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# Effects of Estrogen on Muscle Damage in Response to an Acute Resistance Exercise Protocol

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Creatine Kinase (CK) is used as a measure of exercise-induced muscle membrane damage. During acute eccentric (muscle lengthening) exercise, muscle sarcolemma, sarcoplasmic reticulum, and Z-lines are damaged, thus causing muscle proteins and enzymes to leak into the interstitial fluid. Strenuous eccentric exercise produces an elevation of oxygen free radicals, which further increases muscle damage. Muscle soreness and fatigue can be attributed to this membrane damage. Estradiol, however, may preserve membrane stability post-exercise (Brancaccio, Maffulli, & Limongelli, 2007; Carter, Dobridge, & Hackney, 2001; Tiidus, 2001). Because estradiol has a similar structure to Vitamin E, which is known to have antioxidant properties, and both are known to affect membrane structure, researchers have proposed that estrogen acts as an antioxidant to provide a protective effect on the post-exercise muscle of women (Sandoval & Matt, 2002). As a result, it has been postulated that muscles in women incur less damage in response to an acute strenuous exercise as compared to men. PURPOSE: To determine if circulating estrogen concentrations are related to muscle damage, as measured by creatine kinase activity and to determine gender differences in creatine kinase as a marker of muscle damage in response to an acute heavy resistance exercise protocol. METHODS: 7 healthy, resistance-trained, eumenhorrheic women (23±3 y,

169±9.1 cm, 66.4±10.5 kg) and 8 healthy, resistance-trained men (25±5 y, 178±6.7 cm, 82.3±9.33 kg) volunteered to participate in the study. Subjects performed an Acute Resistance Exercise Test (ARET) consisting of 6 sets of 5 repetitions Smith machine squats at 90% of their previously determined 1-RM. Blood samples were taken pre-, mid-, post-, 1 hour post-, 6 hours post-, and 24 hours post-exercise. Samples were stored at -80ºC until analyzed. Serum creatine kinase was measured using an assay kit from Genzyme (Framingham, MA). Serum estradiol was measured by an ELISA from GenWay (San Diego, CA). Estradiol β-receptor presence on granulocytes was measured via flow cytometry using primary antibodies from Abcam (Cambridge, MA) and PeCy7 antibodies (secondary) from Santa Cruz (Santa Cruz, CA). RESULTS: No significant correlations between estrogen and CK response were found after an acute resistant exercise protocol. Moreover, no significant change in estradiol receptors were expressed on granulocytes after exercise. Creatine Kinase response, however, differed significantly between genders. Men had higher resting CK concentrations throughout all time points. Creatine Kinase response increased significantly after exercise in both men and women ($p=0.008$, $F=9.798$). Men had a significantly higher CK response at 24 hours post exercise than women. A significant condition/sex/time interaction was exhibited in CK response ($p=0.02$, $F=4.547$). Perceived general soreness presented a significant condition, sex interaction ($p=0.01$, $F=9.532$). DISCUSSION: Although no estradiol and CK response correlations were found in response to exercise, a significant difference in creatine kinase activity was present between men and women. This discrepancy of our results and findings in the literature may be due to the high variability between subjects in creatine kinase activity as well as estrogen concentrations. The lack of significance in

change of estradiol receptor expression on granulocytes in response to exercise may be due to intracellular estradiol receptor staining and non-specific gating for granulocytes rather than additional staining for neutrophil markers. Because neutrophils are the initial cells present in the inflammatory response after strenuous exercise, staining for estrogen receptors on this cell type may allow for a better understanding of the effect of estrogen and its hypothesized protective effect against muscle damage. Furthermore, the mechanism of action may include estradiol receptor expression on the muscle fiber itself may play a role in the protective effects of estradiol rather than or in addition to expression on neutrophils. We have shown here that gender differences occur in CK activity as a marker of muscle damage in response to strenuous eccentric exercise, but may not be the result of estradiol concentration or estradiol receptor expression on granulocytes. Other variables should be examined in order to determine the mechanism involved in the difference in creatine kinase as a marker of muscle damage between men and women after heavy resistance exercise.

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# CHAPTER 1:

# INTRODUCTION

During acute eccentric (muscle lengthening) exercise, muscle sarcolemma, sarcoplasmic reticulum, and Z-lines are interrupted, thus causing muscle protein and enzymes such as creatine kinase (CK) to leak into the interstitial fluid. Eccentric exercise results in the greatest strain and muscle damage (Brancaccio et al., 2007; Clarkson & Hubal, 2001).

