et al., 2000; Razeghi & Taegtmeyer, 2006; Hill & Olson, 2008).

 Pregnancy is an ideal model for the study of both cardiac hypertrophy and regression. Although cardiac hypertrophy during pregnancy has been thoroughly examined by several investigators (Kametas et al., 2001; Eghbali et al., 2005; Chung et al., 2012; Chung, Yeung, & Leinwand, 2013), it is unknown how lactation affects cardiac hypertrophy. Throughout pregnancy and lactation, there are many physiological changes that occur. For instance, in rats, food intake increases by 40-50% during pregnancy, and during lactation, food consumption increases by two or three times greater than that of non-lactating, non-pregnant animals (Anderson & Turner, 1963; Cripps & Williams, 1975). In addition, blood volume increases during pregnancy, but it increases even more during lactation, especially during the first half of lactation (Bond, 1958). It has been shown that an increase in blood volume induces cardiac hypertrophy (Rossi & Carillo, 1991).

Signaling pathways are different series of proteins in the cell that communicate a signal from a receptor on the outside of the cell to the nucleus, which will then initiate protein turnover. In this study, the signal comes from pregnancy and lactation and the changes, such as an increase in blood volume, that cause the cardiac muscle to increase in size. Certain signaling pathways initiate physiological cardiac hypertrophy, including ERK, AKT, and STAT (Wang et al., 1998; Pan et al., 1999; Molkentin & Dorn, 2001; Skurk et al., 2005; Heineke & Molkentin, 2006; Shiojima & Walsh, 2006; Yu, Chen, Fu, Wang, & Wang, 2010; Chung et al., 2012). Moreover, during pregnancy-induced cardiac hypertrophy, previous studies observed an increase in AKT and ERK (Chung et al., 2012). These are different from pathological inducers such as MAPK pathways, which include JNKs and p38 (Yamaguchi et al., 2003). On the other hand, pro-atrophic pathways, such as FoxO, have been shown to induce cardiac regression (Skurk et al., 2005; Li et al., 2007; Schips et al., 2011; Wang & Su, 2011).

Statement of the Purpose

 The purposes of this investigation were to investigate 1) the effects of lactation on cardiac hypertrophy and regression, and 2) the signaling pathways mediating cardiac hypertrophy and regression in postpartum mice.

Significance of the Study

Although cardiac hypertrophy has been studied extensively (Wang et al., 1998; Pan et al., 1999; Konhilas et al., 2004; Ni et al., 2006), only a few have specifically researched pregnancy-induced cardiac hypertrophy (Chung et al., 2012; Eghbali et al., 2005; Chung et al., 2013). Although late pregnancy and the early postpartum period are critical for maternal health, only a few studies have investigated the mechanisms underlying cardiac remodeling during this period. In addition, even within these pregnancy studies (Chung et al., 2012; Eghbali et al., 2005; Chung et al., 2013), cardiac adaptation during postpartum has not been thoroughly investigated. Moreover, current postpartum studies (Gonzalez et al., 2007; Umar et al., 2012; Iorga, Dewey, Partow-Navid, Gomes, & Eghbali, 2012) did not indicate whether lactation took place postpartum.

Postpartum cardiomyopathy (PPCM) is a rare but fatal disorder in which left ventricular dysfunction and symptoms of heart failure occur in late pregnancy and early postpartum in previously healthy women (Abboud, Murad, Chen-Scarabelli, Saravolatz, & Scarabelli, 2007; Hilfiker-Kleiner et al., 2007). Previous studies suggest that breastfeeding or the hormones associated with breastfeeding, such as prolactin, could be possible risk factors for PPCM (Hifliker-Kleiner et al., 2007; Abboud et al., 2007). However, others have found positive effects associated with lactation on PPCM (Schwarz et al., 2009; Murata et al., 2013). Thus, it is important to understand the effect of lactation during the postpartum period and regression during the weaning period.

Hypotheses

 The experimental design of this study allowed for the comparison of several different stages of pregnancy and lactation. The design of this study includes the following groups: a 17 days of gestation (late stage of pregnancy group: LP); a one week after postpartum group (1PP); a three weeks after postpartum, time of weaning group (3PP); a one week after weaning group (1W); a three weeks after weaning group (3W); and a non-pregnant diestrus control group (C).

Hypothesis I.

Cardiac hypertrophy associated with pregnancy is further increased with lactation while regression is initiated with the onset of weaning.

**Hypothesis II.**

Hypertrophy in groups LP, 1PP, and 3PP would be caused by an increase in pro-hypertrophic signaling pathways, such as AKT, ERK, and STAT expression, while regression in groups 1W and 3W would be caused by an increase in pro-atrophic signaling pathways, such as the FoxO family.

Delimitations

 This study is delimited to the following:

1. Thirty-two virgin C57Bl/6 mice at the age of seven to ten weeks purchased from Charles River.
2. The manipulated variables were the different stages of pregnancy and lactation versus the non-pregnant control group.
3. Body weight and food intake were measured at least twice a week.
4. Mice that killed their pups during postpartum were excluded from the study.
5. Mice that never carried full term were excluded from the study.
6. Control mice were sacrificed during diestrus cycle. If the mouse was not in diestrus cycle, the mouse was excluded from the study.

Limitations

 The following are potential limitations of the study:

1. C57Bl/6 mice have a 75% success rate up to 12 days gestation; therefore, spontaneous abortions may have occurred, and this could activate the signaling molecules.
2. If stressed during the first week of postpartum, C57Bl/6 mice occasionally kill their young, which would decrease the sample size per group
3. As C57Bl/6 mice age, they increase in body weight, which can increase the heart size. Since this is the primary data collected, the ending age of the mice could have affected the results of the study.
4. When measuring body weight throughout the study, mice were not sedated; therefore, movement could have affected the reliability of the measurements.

Assumptions

 The following assumption was made during this study:

1. The mice did not have complications during pregnancy and lactated properly postpartum.

Definition of Terms

1. Concentric – relatively greater increase in wall thickness with small cavities (Chung & Leinwand, 2014)
2. Eccentric – enlarged cavities with relatively thin walls (Chung & Leinwand, 2014)
3. AKT – Protein Kinase B; shown to be activated by phosphorylation during physiological cardiac hypertrophy, including pregnancy; important mediator for physiological cardiac hypertrophy (Shiojima & Walsh, 2006; Dobson et al., 2001; Chung & Leinwand, 2014)
4. FoxO – Forkhead box O, Forkhead family of Transcription Factors; downstream target of AKT; inactivated by phosphorylation during physiological cardiac hypertrophy; under atrophic condition, it is activated (not phosphorylated), moves to the nucleus, and activates transcriptional atrogene, such as Atrogin-1 (Li et al., 2007; Schips et al., 2011; Wang & Su, 2011)
5. ERK – Extracellular Signal-regulated Kinases; when activated, ERK results in long-standing concentric physiological hypertrophy that is associated with enhanced cardiac pump function; increased during pregnancy-induced cardiac hypertrophy (Bueno et al., 2000;Chung et al., 2012)
6. STAT – Signal Transducers and Activators of Transcription; activated during physiological cardiac hypertrophy (Pan et al., 1999; Molkentin & Dorn, 2001); specifically shown to protect hearts from PPCM during pregnancy-induced cardiac hypertrophy (Hilfiker-Kleiner et al., 2007)
7. p38 – p38 Mitogen-activated Protein (MAP); downstream target of MAPKs (mitogen-activated protein kinases); associated with pathological cardiac hypertrophy and negatively regulates physiological cardiac hypertrophy by possibly inhibiting the AKT pathway (Taniike et al., 2008)

CHAPTER II

LITERATURE REVIEW

Cardiac Hypertrophy

Definition.

Frey and Olson (2003) defined cardiac hypertrophy as the cellular response to an increase in biomechanical stress, which can be extrinsic, such as in arterial hypertension or valvular heart disease, or intrinsic, as in familial hypertrophic cardiomyopathy. Some defining features of hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere (Frey & Olson, 2003). Frey and Olson (2003) discussed the difference between physiological hypertrophy, which occurs during postnatal development and in response to exercise, versus pathological hypertrophy, which leads to heart failure. Strategies to stimulate physiological hypertrophy and inhibit pathological hypertrophy would have an obvious therapeutic value in the context of heart failure (Frey & Olson, 2003).

In contrast to Frey and Olson (2003), Razeghi and Taegtmeyer (2006) focused strictly on pathological hypertrophy. They noted that left ventricular hypertrophy is a feature of the failing heart and associated with increased cardiovascular morbidity and mortality. Razeghi and Taegtmeyer (2006) suggested that the reversal of left ventricular hypertrophy in heart failure patients, through the activation of atrophic signaling pathways, could be a more effective therapy than inhibiting physiological hypertrophy. Atrophy, also known as regression of hypertrophy, improves cardiac function even in the presence of a sustained pro-hypertrophic stimulus. Therefore, activating pro-atrophic signaling pathways in the presence of pro-hypertrophic signaling may be an attractive strategy to reverse pathological hypertrophy (Razeghi & Taegtmeyer, 2006).

Dorn (2007) similarly defined cardiac hypertrophy as an abnormal increase in heart muscle mass that is functionally, mechanistically, and histologically distinguished from normal embryonic and postnatal myocardial growth by characteristic changes in cardiac myocyte shape and volume. However, like Frey and Olson (2003), who mentioned exercise and postnatal development as physiological hypertrophy inducers, Dorn (2007) noted there is an analogous condition in humans in which a prolonged but reversible increase in cardiac workload produces reversible cardiac hypertrophy. That condition is pregnancy.