Creatine kinase is used as a measure of exercise-induced muscle membrane damage. Because of the release of the enzyme follows disruption in the sarcolemma, CK is a useful tool in understanding membrane stability following strenuous exercise (Tiidus, 2001). The highest CK concentrationss are found after eccentric muscular contractions (Brancaccio et al., 2007). High variability, however, has been found to occur between subjects. Individual conditions may affect creatine kinase, including age, gender, race, muscle mass, physical activity, and climatic condition (Brancaccio et al., 2007). Trained individuals present a higher concentration of CK activity compared to untrained individuals (Brancaccio et al., 2007; Clarkson & Sayers, 1999). Interestingly, young adult males have higher basal concentration of CK compared to females (Brancaccio et al., 2007); furthermore, several reports have shown that females have lower CK activity following exercise (Carter et al., 2001; Roth, Gajdosik, & Ruby, 2001; Stupka et al., 2000; Tiidus, 2001; Tiidus, 2003). Estrogen has been hypothesized to cause this gender discrepancy in muscle damage in response to a similar stimulus.

Current research has shown significant negative correlations between estrogen concentration and CK response (Carter et al., 2001; Clarkson & Hubal, 2001; Roth et al., 2001; Tiidus, 1999). Several mechanisms have been proposed for the explanation of this protective effect of estrogen. Estrogen may act as an antioxidant (Roth et al., 2001; Stupka et al., 2000; Tiidus, 1999), a membrane stabilizer (Brancaccio et al., 2007; Carter et al., 2001; Tiidus, 1999; Tiidus, 2001), or via estrogen receptor mediation (Tiidus, 1999)(Tiidus, 2001; Tiidus, 2003).

Strenuous exercise results in an increase in oxygen free radicals in the area of muscle injury. This hostile environment is further aggravated by the inflammatory response characterized by the influx of neutrophils and macrophages in the area, thus leading to damage of the surrounding muscle fibers. This inflammatory response is important in order to remove damaged tissue and stimulate muscle regeneration (Clarkson & Hubal, 2001). Estrogen, however, may act as an antioxidant, attenuating the inflammatory response and decreasing the amounts of radical oxygen species (ROS). Like Vitamin E, a known antioxidant with a similar structure to estradiol, estradiol has a hydroxyl group on its "A" ring (Tiidus, 1999; Tiidus, 2000). Therefore, estrogen provides antioxidant effects though the donation of a hydrogen atom from this hydroxyl group to peroxyradicals (Carter et al., 2001).

Estrogen may also allow for the stabilization of membranes during strenuous exercise through receptor-mediated actions (Tiidus, 1999; Tiidus, 2001) or through

nonspecific interactions with membrane phospholipids (Tiidus, 1999; Tiidus, 2003). By stabilizing the membrane and decreasing membrane fluidity, estrogen also may protect membranes from peroxidative damage (Tiidus, 1999; Tiidus, 2001). The overall effect of estrogen on muscle fibers is that muscle membranes decrease in fluidity and increase in stability during strenuous exercise, an observation that may be indicative of the lower CK response found in women.

The purpose of this study was to characterize gender differences in creatine kinase as a marker of muscle damage in response to an acute heavy resistance exercise protocol. Furthermore, we wished to determine if circulating estrogen concentrations are related to muscle damage, as measured by creatine kinase activity.

# CHAPTER 2

# REVIEW OF THE LITERATURE

## *Abstract*

This article reviews the effects of gender on muscle damage in response to strenuous exercise and seeks to highlight possible mechanisms for this difference between men and women. Eccentric (muscle-lengthening) exercise, such as the squat exercise leads to a high level of skeletal muscle damage. Creatine kinase (CK), an enzyme released into the blood after muscle fiber membrane disruption, has commonly been used as a rapid and less invasive method for determining muscle damage. Studies in both humans and animal models have shown that women have an attenuated CK response after strenuous exercise as compared to males. Although this conclusion has been well described, the mechanism that causes this discrepancy has been evasive. Some animal models using estrogen supplementation have concluded that estrogen levels are responsible for the decreased muscle damage in women. To our knowledge, however, this mechanism has yet to be described in humans. Estrogen, specifically 17-β-estradiol, has been proposed to exert its protective affect against muscle damage in response to exercise through its ability to stabilize muscle membrane phospholipids, its antioxidant characteristics, or as a down-regulator of the inflammatory response (specifically neutrophil infiltration).

## *1. Muscle damage in response to eccentric exercise*

Exercise-induced muscle damage imparts upon the individual a variety of effects that negatively affect further performance. These effects are, most notably, delayed onset muscle soreness (DOMS), disruption of the muscle fiber membrane, release of muscle proteins and enzymes into the blood, an acute-phase immune response, and decreased performance (Stupka et al., 2000). Tiidus (2000) states that these consequences may last for up to ten days after exercise(Tiidus, 2000). Eccentric, or muscle-lengthening exercises, are reported to be the most detrimental to the exercising muscle and require the longest recovery (Clarkson & Hubal, 2001); (Clarkson & Sayers, 1999). Furthermore, weight-bearing training exacerbates this damage (Brancaccio et al., 2007). Because of the emphasis on resistance exercise in sports, understanding the biological mechanism of the body's response to exercise and the differences in that reaction between genders is useful in designing training protocols that would enhance athletic performance.

### 1.1 Creatine kinase as a marker of muscle damage

Strenuous exercise generally causes a disruption of the muscle fiber membrane, the sarcolemma, and the contractile unit, the sarcomere, in general. Creatine kinase ias an enzyme that is located in each sarcomere in the H zone associated with non-contractile proteins. Due to the fact that damage of a fiber must disrupt this middle portion of the sarcomere or CK will not be released, investigators have used this as an indirect marker of skeletal muscle damage. Furthermore, with exercise, the predominant isoform

released is the MM, for BB and MB arising from the brain and heart, respectively, also exist. Because of this damage, muscle proteins and enzymes, including CK, lactate dehydrogenase (LDH), aspartate aminotransferase, and myoglobin (Carter et al., 2001; Clarkson & Sayers, 1999), are leaked into the interstitial fluid and transported into the blood through the lymphatic system. These protein concentrations are then easily and relatively non-invasively tested in the blood. Therefore, CK is theoretically a good indirect indicator of membrane stability and exercise-induced muscle disruption (Tiidus, 2001); (Carter et al., 2001).

Creatine kinase has become a traditional indicator of muscle damage, and studies have characterized the measurement of this enzyme. In accordance with the conclusion that greatest muscle damage occurs after eccentric contraction exercise, the highest CK concentrations are found in prolonged exercise and especially eccentric muscular contractions (Brancaccio et al., 2007). Because CK is initially released into the interstitial fluid, taken up by the lymphatic system, and then released into the blood, the full CK response to muscle damage is delayed. As reviewed by Brancaccio et al. (2007), Hurley et al. (1995) reported that that CK concentration peaked about two-fold above baseline eight hours after strength training. However, in the same review, Hyatt et al. (1998) found that CK peaked at about 96 hours after prolonged exercise (Brancaccio et al., 2007). In another study, Roth et al. (2001) reported that creatine kinase activity began increasing at 24 hours after a unilateral eccentric hamstring exercise and peaked at 72 hours post exercise (their last time point measured) (Roth et al., 2001). Carter et al. (2001) also reported significant increases in CK after a downhill run at 24, 48, and 72

hours post exercise (Carter et al., 2001). Studies have well documented the lag time in CK response and its relation to exercise-induced muscle damage.

However, creatine kinase response can be affected by extraneous variables. CK concentration depends on age, gender, race, muscle mass, physical activity and climatic conditions (Brancaccio et al., 2007). Trained individuals tend to have higher resting concentrations of CK (Clarkson & Sayers, 1999) and significantly higher increases in CK response due to exercise (Brancaccio et al., 2007). In studying creatine kinase response, one must keep in mind a high inter-subject variability (Stupka et al., 2000) and the observation that higher responders (such as trained individuals) have a higher variability in CK activity after exercise (Brancaccio et al., 2007; Carter et al., 2001). The most interesting difference in creatine kinase response (and therefore muscle damage) has been the difference between genders.

## 1.2 Gender differences in exercise-induced muscle damage

Gender differences in muscle damage after strenuous exercise, and, therefore, differences in creatine kinase response, has been well-documented. Males have been shown to have a higher baseline CK activity than females (Brancaccio et al., 2007; Carter et al., 2001; Clarkson & Sayers, 1999; Clarkson & Hubal, 2001; Stupka et al., 2000; Tiidus, 2003). Moreover, after a session of strenuous exercise, the CK response in women is lower than men (Carter et al., 2001; Clarkson & Sayers, 1999; Roth et al., 2001; Stupka et al., 2000; Tiidus, 2001; Tiidus, 2003). Clarkson and Hubal (2001) have

also observed that women are more prone to initial functional losses but have faster recovery rates than men who lost similar initial strength after exercise (Clarkson & Hubal, 2001).

Clarkson and Sayers (1999) reviewed a study conducted using a rat model by Van Der Meulen et al. (1991). Both female and male rats had a significant increase in CK activity after 150 minutes of uphill running; however, male CK concentrations were significantly higher than females. Furthermore, only male rats had a significant increase after 90 minutes of uphill running (Clarkson & Sayers, 1999). Rat models are used extensively in the research of the cause of this gender differential because of the ability to ethically research supplementation and biopsies of exercising muscle. Human studies, though, also portray this discrepancy in CK response to exercise.

Tiidus (2000), in his review, presents the results of Shumate et al. (1979), which described that males had about a six-fold higher increase in creatine kinase activity than females after a moderate intensity running exercise for two hours (Tiidus, 2000). Another study was done using eccentric resistance exercise (leg press and leg extension). These researchers observed a consistant trend differentiating men and women at 48 hours and six days after exercise (Stupka et al., 2000).

Although the differences in muscle damage between men and women have been thoroughly reviewed in both humans and animal models, the question still remains of the

cause of this disparity. A common explanation is that of the presence and effect of estrogen in women.