In addition to the above definition, Hill and Olson (2008) discussed the three major forms of cardiac plasticity that occur during hypertrophy. They include: physiologic growth in response to normal demand, pathologic hypertrophic remodeling in the setting of hemodynamic stress, and cardiac atrophy as an adaptation to ventricular unloading. Similar to Frey and Olson (2003) and Dorn (2007), Hill and Olson (2008) acknowledged different inducers of physiological hypertrophy, which include exercise, pregnancy, and postnatal growth.

 Types of Cardiac Hypertrophy: Pathological and Physiological.

As stated above, there are two types of cardiac hypertrophy: pathological and physiological (Frey & Olson, 2003; Shiojima & Walsh, 2006; Dorn, 2007; McMullen & Jennings, 2007). Pathological cardiac hypertrophy is typically not a reversible condition. It leads to progressive systolic dysfunction and ultimately heart failure (Frey & Olson, 2003; Shiojima & Walsh, 2006; Razeghi & Taegtmeyer, 2006; Dorn, 2007). Examples of pathological hypertrophy are neurohumoral activation, hypertension, and myocardial injury (Hill & Olson, 2008). On the other hand, physiological cardiac hypertrophy induced by exercise and pregnancy is reversible when the stimuli are ceased (Frey & Olson, 2003; Shiojima & Walsh, 2006; Dorn, 2007; McMullen & Jennings, 2007).

 Furthermore, there are two different causes of exercise-induced hypertrophy. Isotonic exercise, such as running, walking, cycling and swimming, involves movement of large muscle groups. The profound vasodilation of the skeletal muscle vasculature that is involved produces eccentric hypertrophy by increasing venous return to the heart and volume overload. This hypertrophy is characterized by chamber enlargement and a proportional change in wall thickness. In contrast, isometric or static exercise, such as weight lifting, involves developing muscular tension against resistance with little movement. Reflex and mechanical changes cause a pressure load on the heart rather than volume load resulting in concentric hypertrophy (McMullen & Jennings, 2007). Although McMullen and Jennings (2007) provided an excellent explanation of the different types of exercise-induced cardiac hypertrophy, they, like Razeghi and Taegtmeyer (2006) and Frey and Olson (2003), did not mention the other physiological inducer, which is pregnancy.

In the past, scientists believed that exercise-induced cardiac hypertrophy was a pathological problem. In the late nineteenth century, the Swedish physician Henschen was the first to recognize exercise-induced cardiac enlargement. He detected dilation and hypertrophy on both sides of the heart and concluded that there was a pathologic enlargement of the heart after exercise (Hill & Olson, 2008). Over time, a different view of cardiac hypertrophy began to emerge in accordance with Laplace’s law, which dictated that afterload-induced increases in systolic wall stress and oxygen consumption are offset by increases in wall thickness. Consequently, hypertrophic growth of the heart was seen as compensatory and hence beneficial (Hill & Olson, 2008).

Pregnancy-Induced Cardiac Hypertrophy

Cardiac hypertrophy occurs during pregnancy as a consequence of both volume overload and hormonal changes (Chung & Leinwand, 2014; Chung et al., 2012). Kametas et al. (2001) used two-dimensional and M-mode echocardiography of the maternal left ventricle and left atrium on 125 pregnant women at 9-42 weeks of gestation and 19 non-pregnant female controls. During pregnancy, left ventricular mass increased by 52% above non-pregnant controls (Kametas et al., 2001) showing that pregnancy-induced cardiac hypertrophy does occur in humans.

In addition to humans, animal models also show cardiac hypertrophy. Eghbali et al. (2005) used C57Bl/6 mice and compared late-pregnancy to non-pregnant controls. The authors reported that the size of the heart increases enormously in late pregnancy. Heart weights in late-pregnancy mice were 178±2 mg compared to non-pregnant mice at 126±2 mg (Eghbali et al., 2005). Similarly, Chung et al. (2012) used three-month-old, virgin, female C57Bl/6 mice and mated them with a proven breeder male C57Bl/6 mouse. Mice were then divided into three groups: non-pregnant diestrus control, mid-pregnancy, and late-pregnancy. Both time points of pregnancy were associated with significant cardiac hypertrophy (Chung et al., 2012). Therefore, both humans and animal models demonstrated pregnancy-induced cardiac hypertrophy (Chung & Leinwand, 2014; Chung et al., 2012; Eghbali et al., 2005; Kametas et al., 2001).

Lactation

Benefits.

There are many benefits associated with breastfeeding. Schwarz et al. (2009) examined the effect of lactation on subsequent risk of obesity, hypertension, diabetes, hyperlipidemia, and cardiovascular disease among 139,681 postmenopausal women. After additional adjustment for lifestyle and family history, within seven to 23 months of lactation, there was a trend toward less obesity (Schwarz et al., 2009). In addition, increased duration of lactation was associated with a reduced prevalence of cardiovascular risk factors, including hypertension, diabetes, and hyperlipidemia, even after adjustment for sociodemographic variables, lifestyle variables, family history, and BMI category (Schwarz et al., 2009). Similarly, according to a Kaiser Permanente study (2009), breastfeeding a child may lower a woman’s risk of developing metabolic syndrome, a condition linked to heart disease and diabetes in women.

Likewise, Schwarz (2013) argued that to effectively fight heart disease, efforts are needed to promote all aspects of a healthy lifestyle, which for women includes breastfeeding. Schwarz (2013) noted that as long as mothers breastfed as recommended, they were, on average, no more obese than women who had never been pregnant, unless they had four or more children. Since obesity is a risk factor for cardiovascular disease, which is a leading cause of death of women around the world (Schwarz, 2013), breastfeeding could help reduce chances of heart disease.

**Risk Factors.**

***Pregnancy-Associated Hypertension.***

On the other hand, there are some risks with breastfeeding, but only in particular circumstances. Murata et al. (2013) investigated postpartum change in cardiac remodeling and the function of pregnancy-associated hypertensive (PAH) mice with and without lactation. Murata et al. (2013) stated that PAH mice are able to breastfeed and that lactation played an important role for the onset of cardiac contractile dysfunction, but lactation did not affect the alteration in cardiac remodeling in postpartum PAH mice. This finding clearly showed that lactation had adverse effects on cardiac function in PAH mice (Murata et al., 2013). Therefore, there are some risk factors involved with breastfeeding if someone experiences pregnancy-associated hypertension.

***Postpartum Cardiomyopathy.***

One pregnancy-related pathological cardiac hypertrophy is postpartum cardiomyopathy (PPCM). PPCM is a disease of unknown etiology, characterized by an acute onset of heart failure in women in the late stage of pregnancy up to several months postpartum, resulting in high mortality despite optimal medical therapy (Hilfiker-Kleiner, 2007; Abboud et al., 2007; Safirstein et al., 2012; Patten et al., 2012; Hilfiker-Kleiner & Sliwa, 2014; Ricke-Hoch et al., 2014). One study done by Abboud et al. (2007) suggested that breastfeeding is a possible risk factor for PPCM. Because lactation is an energetically expensive process, maternal metabolic changes occur during lactation in peripheral tissues, including mammary gland, adipose tissue, liver, skeletal muscle, and the heart (Murata et al., 2013). In addition, Hilfiker-Kleiner et al. (2007) reported the association between onset of PPCM and the lactation hormone prolactin. However, it is also suggested that breastfeeding is associated with recovery of left ventricular systolic function in PPCM patients (Sarfirstein et al., 2012). Therefore, further investigation is required regarding the effect of lactation on postpartum cardiac function (Murata et al., 2013).

Physiological Changes during Pregnancy and Lactation.

 During pregnancy and lactation, various changes, including increases in both food consumption and blood volume, occur that can affect cardiac hypertrophy. Cole and Hart (1938) reported that food intake increases throughout pregnancy with further increases through lactation. Specifically, by day 12 of lactation, rats ate about three times as much as non-lactating controls, and this food consumption level remained constant until weaning (Cole & Hart, 1938). Anderson and Turner (1963) also used rats and measured their food each day. The authors found that food consumption increased gradually throughout the 20 days of lactation, and then reduced rapidly when the pups were separated from their mother (Anderson & Turner, 1963). In addition, Cripps and Williams (1975) reported an increase in food intake by 40-50% during pregnancy. During lactation, an increase of two to three times greater than that of the non-lactating and non-pregnant rats was observed (Cripps & Williams, 1975). However, it is not only food intake that increases during pregnancy and lactation.

In addition to food consumption, Pritchard (1965) found an increase in blood volume during pregnancy. Pritchard (1965) measured the apparent maternal blood volume and the red blood cell volume during the last two trimesters of pregnancy, before and after delivery, at the end of the first week postpartum, and again two months or more after delivery. He found that the volume of circulating blood in normal pregnant women was usually considerably greater than non-pregnant women. There was a moderate increase in blood volume during the first trimester, a more marked rise during the second trimester, and a slight increase throughout the third trimester of pregnancy (Pritchard, 1965). In addition, Faupel-Badger, Hsieh, Troisi, Lagiou, and Potischman (2007) stated that during pregnancy, maternal plasma volume increases to meet the greater circulatory needs of the placenta and maternal organs, such as the uterus, breasts, skin, and kidneys, with an average increase of about 45%. These increases in blood volume lead to an increase in stress on the heart and therefore induce cardiac hypertrophy (Rossi & Carillo, 1991).

 Although neither Pritchard (1965) nor Faupel-Badger et al. (2007) mentioned lactation changes, Bond (1958) demonstrated an increase in blood volume during pregnancy in rats, which then steadily increased during lactation. Measurements were made on the days nine, ten, 20, and 21 of lactation and days 13-15 post-lactation (Bond, 1958). These data showed an increase in blood volume during lactation, which could increase cardiac hypertrophy.