## *2. Estrogen has protective effects against exercise-induced muscle damage*

Gender differences in creatine kinase activity have been theorized to be caused by estrogen, specifically estradiol. Although still under dispute, estradiol has been shown by many researchers to attenuate CK response after exercise in females versus males (Carter et al., 2001; Clarkson & Sayers, 1999; Clarkson & Hubal, 2001; Roth et al., 2001). Furthermore, Carter et al. (2001) reported that women using a higher estrogen dose in oral contraceptives had a lower CK response following exercise as compared to women taking lower levels of estrogen in their oral contraceptives (Carter et al., 2001). This effect may also be seen at different stages of the menstrual cycle. At mid-luteal phase, women have high circulating estrogen, whereas during mid-follicular phase, estrogen is lower in the system. Researchers have studied the effects of estrogen mainly in rats, but some human studies have also found correlations between estradiol and muscle damage.

### 2.1 Animal models

In many of the *in vivo* rat model studies, researchers have compared male rats supplemented with estradiol, ovariectomized female rats, and normal males and females

in their response to exercise. In a series of studies highlighted by several reviews, Bär, Amelink and collegues (1986, 1988, 1990, 1991) tested the differences between male and female rats, ovariectomized female rats, and male and ovariectomized female rats supplemented with estradiol after a two hour treadmill run. The results showed that CK activity increased about four-fold for male and ovariectomized female rats (estrogen-deficient rats), but that male and ovariectomized female rats pre-treated with estradiol for 21 days prior had less than a one-fold increase in creatine kinase activity post exercise. Moreover, they found that an increase in length of exposure to supplemented estradiol further attenuated the CK response (Carter et al., 2001; Clarkson & Sayers, 1999; Clarkson & Hubal, 2001; Roth et al., 2001; Tiidus, 2000; Tiidus, 2003).

An *in vitro* study also performed by Amelink et al. (1990) was reviewed by Tiidus, stating that an inverse relationship between estrogen and CK release could be seen after electrical stimulation (Tiidus, 1999). These studies using rat models have thoroughly described a link between estrogen and attenuation of muscle damage after exercise.

## 2.2 Human studies

Human studies have also examineed the implications of the interaction between estrogen and exercise-induced muscle damage. Roth and colleagues (2001) examined the effects of muscle damage between women who used oral contraceptives and those who did not. Women who took oral contraceptives, and therefore had a higher concentration

of circulating estradiol, had a lower CK activity following an eccentric hamstring exercise protocol (Roth et al., 2001).

Another study conducted by Carter et al. (2001) also looked at the effects of an eccentric exercise downhill treadmill run on creatine kinase response between eumenorrheic women who did not use oral contraceptives and those who did. Eumenorrheic women were in mid-follicular phase, when estrogen was lowest, and women taking the oral contraceptive were in the mid-luteal phase, when estrogen concentration was the highest; this distinction provided a further differential in circulating estrogen concentration between the groups. The researchers found that at 72 hours after exercise, the CK activity in the eumenhorreic group was significantly higher than the oral contraceptive group (Carter et al., 2001). These results in human studies have led researchers to believe that estrogen does have an affect on exercising creatine kinase concentration in the blood. Nevertheless, a more aggressive exercise regimen may overwhelm this protective effect of estrogen (Tiidus, 2000).

Estrogen has been shown to have a protective effect, as seen through the attenuation of muscle damage in response to exercise. What is left to be discovered is the mechanism in which estrogen provides this effect. Researchers are beginning to analyze this mechanism in order to more fully understand the actions of this vital hormone.

## *3. Estrogen: Potential mechanisms of action*

### 3.1 Membrane stability

Several reviews have hypothesized that different characteristics of estrogen may contribute to the protective effect against muscle damage that has been observed after strenuous exercise. The first of these ideas has suggested that estrogen influences membrane fluidity and membrane integrity. Because membrane disruption is followed by an increase in CK efflux out of the muscle fiber, the stabilization of the sarcolemma by estrogen may cause the correlations seen between estrogen and CK concentrations in the blood (Brancaccio et al., 2007; Carter et al., 2001; Tiidus, 2001).

Several methods of interaction with the sarcolemma have been theorized. First of all, skeletal muscle has a membrane-bound estrogen receptor-$\alpha$ (Tiidus, 2003); therefore, estrogen may exert its effect in a receptor-mediated system (Tiidus, 1999; Tiidus, 2001). On the other hand, estrogen may directly interact with membrane phospholipids and fatty acids, thus optimizing membrane fluidity (Tiidus, 1999; Tiidus, 2003).

Whichever the method, by stabilizing membrane fluidity and thus protecting membrane disruption by stressors such as exercise, estradiol may be suggested to have a direct interaction with attenuating muscle membrane damage as shown through CK release into the blood.

## 3.2 Antioxidant effects

Strenuous muscular exercise leads to an increase in the formation of oxygen free radicals (Tiidus, 1999), peroxidative muscle damage, and oxidative stress (Tiidus, 2000). Tiidus (1999) explains this mechanism in his review. An increase in oxygen free radical formation is due to exercise-induced mitochondrial oxygen consumption, which overwhelms the system. Furthermore, iron may be unavailable to catalyze the formation of these hydroxyl radicals (the most likely cause of oxygen-radical induced muscle fiber damage). These hydroxyl radicals may further interfere with the excitation-contraction coupling in muscles to cause a decrease in force generation and the associating muscular fatigue (Tiidus, 1999).