Signaling Pathways Mediating Cardiac Hypertrophy

Molkentin and Dorn (2001) reviewed the field of cardiomyocyte signal transduction and the regulation of the hypertrophic response. Since sustained cardiac hypertrophy is a leading predictor of heart failure, many are attempting to find the particular pathways involved in the process. Molkentin and Dorn (2001) suggested that ERK 1 and 2 signaling factors are involved with the hypertrophic response. Heineke and Molkentin (2006) expounded upon this, agreeing that ERK 1 and 2 do contribute to hypertrophy, but specifically concentric hypertrophy, which occurs with uniform profile of an increased heart-to-body ratio of approximately 25-30%. This is considered physiological hypertrophy, which is also known as compensated hypertrophy since cardiac specific overexpression of ERK 1 and 2 induces cardiac hypertrophy with enhanced function (Bueno et al., 2000).

Another cardiac hypertrophy related pathway is AKT. Heineke and Molkentin (2006) discussed that the AKT/PKB pathway is required for physiological heart growth. Similarly, Shiojima and Walsh (2006) expressed that AKT is involved in cardiac hypertrophy. DeBosch et al. (2006) also concluded that AKT is expressed during cardiac hypertrophy by using AKT1-deficient mice to look at AKT’s influence on exercise-induced cardiac hypertrophy. They found that these mice were resistant to swimming training-induced cardiac hypertrophy, therefore providing evidence that AKT expression induces physiological cardiac hypertrophy (DeBosch et al., 2006). Chung et al. (2012), who specifically looked at pregnancy-induced cardiac hypertrophy, showed an increase in activity of AKT in both mid-pregnancy and late-pregnancy. In addition, they showed that increased activities of downstream targets of AKT, including GSK3ß, p70SK, and mTOR in mid-pregnancy are significantly increased and therefore may help induce cardiac hypertrophy (Chung et al. 2012).

The other downstream target of AKT is the FoxO family of transcription factors (Shiojima & Walsh, 2006; Skurk et al., 2005; Yu et al., 2010). The phosphorylation of FoxO3 is increased in the heart in response to pressure overload or AKT overexpression. Therefore, AKT-induced hypertrophy is partly mediated by the inhibition of FoxO factors (Shiojima & Walsh, 2006). Furthermore, Ni et al. (2006) showed that cardiac specific FoxO1 overexpression blunts hypertrophic growth of cardiomyocytes by inhibiting calcineurin/NFAT, an important mediator of pathological cardiac hypertrophy.

Molkentin and Dorn (2001) also mentioned the effects of the transgenic overexpression of STAT3 in the heart, which induces cardiac hypertrophy. Similarly, Pan et al. (1999) found that mechanical stretch induced the rapid phosphorylation of STAT1 and STAT3. By using neonatal rat cardiomyocytes that were cultured on malleable silicone dishes, they stretched the dishes by 20% and after just two minutes, phosphorylation of STAT1 and STAT3 was seen with a peak at five to 15 minutes (Pan et al., 1999).

In contrast, during pathological hypertrophy, such as human hearts with failure secondary to advanced coronary artery disease, this hypertrophy is accompanied by increased expression of p38 MAPK activity (Molkentin & Dorn, 2001; Chung & Leinwand, 2014). Wang et al. (1998) stated that the direct involvement of p38 pathways in cardiac hypertrophy and apoptosis suggests a significant role for p38 in the pathophysiology of heart failure. Therefore, p38 may be specifically involved with pathological cardiac hypertrophy.

Cardiac Regression

Definition.

Simply put, cardiac regression is the reversal of cardiac hypertrophy (Sen, 1983; Friddle et al., 2000; Razeghi & Taegtmeyer, 2006). However, the primary implication of regression for pathological hypertrophic patients would be a better therapeutic achievement than simply arresting hypertrophy's progress (Sen, 1983). In an early study, Friddle et al. (2000) were unsure of how regression occurred. Did it involve a simple reversal of the induction program, or activation of a separate transcription program (Friddle et al., 2000)? According to Razeghi and Taegtmeyer (2006), regression occurs through the activation of atrophic signaling pathways and by inhibiting pro-hypertrophic signaling. But, when does regression occur? As stated by Hill and Olson (2008), pregnancy-induced heart growth regresses over a period of months after delivery. However, they failed to mention whether or not breastfeeding would increase hypertrophy, therefore delaying regression or increasing the amount of time it would take to regress completely back to normal size. In addition, athletes who train seasonally will have seasonal variation in left ventricular dimensions (Hill & Olson, 2008). Consequently, during their periods of detraining they will experience regression and then hypertrophy will occur again once their training begins.

Regression of Exercise-Induced Cardiac Hypertrophy.

 Hickson, Hammons, and Holloszy (1979) studied both the progression of exercise-induced cardiac hypertrophy and regression in rats. Adult female rats were exercised by daily swimming for 21 days. The exercise program resulted in a 30% increase in heart weight. The regression groups of animals were sacrificed on days 1, 3, 7, 14, 21, 35, and 50 after the last exercise session. Heart weight and total protein content decreased rapidly during the first seven days after ending the exercise program. During this period, approximately 60% of total regression of cardiac hypertrophy occurred. However, heart weight and protein content remained practically unchanged from day seven through day 14 after stopping exercise, even after repeating the procedure again. After day 14, heart weight returned back to the base line of sedentary control values (Hickson et al., 1979). Therefore, cardiac regression was shown to occur after cessation of exercise.

Regression of Pregnancy-Induced Cardiac Hypertrophy.

 Umar et al. (2012) observed cardiac structural changes associated with pregnancy-induced cardiac hypertrophy and regression. Three groups of female C57Bl/6 mice were used including non-pregnant diestrus, late pregnant, and seven days of postpartum (Umar et al., 2012). As expected, late pregnancy was associated with significant heart hypertrophy. In addition, this hypertrophy was reversed by day seven of postpartum (Umar et al., 2012). However, they did not indicate whether postpartum mice were lactating or not.

Signaling Pathways Underlying Cardiac Regression

Gonzalez et al. (2007) noted that after pregnancy in rats, there was a rapid reversal of cardiac hypertrophy. During this time, left ventricular mass returned to normal by 12-24 weeks postpartum. In addition, Gonzalez et al. (2007) observed that the reversal of left ventricular hypertrophy was accompanied by an increase in the signaling molecules such as phosphorylated p38 and AKT. Phosphorylated ERK decreased immediately after parturition and returned to normal values by two weeks after birth (Gonzalez et al., 2007). However, they did not mention the status of lactation during postpartum.

In addition, downstream targets such as FoxO transcription factors can inhibit crucial hypertrophic pathways (Li et al., 2007; Schips et al., 2011). According to Schips et al. (2011), there are two members of the FoxO family, FoxO1 and FoxO3, which show high expression in order to decrease hypertrophic pathways leading to regression. Wang and Su (2011) agreed with Schips et al. (2011) about FoxO3, and mentioned that the activation of FoxO3 is sufficient to activate regression under baseline conditions. Interestingly, cardiac overexpression of FoxO3 does not reduce pathological hypertrophy in adult mice (Schips et al., 2011).

**Summary**

Cardiac hypertrophy has been extensively researched (Kametas et al., 2001; Eghbali et al., 2005; Shiojima & Walsh, 2006; Chung et al., 2012; Chung et al., 2013). There are two types of cardiac hypertrophy: pathological and physiological. Of these, physiological cardiac hypertrophy has two different inducers. Both exercise and pregnancy can induce cardiac hypertrophy (Frey & Olson, 2003; Razeghi & Taegtmeyer, 2006; McMullen & Jennings, 2007; Hill & Olson, 2008; Chung & Leinwand, 2014; Chung et al., 2012; Eghbali et al., 2005; Kametas et al., 2001). The signaling pathways that control physiological cardiac hypertrophy include ERK, AKT, and STAT (Wang et al., 1998; Pan et al., 1999; Molkentin & Dorn, 2001; Skurk et al., 2005; Heineke & Molkentin, 2006; Shiojima & Walsh, 2006; Yu et al., 2010; Chung et al., 2012). In addition, after continuous exercise ends or during postpartum , both conditions will experience cardiac regression (Hickson et al., 1979; Umar et al., 2012). The FoxO family has been shown to mediate cardiac regression (Li et al., 2007; Schips et al., 2011; Wang & Su, 2011). The physiological changes of pregnancy and lactation include increased food intake and blood volume (Cole & Hart, 1938; Anderson & Turner, 1963; Cripps & Williams, 1975; Pritchard, 1965; Faupel-Badger et al., 2007; Bond, 1958).

However, the effect of lactation on cardiac hypertrophy has not been thoroughly researched. Thus, in this study the purposes were to investigate 1) the effects of lactation on cardiac hypertrophy and regression; and 2) the signaling pathways responsible for cardiac hypertrophy and regression in in postpartum mice.

CHAPTER III

METHODOLOGY

**Animals**

 This study took place at Texas Tech University according to an approved Institutional Animal Care and Use Committee protocol (ACUC Approval Number: 13091-10; see Appendix). C57Bl/6 mice (N=32) were used for this study. The mice were purchased from Charles River at seven to ten weeks of age.