If estrogen has the ability to decrease this affect, less damage would be incurred post exercise. Barclay and Hansel (1991), as outlined by Tiidus (1999), described that use of an antioxidant—superoxide dismutase—delayed the onset of fatigue in a canine gastrocnemias plantaris muscle *in vitro* (Tiidus, 1999). Estrogen has been hypothesized to work in a similar fashion.

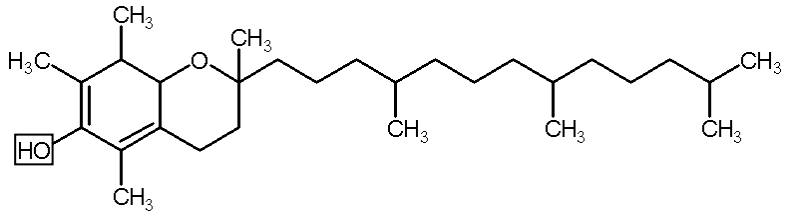
As shown in Figure 1, estradiol and vitamin E, a known antioxidant, have similar structures, with a hydroxyl group on their "A" rings (Tiidus, 1999; Tiidus, 2000). This hydroxyl group can donate the hydrogen atom to stabilize peroxyradicals (Carter et al., 2001); (Tiidus, 2000). Moreover, estrogen as an antioxidant may be more potent when interfering with iron-associated free radical formation (Tiidus, 1999). The membrane-

stabilizing effects of estrogen may also protect membranes from further peroxidative damage, thus decreasing oxidative stress of muscle (Clarkson & Sayers, 1999; Tiidus, 2001).

Tiidus reviewed his own previous work (Tiidus et al. 1993a, Tiidus et al. 1993b, Tiidus and Houston 1993, Tiidus and Houston 1994) on vitamin E depleted rats. Female rats were not as susceptible to oxidative stress after strenuous exercise (Tiidus, 2000), presuming that estrogen may have rendered a protective effect in light of the vitamin E deficiency. A similar effect was not observed in male rats.

The theory of estrogen as an antioxidant appears as a relatively viable explanation for estradiol as the source of a protective effect against muscle damage. Through these antioxidant properties, though, estrogen may also provide an effect on the post-damage inflammatory process seen in post-exercise muscle.

**α-tocopherol (Vitamin E)**




**17-β-estradiol**

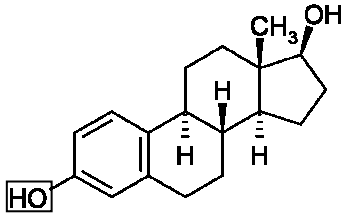



**Figure 1.** Structures of Vitamin E and 17-β-estradiol. Similar placement of hydroxyl group is conducive for antioxidant effects.

## 3.3 Estrogen's role in the inflammatory response

Exercise-induced muscle damage is characterized by an inflammatory response associated with granulocyte (specifically neutrophil and macrophage) infiltration of the damaged muscular tissue. Neutrophils, however, can further damage bystander muscle tissue in an effort to clear damaged tissue and promote repair; they catalyze this process through the formation of more reactive oxygen species through the enzyme NADPH oxidase and myeloperoxidase reactions (Tiidus, 2000). By increasing the amount of damage, inflammation can add to the decreased generation of force by the muscles (Tiidus, 1999). Researchers have speculated that estrogen may attenuate this inflammatory response, thus leading to the gender difference in observed muscle damage and the ability to recover after strenuous exercise.

Studies have shown a lower granulocyte infiltration in the muscles of exercised females than males. Stupka et al. (2000) showed that after an eccentric leg press and leg extension exercise protocol, plasma granulocyte counts increased 48 hours after exercise in men but decreased at the same time point for women (Stupka et al., 2000). This result is an indication of a reduced inflammatory response in women after strenuous exercise.

Estrogen may be the cause of the attenuation in the inflammatory response after exercise (Timmons, Hamadeh, Devries, & Tarnopolsky, 2005). In a study highlighted by Tiidus (2001) by Gregory et al. (2000), estrogen was shown to be the primary cause of a suppressed inflammatory response in females who suffered burn injuries (Tiidus, 2001).

A similar mechanism may be involved in the attenuation of inflammation after exercise in women.

Myeloperoxidase (MPO) activity indicates neutrophil infiltration and has been used as a measure to study the inflammatory response between genders. Tiidus et al. (1999), as described in Carter et al. (2001), presented evidence that two weeks of treatment with estrogen in male rats resulted in an attenuated post-exercise MPO activity (Carter et al., 2001). Furthermore, Tiidus (2001) also reviewed a study by St. Pierre-Schneider and colleagues (1999) in which female mice were found to have a delayed macrophage invasion following eccentric exercise (Tiidus, 2001). Through the use of MPO, estrogen studies in rodents may simulate the mechanism occurring in humans following exercise.

Several mechanisms have been proposed as to the mechanism by which estrogen contributes to the attenuation of the inflammatory response. Tiidus (2003) reports that the attenuation of neutrophil infiltration may be due to estrogen's ability to stabilize the sarcolemma (Tiidus, 2003). By stabilizing the muscle fiber membrane, exercise-induced damage would be reduced, causing a smaller inflammatory response in females.

The inflammatory response after strenuous exercise is theorized to be caused by a disturbed calcium homeostasis in the muscle fiber due to disruption of the membrane. Disruption in calcium homeostasis can activate proteases and phospholipids that can cause increased organelle damage (Stupka et al., 2000). Furthermore, this disruption of

the membrane also causes a degradation pathway beginning with activation of calpain, a non-lysosomal protease (Tiidus, 2001). Calpain then signals for neutrophil infiltration of the damaged muscle tissue (Tiidus, 2003), thus causes the negative effects described previously. Moreover, muscle fatigue occurs as a result of the disrupted calcium homeostasis because the sarcoplasmic reticulum has a decreased ability to uptake calcium in response to stimulus (Tiidus, 1999).

Estrogen, however, may reduce this level of neutrophil infiltration by limiting membrane disruption, thus diminishing calpain activation, and so on. Calcium homeostasis is important in the role of the inflammatory response, and estrogen may mediate this pathway in order to attenuate the response in women after exercise.

## 3.4 Muscle fiber repair

Estrogen may not only have a role in attenuating muscle damage initially following strenuous exercise, but it also may have a role in repair of the muscle fibers. Heat shock proteins play a role in the protein synthesis involved in muscle repair; moreover, these proteins may protect the muscle fibers from subsequent damage (Tiidus, 2003). Thus, a decrease in these proteins is indicative of diminished muscle damage after a strenuous exercise regimen.

Tiidus (2003), in his review, describes that Paroo and colleagues (2002) found that estrogen-supplemented male rodents and estrogen-supplemented ovariectomized

females rodents exhibited a suppression in HSP70 and HSP70 mRNA synthesis after exercise (Tiidus, 2003). These heat shock proteins were not being synthesized because damage to the muscle tissue was diminished. Estrogen was therefore shown to be correlated with the decrease in heat shock protein synthesis.

## *Conclusions*

The gender differences in muscle damage after strenuous exercise has been well documented. However, the reasons for this disparity between men and women are still being disputed. Estrogen appears to be the likely candidate for mediating the response to exercise in women, and this hypothesis is well supported in the literature. First of all, estrogen may be able to stabilize the sarcolemma, thus preventing muscle damage during exercise. Through its membrane-stabilizing and antioxidant effects, estrogen may act to further diminish damage to the muscle tissue created by free radicals produced in response to a high-intensity work out. Finally, estrogen may use these previously noted effects to reduce the inflammatory response, and the secondary damage incurred by neutrophil-mediated increases in reactive oxygen species. Although a number of studies using animal models have substantially characterized these effects of increased circulating estrogen, further human studies are needed to begin to characterize estrogen's suggested protective effects on the female muscle. Appropriately designed studies examining exercise regimens that result in the most acute muscle damage, such as weight-bearing eccentric exercises, and the examination of women in different stages of the menstrual cycle or being administered a variety of oral contraceptives may be

undertaken in order to study the effects of estrogen on human muscle damage in more depth.

# CHAPTER 3

# METHODS

## *Subject Characteristics*

Seven healthy women and 8 healthy men volunteered to participate in the study. Individuals were then medically screened by providing a past health history and were screened by a physician prior to participation. Table 1 shows the descriptive characteristics of each group. Women were significantly smaller and weighted significantly less than men. Men had a significantly higher 1-RM load compared to women ($P < 0.05$). Subjects were resistance trained and women were classified as eumenorrheic (De Souza et al., 1989). Resistance trained was operationally defined as having been involved with a resistance training program at least 3 times a week for the past 6 months. Women were tested in the follicular phase of their menstrual cycle. Participants had no previous musculoskeletal conditions and did not take any hormonal substances; prescribed or over the counters drugs; and were not smokers. This study was approved by the Institutional Review Board at the University of Connecticut. The risks and benefits of the study were explained to each subject and after reading the informed consent document, subjects who wanted to participate in the study signed the documents (Appendix A).

| | Women (n=7) | | | | Men (n=8) | | |
|---|---|---|---|---|---|---|---|
| Body Mass (kg) | 65.60 | ± | 10.01 | * | 82.27 | ± | 9.33 |
| Height (m) | 1.69 | ± | 0.084 | * | 1.78 | ± | 0.067 |
| BMI (kg/m$^2$) | 22.63 | ± | 2.033 | * | 26.09 | ± | 2.207 |
| 1-RM squat (kg) | 75.28 | ± | 13.05 | * | 135.51 | ± | 14.47 |
| Age (y) | 22.13 | ± | 3.09 | | 24.63 | ± | 5.069 |
| Training Experience (y) | 4.7 | ± | 4.4 | | 7.8 | ± | 6.0 |

Values are reported as means ± SD.
*P ≤ 0.05 between women and men

**Table 1.** Subject characteristics between groups.

## *Exercise Protocol*

Subjects performed an Acute Resistance Exercise Test (ARET), consisting of 6 sets of 5 repetitions Smith Machine squats at 90% of their previously determined 1-RM, with a 3 minute rest between each set. Subjects were supervised by a National Strength and Conditioning Association (NSCA) Certified Strength and Conditioning Specialist (CSCS), who is also certified in CPR, First Aid, and the use of an AED.