**Procedures**

 All of the mice were given a week for acclimatization before the study commenced. The mice were kept in a temperature-controlled room at 22.78°C (73°F) and 16% humidity, with food and water available *ad libitum*. Light was given from seven o’clock in the morning till seven o’clock at night. Mice were randomly assigned to various groups including:

1. C (Non-pregnant diestrus controls) (N=7)
2. LP (17 days of gestation, 3 days before parturition) (N=5)
3. 1PP (Seven days of postpartum) (N=4)
4. 3PP (21 days of postpartum, time of weaning) (N=5)
5. 1W (Seven days after weaning) (N=5)
6. 3W (21 days after weaning) (N=6)

Group C was never in the presence of the male mice. The occurrence of the estrus cycle was checked by the appearance of the vagina. Mice in the diestrus cycle were sacrificed, as estradiol levels are an important mediator of cardiac morphology and function (Kilic, Javadov, Karmazyn, 2009).

Mice in all groups except C were mated with male mice. Day one of pregnancy was recognized as the day of the presence of the copulatory plug. After the plug was detected, the male mouse was removed. However, at day 13 if any mouse’s body weight had not changed, that particular mouse was placed back with the male. LP mice were allowed to continue through gestation until day 17 and then were sacrificed. Postpartum mice (1PP and 3PP) were allowed to deliver their babies, and sacrificed on day seven of postpartum (1PP) and on day 21 of postpartum (3PP). Since pups at this age, seven days after delivery, cannot care for themselves, they were euthanized at this time by cervical dislocation because carbon dioxide is ineffective on pups. Weaning mice (1W and 3W) were allowed to deliver and raise their babies. These mice were sacrificed on day seven after weaning (1W) and on day 21 after weaning (3W). Pups were weaned from their mothers on day 21 of postpartum and were used for snake food for the Texas Tech University Biology Department. Food intake was measured throughout the study and body weight was measured twice a week for all mice.

After parturition, if mothers are stressed, they sometimes kill their pups. Mice that killed their pups were excluded from the study (N=7). Also, at the end of the study, one control mouse was never observed in diestrus cycle and was therefore sacrificed on estrous cycle and was excluded from the study. In addition, there was one mouse that never carried full term even after three attempts and was consequently excluded from the study. Furthermore, there was also one mouse that was accidentally sacrificed by someone not within the study and therefore the data was excluded. All mice were sacrificed between the ages of 16 to 21 weeks.

Four hours before being euthanized, all mice were placed in a cage without food for fasting. Fasting was performed because blood was taken in order to measure prolactin and insulin. Blood was collected from the posterior vena cava, while mice were deeply anesthetized with 3% isoflurane. All mice were euthanized within the Experimental Sciences Building animal facility of Texas Tech University, by being placed in an isoflurane-induction machine, followed by cervical dislocation after blood was drawn. Hearts were then rapidly excised and washed in phosphate buffered saline solution (PBS) to allow the blood to be pumped out of the cardiac chambers and coronary vessels. The hearts were then trimmed of connective tissue, vascular tissue, and atria, and measured. After these measurements were taken, the left ventricle was immediately frozen in liquid nitrogen and stored at -80°C for RNA or protein extraction.

**Western (immunoblot) Blot Analysis**

**Homogenization.**

 Left ventricular tissues were homogenized in RIPA lysis buffer (150 mM NaCl, 1.0% NP40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 0.4 mM EDTA, 10% glycecol, 1 mM Dithiothreiotol , DTT) with protease/phosphate inhibitors (Thermo Scientific). After homogenization, samples were rotated at 4°C for 30 minutes. Then, samples were centrifuged at 12,000 x g for 20 minutes at 4°C, and the supernatant were transferred to a 1.5 ml microcentrifuge tube. Samples were then stored at -80°C for further analyses.

**Protein Assay.**

 Pre-diluted protein assay standards (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 μg/μl) were used (Thermo Scientific), and all tissue samples were diluted to 1 to 10 dilutions to measure protein concentration. Standards and unknown samples were placed in a 96-well plate. Last, in all wells, 150 μl of Pierce 660 nm (Thermo Scientific) was added. The plate then sat in the dark for five minutes, then was placed in spectrothe SpectraMax Plus384 microplate reader (Molecular Devices) and the comupter software, SoftMax Pro 6.3, was used for the analysis.

 **Electrophoresis Sample Preparation.**

Homogenized samples were prepared with LDS sample buffer (Invitrogen) with 1 mM DTT. Samples were then centrifuged for five seconds before being denatured for ten minutes at 70°C. After denaturing, the samples were centrifuged again for ten seconds and then loaded into the gel. 20-30 μg of protein was used.

**Gel Electrophoresis.**

 Tris/glycine (8%) gels were used. The separating gel was prepared by combining 4.0 ml acrylamide/BIS, 9.4 ml of pH 8.8 Tris-HCL, 250 μl of 10% SDS, 4.0 ml 50% sucrose, 7.1125 ml of H2O, 312.5 μl 10% APS, and 6.25 μl tetraethyl (TEMED). Once the separating gel was delivered into gel casettes, 20% ethanol was pippetted on top and then it was allowed to rest for one hour. Then the ethanol was poured out and completely removed from the casettes, before the stacking gel was prepared. The 4% stacking gel consisted of 1.0 ml 50% Acrylamide/BIS, 4.2 ml pH 6.8 Tris, 125 μl 10% SDS, 7.3 ml of water, 500 μl of 10% APS, and 5.0 μl TEMED. The mixture was then pipetted on top of the separating gel inside the casettes, and combs were placed to create wells.

 An XCell SureLock electrophoresis machine (Invitrogen) was used for the electrophoresis process. A 1x MES running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) was placed in the inner and outer tanks of the apparatus. 500 μl of antioxidant was added into the inner tank and then the wells were washed with a pipette. Eight μl of pre-stained Molecular Weight Standard (MWS) was always placed in the first well of each gel, then the samples were placed in the rest of the wells. The gel generally ran at 150 volts for at least 1.5 hours. After the 25 kDa band reached the bottom of the gel, the electrophoresis process was stopped.

**Transfer to Membranes.**

 A wet transfer method was used for all transfers to membranes. All sponges were soaked in transfer buffer (Tris/Glycine Buffer (Bio Rad) with 10% Methanol) and rolled out to remove any bubbles before transfer. A sandwich was created inside the transfer apparatus in this order: two sponges, filter paper, gel, membrane, filter paper, and then two more sponges. The last two sponges were placed onto the filter paper after it was rolled to remove bubbles in between the gel and the membrane. Once the sandwich was made, the apparatus was locked and extra transfer buffer was poured inside the inner tank. Cold water was added to the outside tank and then the transfer took place at 30 volts for one hour.

**Primary and Secondary Antibodies.**

 Membranes were blocked in 5% milk in TBS (100mM pH 8 Tris Base, 500mM NaCl, 100mM KCl, water) with 0.01% Tween 20 solution for 30 minutes at 37°C before being treated with the primary antibodies. The membranes were incubated with the following primary antibodies: AKT, FoxO3a, STAT, ERK, and their corresponding phosphorylated forms. After stripping the phosphorylated blots, total protein content were examined and the amount of phosphorylated to total protein were used to represent the degree of activity of corresponding proteins. Primary antibodies were incubated overnight at 4°C. The next day, the membranes were washed with TBST (3 times), and then applied with specific secondary antibodies, depending on the host of primary antibody.

**Developing Membranes.**

 Mixed substrates with a 1:1 ratio (Bio Rad Clarity Western ECL) were applied to the membranes for five minutes before developing. After this application, the membranes were placed on a glass plate for the picture in the Bio Rad ChemiDoc MP Imaging System. Western blot band intensity was analyzed by using the computer software, Image Lab 6.8 (Bio-Rad).

**Data Analysis**

 Data were expressed Mean ± SEM. The differences among groups were analyzed using one-way ANOVAs followed by Tukey test for multiple group comparison. P<0.05 was considered as significant.

CHAPTER IV

RESULTS

 The purpose of this study was to identify the effects of lactation and weaning on cardiac hypertrophy and regression during postpartum as well as to distinguish signaling pathways mediating cardiac hypertrophy and regression in postpartum mice.

Body Weight

Body weight was measured twice a week. Figure 1A shows the body weight changes throughout the study. Body weight increased gradually during gestation, and sharply dropped after parturition. Body weight increased again during lactation but decreased slightly before weaning. As shown in Figure 1B, there was not any difference between body weight and the number of pups throughout lactation. Figure 1C shows final body weights compared to each group. Body weight during LP was significantly increased compared to every other experimental group (^p<0.05 vs. LP). In addition, LP and 1PP were significantly increased compared to C (\*p<0.05 vs. C).



*Figure 1A*. Changes of body weight throughout the study. This figure illustrates the change in body weight throughout pregnancy, lactation, and after weaning.



*Figure 1B*. Body weight versus number of pups during lactation. This figure shows the distribution of body weight throughout lactation based on the number of pups.



*Figure 1C*. Endpoint body weight. This figure displays body weight at the time of sacrifice (\*p<0.05 vs. C, ^p<0.05 vs. LP).

Food Intake

Food intake was also measured throughout the study. Figure 2A shows the trend of food intake over time. Food intake increased throughout gestation, then further increased as postpartum progressed, and finally decreased noticeably after weaning. As shown in Figure 2B, there was not any difference between food intake and the number of pups throughout lactation. In addition, Figure 2C shows the average food intake. Both lactation groups (1W and 3W) had a significantly higher food intake than C (\*p<0.05 vs. C). Furthermore, both 1PP and 3PP also had a significantly higher food intake than LP (^p<0.05 vs. LP). Also, both 1W and 3W showed a significant decrease in food intake compared to that of both lactation groups (~p<0.05 vs. 1PP, #p<0.05 vs. 3PP).



*Figure 2A*. Changes of food intake throughout the study. This figure illustrates food intake throughout pregnancy, lactation, and after weaning.



*Figure 2B*. Food intake versus number of pups during lactation. This figure shows food intake throughout lactation based on the number of pups.



*Figure 2C*. Average food intake. This figure demonstrates the average food intake per group (\*p<0.05 vs. C, ^p<0.05 vs. LP, ~p<0.05 vs. 1PP, #p<0.05 vs. 3PP).

Heart Weight

Figure 3A shows the absolute heart weight of each group. All groups were significantly increased when compared to that of C (\*p<0.05 vs. C), with the highest degree of hypertrophy in 3PP. While absolute heart weight was significantly higher in 3PP compared to LP (^p<0.05 vs. LP), 3W was significantly regressed compared to 3PP (#p<0.05 vs. 3PP). However, the heart weight was still significantly larger than C (\*p<0.05 vs. C). Figure 3B displays relative heart weight, heart weight normalized to tibia length (HW/TL). The changes in HW/TL had as similar pattern as absolute heart weight. Moreover, Figure 3C shows the percentage increase in HW/TL relative to C. 3PP had the highest degree of cardiac hypertrophy showing a 32.15% increase in HW/TL compared to C. Although heart weight began to regress after weaning, the percent increase in HW/TL was still 15.81% higher than C at 3W. A summary of the morphological data was reported in Table 1.



*Figure 3A*. Heart weight. This figure shows the absolute heart weight of each group at the time of sacrificing (\*p<0.05 vs. C, ^p<0.05 vs. LP, #p<0.05 vs. 3PP).



*Figure 3B*. Heart weight normalized to tibia length. This figure shows the relative heart weight of each group at the time of sacrificing (\*p<0.05 vs. C, ^p<0.05 vs. LP, #p<0.05 vs. 3PP).



*Figure 3C*. Percent increase of heart weight compared to control. This figure illustrates the percent increase of heart weight for each experimental group compared to the control group (^p<0.05 vs. LP, #p<0.05 vs. 3PP).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Groups | C | LP | 1PP | 3PP | 1W | 3W |
| N | 7 | 5 | 4 | 5 | 5 | 6 |
| BW (g) | 23.39 ± 0.5704 | 34.32 ±1.252\* | 27.71 ± 0.7922\*^ | 26.53 ± 0.7217^ | 26.36 ± 0.7611^ | 25.49 ± 0.7619^ |
| HW (mg) | 97.88 ± 1.922 | 112.4 ± 4.545\* | 122.3 ± 4.151\* | 131.2 ± 4.283\*^ | 119.8 ± 3.441\* | 114.0 ± 3.559\*# |
| % HW/TL (mg/mm) | n/a | 15.61 ± 4.456 | 24.00 ± 3.376 | 32.15 ± 4.190^ | 21.55 ± 2.796 | 15.81 ± 3.331# |
| Food Intake | 3.143 ± 0.03612 | 5.672 ± 0.1627 | 11.51 ± 0.9818\*^ | 14.53 ± 1.668\*^ | 4.951 ± 0.2~# | 3.868 ± 0.492~# |

Table 1 Summary data for physical changes during pregnancy and postpartum (\*p<0.05 vs. C, ^p<0.05 vs. LP, ~p<0.05 vs. 1PP, #p<0.05 vs. 3PP)

**Signaling Pathways Responsible for Cardiac Hypertrophy and Regression**

 **AKT.**

 Increased AKT activity by phosphorylation has been implicated as an important mediator for physiological cardiac hypertrophy, and also changes during pregnancy (Chung et al., 2012). Figure 5 shows the change in phosphorylated AKT versus total AKT in each group. However, there was not a significant difference between each group.



*Figure 4*. Changes in phosphorylated AKT versus total AKT. This figure displays that AKT activity was not changed among groups.

 **FoxO** **Family.**

 FoxO is one of the downstream targets of AKT. FoxO is inactivated by phosphorylation via AKT and results in cardiac hypertrophy (Shiojima & Walsh, 2006; Skurk et al., 2005; Yu et al., 2010). Figure 5A shows that phosphorylated FoxO at phosphorylation site threonine 24 increased significantly versus total FoxO in 1PP and 3PP when compared to C (\*p<0.05 vs. C). Also, when compared to C, Figure 5B illustrates that 1PP had an increase in phosphorylated FoxO at phosphorylation sites serine 318 and 321 versus total FoxO (\*p<0.05 vs. C). Figure 5C displays an increase in phosphorylated FoxO at phosphorylation site serine 253 versus total FoxO in 1W compared to C (\*p<0.05 vs. C). Thus, our results suggest that the inactivation of FoxO in various phosphorylation sites is important in cardiac hypertrophy during the postpartum period.



*Figure 5A*. Changes in phosphorylated FoxO at phosphorylation site threonine 24 versus total FoxO. This figure displays the changes in phosphorylated FoxO at phosphorylation site threonine 24 versus total FoxO for each group (\*p<0.05 vs. C).



*Figure 5B*. Changes in phosphorylated FoxO at phosphorylation sites serine 318 and 321 versus total FoxO. This figure displays the changes in phosphorylated FoxO at phosphorylation sites serine 318 and 321 versus total FoxO for each group (\*p<0.05 vs. C).



*Figure 5C*. Changes in phosphorylated FoxO at phosphorylation site serine 253 versus total FoxO. This figure displays the changes in phosphorylated FoxO at phosphorylation site serine 253 versus total FoxO for each group (\*p<0.05 vs. C, ^p<0.05 vs. LP).

 **ERK.**

 In addition to phosphorylated AKT, ERK has also been shown to stimulate physiological cardiac hypertrophy (Molkentin & Dorn, 2001; Heineke & Molkentin, 2006) including pregnancy-induced cardiac hypertrophy (Chung et al., 2012). Figure 6 displays the change in phosphorylated ERK versus total ERK in each group, and 1PP had a significant increase in phosphorylated ERK when compared to LP (^p<0.05 vs. LP).



*Figure 6*. Changes in phosphorylated ERK versus total ERK. This figure displays the changes in phosphorylated ERK versus total ERK for each group (^p<0.05 vs. LP).

 **STAT.**

 STAT has previously been indicated to induce cardiac hypertrophy (Pan et al., 1999; Molkentin & Dorn, 2001); furthermore, it has specifically been shown to protect hearts from PPCM during pregnancy-induced cardiac hypertrophy (Hilfiker-Kleiner et al., 2007). Figure 7 shows the change in phosphorylated STAT versus total STAT in each group. However, there were no differences between each group.



*Figure 7*. Changes in phosphorylated STAT versus total STAT. This figure illustrates that STAT activity was not changed among groups.

CHAPTER V

DISCUSSION

This study provides evidence of an increase in cardiac hypertrophy during lactation. In agreement with previous studies (Chung et al., 2012; Chung et al., 2013), the LP group experienced significant increase in the size of the heart compared to group C (Figures 3A & 3B). Both lactation groups (1PP & 3PP) further increased pregnancy-induced cardiac hypertrophy showing that 3PP induced the highest degree of cardiac hypertrophy (Figures 3A & 3B). In addition, our results showed that lactation induced greater percent increases in HW/TL than both exercise (Konhilas et al., 2004) and late pregnancy (Chung et al., 2012). After 21 days of exercise, female C57BL/J6 mice undergo about 15.90% increase in HW/TL compared to sedentary controls (Konhilas et al., 2004). In this study, LP showed 15.61% increase in HW/TL (Table 1). Interestingly, 1PP had an increase of 24.00% and 3PP had an increase of 32.15% compared to C, which was much greater than LP (Table 1 & Figure 3C). Therefore, lactation does significantly increase cardiac hypertrophy after pregnancy.

 In addition, this study also offers evidence for cardiac regression after lactation. As seen in Figures 3A, 3B, and 3C, 1W and 3W started regressing in heart weight. Therefore, just one week after weaning, the heart was decreasing in size. At this point, the heart was regressed by 8.69% compared to 3PP. In addition, by 3W the heart was further decreased by 13.11% compared to 3PP, which was significantly less than 3PP (Table 1 & Figure 3C). However, this is still significantly larger than the control heart (Figures 3A & 3B). Therefore, our results suggest that more time is needed for the heart to regress completely back to C.

 It is important to note that possible influences on heart size were considered, including age and number of pups per dames. Age or growth were accounted for by normalizing heart weight by tibia length (Figure 3B). However, both absolute heart weight (Figure 3A) and relative heart weight (heart weight normalized by tibia length) (Figure 3B) showed the same degree of cardiac hypertrophy; therefore, age was not a factor in these results. In addition, there was not any significance in the number of pups and the size of the heart (data not shown).

 Next, we investigated the possible signaling pathways underlying cardiac hypertrophy and regression. Unlike in a previous study (Chung et al., 2012), AKT activity expressed as phosphorylation of AKT to total AKT did not change among groups (Figure 4). However, the downstream target of AKT, FoxO (Shiojima & Walsh, 2006; Skurk et al., 2005; Yu et al., 2010), was significantly inactivated (Figures 5A, 5B, & 5C).