## *Blood Draws*

Blood samples were taken pre-, mid-, post-, 1 hour post- (+1HR), 6 hours post- (+6HR), and 24 hours post- (+24HR) exercise. Blood samples were obtained through an indwelling Teflon cannula, inserted by a trained phlebotomist into a superficial antecubital forearm vein. About 20 mL of blood was collected into Vacutainer tubes. Tubes were centrifuged for 15 minutes at 1500 g at 4 C, and were aliquoted into plasma/serum eppendorf tubes for storage. Samples were stored at -80 C until analyzed. Blood was unthawed only once. Subjects also completed general soreness scale from one to five at pre-, post-, +6HR, and +24HR time points.

## *Control Condition*

The control condition required the subjects to sit for 2 hours under similar conditions and at the same time of day as the exercise, with blood draws and scale sets provided at the same time points.

## *Creatine Kinase Assay*

Creatine Kinase activities were measured at pre-, +6HR and +24HR. The CK concentrations were determined using a kit from Genzyme Diagnostics (Charlottetown, PE Canada). CK activity was measured on a BioMate spectrophotomer from Thermo

Scientific (Waltham, MA). Each sample was run in duplicate. The coefficient of variation for creatine kinase analyses was < 3% for all samples.

## *Estradiol ELISA*

17-β-estradiol concentrations were measured at pre-, +6HR, and +24HR. An enzyme-linked immunosorbent assay (ELISA) from GenWay Biotech, Inc. (San Diego, CA) was performed in order to measure 17-β-estradiol concentration. We followed the ELISA as outlined in the kit insert, using 50 μL of unknown samples with estradiol conjugated to horseradish peroxidase and anti-estradiol serum in a protein-based buffer in microtitration wells coated with goat anti-rabbit gamma globulin serum. An incubation with substrate tetramethylbenzidine (TMB-$H_2O_2$) followed, and $H_2SO_4$ was used to stop the reaction. Absorbance was read at 450 nm. The sensitivity of this assay was 5 ± 2 pg/mL, and the coefficient of variance was 6.1%.

## *Flow Cytometry*

EDTA-treated blood was used for the quantification of cell types through direct immunofluorescence and flow cytometry. Cells were tagged with antibodies to detect Estradiol β receptor presence on leukocytes. Primary antibodies were from Abcam (Cambridge, MA) and PeCy7 antibodies (secondary) were from Santa Cruz (Santa Cruz, CA). Reagents were purchased from BD Biosciences (Franklin Lakes, NJ). Samples

were stained using the manufacturer's instructions. Compensation corrections were analyzed using compensation beads (Anti-Mous Ig, κ/Negative Control (FBS) Compensation Particles (BD Biosciences)). Flow cytometry data was analyzed using FlowJo software (Ashland, OR). Gating through forward-scatter versus side-scatter characteristics were used to identify granulocyte subsets in FlowJo. Receptor staining was compared by overlaying the histogram plots to the control samples.

## *Statistical Analyses*

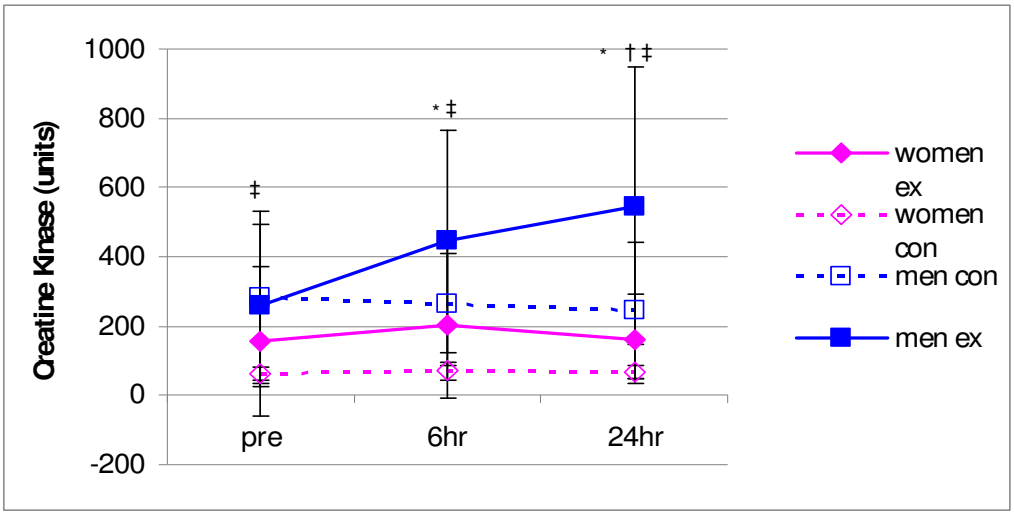
Data are presented as mean ± SD. Data were analyzed using a gender x condition x time repeated measures ANOVA. Fisher's least significant difference post hoc test was used to determine pairwise differences when an *F* score was significant. Mauchly's Test of Sphericity was used to assess homogeneity of variance in the repeated measures design. A Greenhouse-Geisser correct was used if Mauchly's Test was significant. A sample size *n* or 7 was determined to be sufficient to defend the 0.05 alpha level of significance with a Cohen probability level of at least 0.80 for each dependent variable (nQuery Advisor software; Statistical Solutions, Saugus, MA). One-way ANOVA was used to analyze significant between men and women in physical characteristics and the baseline characteristics in exercise and control conditions. $P \leq 0.05$ was considered significant.