The FoxO family, when it is activated, is associated with cardiac regression (Li et al., 2007; Schips et al., 2011; Wang & Su, 2011). On the other hand, FoxO can be inactivated by AKT by phosphorylating multiple sites, including threonine 24, serine 318, 321, and 253, which results in cardiac hypertrophy (Brunet et al., 2001; Skurk et al., 2005; Ni et al., 2006; Ronnebaum & Patterson, 2010; Dobson et al., 2001). The phosphorylation sites of threonine 24, serine 318, 321, and 253 have previously been shown to be the downstream target of the AKT pathway resulting in cardiac hypertrophy (Skurk et al., 2005; Ni et al., 2006; Ronnebaum & Patterson, 2010). Among all phosphorylation sites, the phosphorylation site of serine 253 has a higher affinity than the other two sites (Brunet et al., 2001; Ronnebaum & Patterson, 2010). In this study, phosphorylated FoxO at phosphorylation site threonine 24 was significantly increased at both 1PP and 3PP when compared to C (Figure 5A), while phosphorylation sites of serine 318 and 321 was significantly increased at 1PP when compared to C (Figure 5B). Phosphorylated FoxO at phosphorylation site of serine 253 was significantly increased during 1W when compared to both LP and C in this study, but decreased at 3W (Figure 5C). The decreased phosphorylation of FoxO at serine 253 in conjunction with the other sites may lead to cardiac regression at 3W (Figures 3A, 3B, & 3C). Taken together, other results suggest that FoxO inactivation by phosphorylation partially mediates cardiac hypertrophy during postpartum. However, it is out of the scope of this study to investigate the role of each phosphorylation site on cardiac hypertrophy.

ERK has been shown to be activated during pregnancy-induced cardiac hypertrophy (Chung et al., 2012; Chung & Leinwand, 2014) and 1PP was significantly increased in ERK when compared to LP (Figure 6). Therefore, ERK may also be an important mediator for cardiac hypertrophy during lactation. STAT did not have any significance among groups (Figure 7) even though it has previously been shown to increase during cardiac hypertrophy (Pan et al., 1999; Molkentin & Dorn, 2001).

In addition to the pathways above, an attempt to observe phosphorylation of p38 MPAK was made; however, it was not detected even after multiple attempts. Since p38 is associated with pathological cardiac hypertrophy (Wang et al., 1998; Molkentin & Dorn, 2001; Chung & Leinwand, 2014), the absence of p38 signaling suggests that pregnancy and lactation may not be forms of pathological cardiac hypertrophy.

 Unfortunately, there were many variations in AKT and STAT. However, these could have been caused by the differences in body weight gain and number of pups in each group. In addition, the time constraints of this study led to a lower sample size used for the signaling pathways data for each group (N=3). Therefore, with the addition of more samples, there may be more concrete results.

Future studies should include different groups from those used in this study, such as adding an immediate postpartum group, a two weeks postpartum group, and a four weeks postpartum group. Since food intake and body weight begin to decrease right before weaning (Figure 2A), it is possible that two weeks after postpartum is the peak of hypertrophy during lactation. This study did not have a two weeks postpartum group; therefore, it is unknown whether two weeks of lactation is the peak of hypertrophy or if hypertrophy continues to increase until the time of weaning. In order to compare these groups, future studies should include a two weeks postpartum group. Also, in order to see the progression of regression, a two weeks after weaning group could be implemented. Lastly, future studies should include at least a four weeks after weaning group. The data acquired from this study showed that at three weeks after weaning, the hearts were still 15.81% more increased than the controls (see Table 1). This is about the same increase as the late pregnancy group, meaning that the complete regression of the hearts had not occurred yet and needed more time to regress back to normal size.

In addition to more groups, future studies should also focus on signaling pathways that mediate cardiac regression. Previous studies show that proteasome activity greatly influences cardiac regression (Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001; Li et al., 2007; Galasso et al., 2010). When the FoxO family is activated, or not phosphorylated, its downstream target, Atrogin-1, has been shown to inhibit cardiac hypertrophy (Gomes et al., 2001; Li et al., 2007; Wang & Su, 2011). Therefore, future studies are needed to investigate the role of proteasome pathways in the regression phase such as after weaning.

 Overall, this study demonstrated that lactation significantly further increases pregnancy-induced cardiac hypertrophy. In addition, signaling pathways that are used during pregnancy-induced cardiac hypertrophy, including phosphorylated FoxO and ERK, are used during lactation. Furthermore, pathological signaling pathways, such as p38, are not activated during pregnancy or lactation. In addition, cardiac regression does occur after lactation ends; however, the role of FoxO during cardiac regression needs to be further investigated.

**CHAPTER VI**

**CONCLUSION**

 In this study, we hypothesized that pregnancy increased cardiac hypertrophy would further increase with lactation, while regression would be initiated with weaning. Also, hypertrophy in groups LP, 1PP, and 3PP would be caused by an increase in pro-hypertrophic signaling pathways, such as AKT, ERK, and STAT expression, while regression in groups 1W and 3W would be caused by an increase in anti-hypertrophic signaling pathways, such as the FoxO family.

 In agreement with our hypotheses, pregnancy-induced cardiac hypertrophy is further increased during lactation. In addition, cardiac regression begins to occur after weaning, however, our results suggest that more time is needed in order to see the full regression. Also, the increase of cardiac mass during lactation may be partially due to the inactivation of the FoxO family.

APPENDIX

This is the protocol used by the lab, which was written by Dr. Eunhee Chung.

***TEXAS TECH UNIVERSITY***

***INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE***

Instructions for Completing

PROTOCOL FOR THE USE OF LIVE ANIMALS

FOR RESEARCH, TEACHING OR DEMONSTRATION

Animal Use Form

Form Revised June, 2013

Information provided on the Animal Use Form will be used by the Animal Care and Use Committee to evaluate your proposed use of live animals in research, teaching, or demonstration. ***Review of your animal use protocol will be based on compliance with the Animal Welfare Act, The Public Health Service Policy on Humane Care and Use of Animals by Awardee Institutions, the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agriculture Animals in Agricultural Research and Teaching.*** Approval by the committee is required before any animal can be ordered or used within Texas Tech University.

The principal investigator (faculty member) submitting a protocol is responsible for the actions of all personnel involved with the care and use of animals. Failure to comply with current regulations will result in suspension of your use of animals at Texas Tech University.

1. Answer all questions.

2. Completed form should be submitted electronically as a Word document attachment to:

 iacuc@ttu.edu

#####

1. The Animal Care and Use Committee meets once per month to review all animal use forms. Meeting schedules and protocol deadlines are available at <http://www.iacuc.ttu.edu>

Completed forms must be submitted seven days before the scheduled meeting.

4. Protocols may be approved for three years, but regulations require a review and reapproval every 12 months.

5. Protocols that involve no pain or distress may be reviewed through a “Designated Member Review” process.

6. Responses to question #3 below may require input from/consultation with other Texas Tech University compliance and/or safety-related committees. Contact information for those committees is listed below:

 Radiation/Laboratory Safety Manager

 <http://www.depts.ttu.edu/ehs/Web/RadLabSafety.aspx>

 Institutional Biosafety and Hazardous Materials Committee

 <http://www.depts.ttu.edu/ehs/Web/BioISafety.aspx>

***TEXAS TECH UNIVERSITY*** (ACUC Use Only Revised

***Institutional Animal Care & Use Committee*** ACUC APPROVAL NO. 13091-10

 Expiration Date 10/7/2016

 Category C

PROTOCOL FOR THE USE OF LIVE ANIMALS

FOR RESEARCH, TEACHING OR DEMONSTRATION

#### Animal Use Form

A protocol can be reviewed only after all questions have been answered completely. Do not refer to, or attach passages from grants.

Date Filed: September 27, 2013

TITLE OF PROTOCOL: (There may be multiple titles) Cardiac hypertrophy and regression in response to pregnancy and exercise; crosstalk between cardiac and skeletal muscle in response to exercise and pregnancy

Principal Investigator: Dr. Eunhee Chung

Department: Health, Exercise, and Sport Sciences Telephone Number: 806-834-3392

E-mail: eunhee.chung@ttu.edu

Emergency Contact Name: Eunhee Chung Emergency Contact Phone Number: 303-345-4366

Proposed funding source: NIH and American Heart Association

ORS Proposal Number:

Expected starting date of project: November, 2014 Expected completion date of project: October, 2016

Project type: Biomedical x Food/Fiber Production Teaching/Demonstration Wildlife

Aquatic Production Other -please describe:

Does this project involve human subjects and need IRB approval? Yes No x1. Animal model(s): mice

1. Common name, scientific name, sex, age: C57/Bl6 mice, Male and Female, 3 to 15 month age

B. Total number of animals requested for the entire project: 310

Number of Animals in Year 1 \_387\_\_\_\_+ Year 2 \_\_\_\_\_ + Year 3 \_\_\_ = Total Requested \_387\_\_\_\_

\*If animals will be carried over from year-to-year then they only need to be counted the first year they are implemented into the study.

C. Location of animals and project: Biological Science 617BF (housing)/Experimental Science Animal Facility (euthanasia)

###### D. The animals will be maintained in what type of caging/housing?

 Cage type for both group housing and individual housing will be micro-isolator cages.

The mice will be initially group-housed (5 per cage) in micro-isolator cages, before being used for the experiments.

1. ***Cardiac regression after exercise cessation***: Each mouse subjected to voluntary wheel running will be placed in a clear plastic cage that contained a free wheel for either 3 weeks or 5 weeks. Each mouse will be housed individually because total running distance, speed, and duration of running per day will be used for normalization of body characteristics (such as body weight change and heart weight change per total running distance). Animals in detraining groups will be given voluntary wheel running either for 3 weeks or 5 weeks, and then will be taken out from the wheel cage and housed by group so that they will be remained sedentary for certain time periods (3- , 5-, 14- and 21days).
2. Pregnancy and postpartum: Mice becoming pregnant on the same day will be housed together in micro-isolator cages.

E. Source of animals (e.g., purchased, institutionally bred, captured from wild):

All mice will be purchased from Taconic.

1. Does this project involve wild-captured animals? Yes No x
2. If yes, have required permits been obtained? Yes No Not Applicable
	* 1. Please provide permit numbers for appropriate state, federal, or international permits under which these animals are being used.
		2. If permits are not required, please explain:

3. Will animals or humans be exposed to:

A. Radiation or radioactive materials? Yes No x

If yes, are you licensed to use material? Yes No Not Applicable

NOTE: Protocols that include use of radioactive materials must have prior approval from the Radiation/Laboratory Safety Manager.

B. Recombinant DNA or BSL 2 or 3 organisms? Yes No x

NOTE: Protocols that include use of any Recombinant DNA or BSL 2 or 3 organisms must have prior approval from the Institutional Biosafety Committee.

C. Hazardous chemical, carcinogens, toxins or noxious agents? Yes No x

1. If yes list the agent(s):
2. Has EH&S been consulted on the use of the agent(s)? Yes No Not Applicable

EH&S Personnel Contact:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Outcome:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

D. Has use of radioactive substancesor recombinant DNA received approval from the appropriate Safety Committee? Yes No Not Applicable x

NOTE: Animal Use Protocols will not be approved until the IACUC receives notification of approval from appropriate Safety Committee(s).

Approval Number: Date of approval from the appropriate Safety Committee: \_\_\_\_\_

E. Will this project necessitate use of DEA Controlled Substances? Yes No X

If yes, have you obtained a Federal and State DEA License? Yes No Not Applicable

Instructions for obtaining Federal and State DEA License can be obtained from <http://www.iacuc.ttu.edu>.

4. Pharmaceutical grade chemicals and other substances should be used for all animal-related procedures. (please see proposed ***TTU IACUC Policy and Guidelines for the Use of Non-Pharmaceutical Compounds in Animal Studies***):

Does this proposed project involve exposure of animals to non-pharmaceutical grade chemicals or substances? Yes No X

If yes, plans to use non-pharmaceutical grade chemicals or substances should be described and justified. For example, the use of a non-pharmaceutical-grade chemical or substance may be necessary to meet the scientific goals of a project or when a veterinary or human pharmaceutical-grade product is unavailable. Consideration should be given to the grade, purity, sterility, pH, pyrogenicity, osmolality, stability, site and route of administration, formulation, compatibility, and pharmacokinetics of the chemical or substance to be administered, as well as animal welfare and scientific issues relating to its use.

Non-Pharmaceutical Grade

 Chemical/Substance Description/Justification for Use

|  |  |
| --- | --- |
|  |  |
|  |  |
|  |  |
|  |  |

 Refer to IACUC Policy and Guidelines

 Expand table as necessary to accommodate all non-pharmaceutical chemicals/substances to which animals will be exposed

5. Provide the rationale and purpose of the proposed use of this animal species. State why living vertebrates, and specifically the species you are using, are required rather than some alternative model:

 This proposal is to investigate the molecular and biochemical mechanisms underlying cardiac regression following pregnancy and exercise. This study cannot be simulated by computer or cell culture. Cell culture cannot be used since pregnancy-and exercise-induced cardiac hypertrophy are associated with volume overload, such as increases in heart rate and blood volume. The mouse is the lowest vertebrate that resembles the physiological features in human exercise and pregnancy including cardiac hypertrophy and regression following the cessation of stimuli. In addition, the use of mouse models compared to cell culture models allows us to examine the integrated response of whole organism as well as signaling pathways responsible for cardiac regression.

6. Provide justification of the number of animals requested:

A. Explain all treatment and /or study groups. (Example: 5 animals/treatment group X 5 treatment groups/study group X 4 study groups = 100 animals required)

i. For the study of cardiac regression following pregnancy, we will use 12 weeks old mice.

[13 mice per group x 7 time course (non-pregnant control, late pregnancy, 1 day postpartum, 7 days postpartum, 14 days of postpartum, at the time of weaning, 21days after weaning)] + (13 x 6 (pregnant time points) x40%) extra for failing pregnancy = 123.

ii. Aging effect on pregnancy, we will use young (12 weeks old) and old (12-14 month old) group

[13 mice per group x 2 age group (young and old) x 2 time course (non-pregnant control, 21 days of weaning)] + 20 (10 mice for expect to fail to pregnant/ age group (have copulatroy plug but reabsorb the pups)) = 72

iii. Male mice for mating (2-3 month old) =10 mice throughout the study

iv. For the study of cardiac regression following exercise cessation:

13 mice per group x 2 sexes (male and female) x 7 time course (controls, 3 weeks of exercise, 5 weeks of exercise, 3 days of detraining, 5 days of detraining, 14 days of detraining, and 21days of detraining)= 182

B. Explain how you determined the total number of animals requested. Whenever possible, the number of animals requested should be justified statistically (research projects only). Two methods are commonly used to statistically justify the numbers of animals: (1) a power test or (2) peer reviewed publication of methods. It is important to include all animals for pilot studies and any expected losses:

Sample size for each study is carefully monitored to assure that no excess animals are used. Using an alpha probability of 0.05, a power of 0.8, and an effect size of 1.2 X SD, the sample size to detect a statistical difference would be 13 per group. Assuring this power analysis, most previous studies1-4 used 9-18 mice/group for pregnancy study. However, from my previous experience about 30-40% of C57Bl/6 mice reabsorb the pups with the presence of copulatory plug, and these mice (pregnant but fail to successfully maintain pregnancy) will not be used from study since many cardiac hypertrophy signaling pathways are already activated. Thus, we will add 40% for expected losses in pregnant study.

7. Perform a literature/database search to address the following issues: **(contact an IACUC member for assistance or clarifications on performing the literature/database search)**

1. Determine if the proposed use of animals will result in unnecessary duplication of experiments:
	* 1. Is the proposed use of animals duplicative? Yes No X
		2. If yes, list the reference(s) and justify the need for duplication
2. If the procedures described do, or could possibly result in more than momentary pain or distress to animals ***you must search for alternatives to those procedures***. Alternatives may include use of less-invasive procedures, lower species, isolated organ preparation, cell or tissue culture, or computer simulation. Your search must include the word “alternative” plus key words describing the painful or distress-causing procedure, and your particular animal model.
	* 1. Were alternative, less invasive procedures, other species, isolated organ preparation, cell or tissue culture, or computer simulation identified in your search?

Yes No Not Applicable X

* + 1. If yes, list the reference(s) and database(s) used:
		2. If the alternative will not be used, please justify:

You must provide details of your search. List the methods and sources used to determine whether alternative procedures are available and do not unnecessarily duplicate previous experiments. Description of methods must include the key words (the word “alternative” plus key word is required) used in the search. Sources can be databases, such as biological abstracts, Index Medicus, Current Research Information Service, Animal Welfare Information Center, etc., or specific references can be cited. Details of your search should include:

1. Sources or databases searched or other sources consulted: PubMEd.gov
2. Date the search was performed: Aug. 20, 2013
3. Years covered by the search methodology (e.g., 1965 – 2012): not restricted by year
4. Key words, key word strings, and (or) search strategy used (use the following format for all key words):

“alternative” + (key word): Number of Hits: Relevance:

Cardiac regression, exercise, signaling pathways 2 none

Detraining, heart, cardiac remodeling 5 human study (2) and hypertensive model

Pregnancy, cardiac regression, signaling pathway 1 my previous study-only studied immediate postpartum.

Pregnancy, hypertrophy, signaling, postpartum 8 4 relevance

Eghbali Mansoureh (author name), pregnancy 15 Eghbali group do similar study (4 papers are relevant); however, Eghbali group did not include the effect of lactation for cardiac hypertrophy and regression

Sex differences, exercise, regression, signaling 2 not relevant

8. Provide a complete, concise, sequential description of procedures involving the use of the animals that will be easily understood by all members of the committee, which includes non-scientists. Begin your description at the point in which live animals enter the protocol through the time they exit it. Describe EXACTLY what you will do to the animals while they are alive. Include all procedures/treatments in your project that will be imposed on the live animals. If animals are to be transported during the project, include a description of animal care during transport (references to SOP are appropriate). If the project involves survival surgery complete question 10.

 We will order mice from Taconic around 8-11 weeks old for the studies of regression following pregnancy and exercise, and around 1 year old mice for aging effect of pregnancy. Previously, we found that phenotypes (body weight and heart weight) of inbred C57Bl/6 mice were different depending on the vendors; we will order mice from Taconic to compare the results from my previous studies. Mice will be acclimated for 1 week before being used for experiments. Mice will be group housed on a 12h/12h light/dark cycle with access to food and water *ad libitum* until experiments begin. For pregnancy study, three month old virgin female C57Bl/6 mice will be mated with a proven breeder male C57Bl/6 mouse. The presence of a copulatory plug will be counted as day 1 of pregnancy, and birth most often occurs at day 20. The male mouse will be removed once a copulatory plug is detected, and mice becoming pregnant on the same day will be housed together in a cage. If we found only one mouse is pregnant on certain day, the mouse will be housed alone. It is critical for us to know the exact time points of pregnancy, so housing will be accommodated.

Pregnant mice will be euthanized at 17-18 days of gestation (late stage of pregnancy), 1 day postpartum, 7 days postpartum, 14 days of postpartum, at the time of weaning, and 21days after weaning. For older mice group, the mating process will be the same, and will be euthanized 21 days after weaning. We checked estrus cycle by the appearance of the vagina5 and diestrus cycle virgin female mice served as non-pregnant controls (NP) since estradiol level is an important mediator of cardiac morphology and function6. Mice with reabsorbed pups will be excluded from the study.

At a given time point, mice will be euthanized in Experimental Science animal facility because this facility is equipped with isoflurane-induction machine. When taking the mice to the Experimental Science building, they will be placed on a cart, and covered with a sheet or some sort of cover (i.e, big cardboard box) as not to be visible to the public.

Mice will be weighed and euthanized by placing mice in isoflurane-induction machine, followed by cervical dislocation. We chose isoflurane as euthanizing method to compare our results with my previous studies. Hearts will be rapidly excised and washed in PBS to allow blood to be pumped out of the cardiac chambers and coronary vessels. The hearts will be trimmed of connective tissue, vascular tissue and atria. The ventricles will be blotted dry and weighed. After right ventricle removal, the left ventricle will be weighed, immediately frozen in liquid nitrogen, and stored at -80°C for RNA or protein extraction. After collect the heart, skeletal muscles (soleus, gastrocnemius, Tibialis anterior, extensor digitorum longus, plantarius) will be collected. Carcasses will be placed in the freezer and disposed of by ACS staff.

Mice that are excluded from study and mice that are weaned from the mother will be euthanized by CO2 inhalation and used for snake food. The pups from the mice that are killed at the late stage of pregnancy, 1-, 7-, and 14 days of postpartum, will be euthanized via decapitation with sharp scissors because CO2 is not effective for pups.

1. ***Cardiac regression after exercise cessation:*** Each mouse subjected to voluntary wheel running will be placed in a clear plastic micro-isolator cage that contained a free wheel for either 3 weeks or 5 weeks. Mouse-sized metal cage wheels with a diameter of 11.5cm (model no. 6208; PetSmart, Phoenix, AZ) will be fitted with digital magnetic counters (model BC,600, Sigma Sport, Olney, IL) and will be placed into 47x 26x 14.5-cm cages for voluntary exercise. The counters will measure maximum running speed, total distance run, and total time run. Each morning, these data will be collected and recorded for each animal and the counter will be reset. The training protocol will be repeated either for 3 weeks or 5 weeks. Training will be done individually (1 mouse per wheel cage) because total running distance, speed, and duration of running per day will be used for normalization of body characteristics (such as body weight change and heart weight change per total running distance). Animals in detraining groups will be given voluntary wheel running either for 3 weeks or 5 weeks, and then will be taken out from the wheel cage and housed by group so that they will be remained sedentary for certain time periods (3- , 5-, 14- and 21days). Mice will be euthanized same way as in pregnancy study.

9. Provide a short (200 words or less) nontechnical, lay summary of the project. Your description must include project objectives and methods in layman’s terms. Your response should be written such that a person unfamiliar with your work and unfamiliar with science can understand what you are doing, how you will do it, and why you are doing it:

The heart is a plastic organ which can change its structure and function in response to various stimuli. For example, in response to high blood pressure, your heart can get bigger, but it is frequently associated with heart dysfunction and disease. This kind of hypertrophy is called pathological hypertrophy. At some point, pathological hypertrophy is irreversible and results in heart failure. In contrast, cardiac hypertrophy induced by exercise and pregnancy is associated with normal or enhanced heart function. In addition, exercise- and pregnancy-induced cardiac hypertrophy is reversible following cessation of stimuli. Reducing heart mass in patients with pathological hypertrophy improves patient outcomes. Thus, it is important to understand what mediates cardiac regression. We will study mediators of cardiac regression in RNA and protein levels.

10. Does this protocol involve survival surgery? Yes No X If yes, contact the University Veterinarian (742-2805 ext. 257) and complete the following:

A. State where the aseptic surgery will be performed:

B. Individual(s) performing the surgery and their training/experience:

C. List the sedation, analgesic and anesthesia and their dosages:

D. Describe the surgical procedure. (Indicate if multiple surgeries are required):

E. List the post-surgical analgesic dosage and duration:

11. Do the proposed animal activities involve potentially painful procedures? (painful procedures include surgery and procedures that may cause more than momentary or slight pain or distress to the animals)

Yes No X If yes, contact the Clinical Veterinarian (742-2805 ext 257) and complete the following:

A. Justify procedures that may cause animal discomfort, distress, pain, and/or injury. Describe how discomfort, distress, pain, and/or injury will be minimized:

B. List signs and/or symptoms of discomfort, distress, or pain that may occur in animals in this study:

C. Describe the frequency with which monitoring for the above listed signs/symptoms of discomfort, distress, and pain will occur and approximate times of day:

D. List all individuals who will monitor animals for signs and symptoms of discomfort, distress, and pain. Describe the training of each person listed with regards to recognizing signs and symptoms of animal discomfort, distress, and pain:

E. If signs or symptoms of discomfort, distress, and/or pain are observed, what intervention will occur (humane endpoints could entail administration of analgesia, anesthesia, euthanasia, etc.)

How quickly after observation of humane endpoints will intervention occur?

List individuals who will intervene if humane endpoints are reached and describe their training/experience:

F. If analgesics, anesthetics and (or) tranquilizing drugs are to be used, list the agent and describe their dosages to minimize discomfort, distress, pain and injury:

G. If any procedure(s) will cause pain or distress and analgesia/anesthesia cannot be administered, list each procedure with justification for the exclusion of analgesia/anesthesia:

H. The attending veterinarian must be involved in planning of projects wherein painful procedures are included. Was the attending veterinarian consulted? Yes No x

12. Provide information on the care of the animals:

## A. List the individuals responsible for the routine daily animal care:

## Taylor Robertson, Tracer Skelton, Kendell Kennedy, Kalli Looten, Eunhee Chung, Dr. Tiffanie Brooks, and ACS Staff

## B. Veterinary care provided by whom?Please provide veterinarian contact information if using someone other than the university veterinarian:

## C. All health, veterinary treatment and surgical records must be available for review by the ACUC, and records should be maintained in close proximity to the animals. If records are not maintained in close proximity to the animals, clearly describe where they will be located and why they must be maintained away from the animals:

## Location of animal records: **Biology 617BF**

13. If euthanasia of any animals is necessary during the project, list the method/agent of euthanasia: (Include dosages where applicable):

At a given time point, mice will be euthanized by isoflurane overdose followed by cervical dislocation.

We chose isoflurane because all previous my pregnancy and exercise studies use isoflurane and it allows us to compare the data with previous results. In addition, we do not want to alter cardiac contractile function due to CO2 inhalation, since CO2 inhalation is commonly associated with hypoxia7.

For the pregnancy study, the group euthanizes at late-pregnancy, the mother will be euthanized same way as above. Since fetuses are not required for study, removal of the uterus with the pups or the pups with the amniotic sac from the dam should ensure rapid cerebral anoxia to the fetus8. However, if at any point the fetuses are allowed to breath, then they will be euthanized by decapitation with surgical scissors. One group of study will be used 21 days after weaning. Thus, the litters will be euthanized by CO2 inhalation followed by cervical dislocation and used for snake food.

We will immediately euthanize the animal by CO2 inhalation followed by cervical dislocation if the animal appears sick. I observed from my previous studies that some female C57Bl/6 mice have a dorsoventral vaginal septum9. It is hard to detect when they are not pregnant, but it was obvious when they reach the full-term. These mice show signs of sick and often die when they deliver pups. All my efforts, I try to exam the mice before pregnancy study begins, but if incidents happen, we will euthanize mice that have a vaginal septum.

Is this method consistent with the recommendation of the 2013 AVMA Euthanasia Guidelines?

Yes X No

If no, provide justification for not following the AVMA Panel’s recommendation:

14. Describe disposition of animals upon completion of the project: Carcasses will be placed in the freezer and disposed by ACS staff, and some euthanized mice will be used for snake food.

15. All individuals that utilize live animals in this project must be appropriately qualified and trained. List personnel that will utilize live animals, including their title/position and describe their training and experience with the procedures used in this project. Give the years of training/experience with each species in this protocol:

 PLEASE NOTE: It is the responsibility of the PI to ensure that all individuals listed below read and understand the final approved AUF, are trained appropriately on procedures included in the AUF, and follow, without deviation, the AUF during the conduct of the project. As assurance to the above, the PI will have each individual listed below sign and initialize a training sheet/signature documentation form which will accompany the final approved AUF. Only signatories of the training/signature documentation form are permitted to work with animals associated with this AUF.

|  |  |  |  |
| --- | --- | --- | --- |
| Name | Tracer Skelton | Title/Position | Graduate assistant |
| Species | Mouse |
| Training | September 6, 2013 |
| Experience | None |

|  |  |  |  |
| --- | --- | --- | --- |
| Name | Taylor Lunsford (Robertson) | Title/Position | URS fellow |
| Species | Mouse |
| Training | September 6, 2013 |
| Experience | None |

|  |  |  |  |
| --- | --- | --- | --- |
| Name | Eunhee Chung | Title/Position | Assistant Professor |
| Species | Mouse |
| Training | September 6, 2013 |
| Experience | Mice and rats since Fall, 2000 in different institutes (University of Wisconsin, Madison; University of Colorado, Boulder) |

16. Investigator Assurance:

I hereby certify that to the best of my knowledge, the statements in this protocol are true and accurate. I further assure Texas Tech University that I am fully aware of our institutional policy, the Animal Welfare Act, the Public Health Service "Guide for the Care and Use of Laboratory Animals," and the "Guide for the Care and Use of Agriculture Animals in Agriculture Research and Teaching" as they pertain to the use of animals in research and teaching. By signing this statement, I am assuring the Animal Care and Use Committee (ACUC) that any and all animal use will be as described in the protocol by trained personnel and in accordance with the above existing policies. Any significant changes in the proposed project or personnel will be submitted in writing by amendment to the IACUC prior to proceeding with any animal use.

 Principal Investigator: Eunhee Chung, Ph.D Date: September 26, 2013

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