# CHAPTER 4

# RESULTS

## *Gender Differences in CK Activity*

The major findings in this study supported the previous literature in that men had a significantly higher basal creatine kinase concentration and women had attenuated CK activity in response to the resistance exercise protocol. As illustrated in Figure 2, both men and women had a significant increase in CK activity in response to exercise 6 hours post and 24 hours post exercise ($P \leq 0.05$). Men had a significant increase in CK activity in response to exercise ($P \leq 0.05$, $F = 4.547$); this increase continued gradually until +24HR. A significantly higher CK response was also shown in men at 24 hours post exercise compared to women ($P \leq 0.05$). Furthermore, basal CK activity was higher for men than women ($P \leq 0.05$).



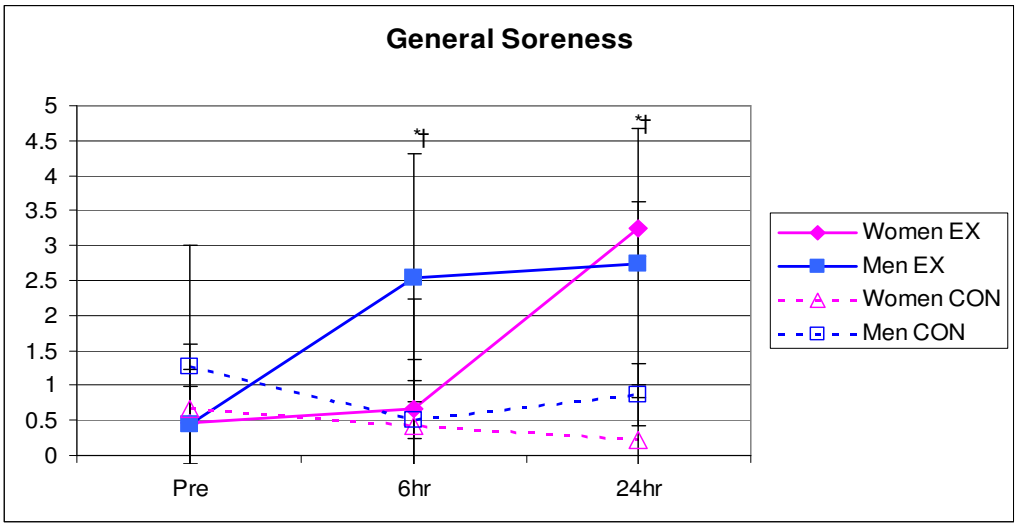

Values are means ± SD

*P ≤ 0.05 between conditions for both men and women
† P ≤ 0.05 between genders after exercise
‡ P ≤ 0.05 between genders at control

**Figure 2.** Creatine Kinase response showed a significant condition x time x sex interaction (p=0.02, F=4.547). Men had significantly higher resting CK levels. CK significantly increased in the exercise condition compared to control in both men and women (p=0.008, F=9.798). Men furthermore had a significantly higher CK response than women at +24HR.

## *Gender Differences in Delayed Onset Muscle Soreness (DOMS)*

Reported general muscle soreness was higher post exercise for both men and women. The exercise protocol resulted in significant muscle soreness over the control condition ($P \leq 0.001$, $F = 89.628$) at 6 hours and 24 hours after exercise (Figure 3). There were no significant correlation between soreness and CK response.




Values are means ± SD
* P ≤ 0.05 vs. corresponding pre-exercise value
† P ≤ 0.05 vs. control condition

**Figure 3.** Perceived general soreness after exercise significantly increased with time (p=0.00, F=17.666). There was also a significant condition x sex interaction (p=0.01, F=9.532).

## *Gender Differences in Estradiol*

No gender differences were found before or after exercise (Figure 4). However, a trend toward a significant difference between genders was shown at basal levels (P = 0.067) and 24 hours after exercise (P = 0.084). There were no significant correlations between estradiol concentration and CK response (Figure 5).

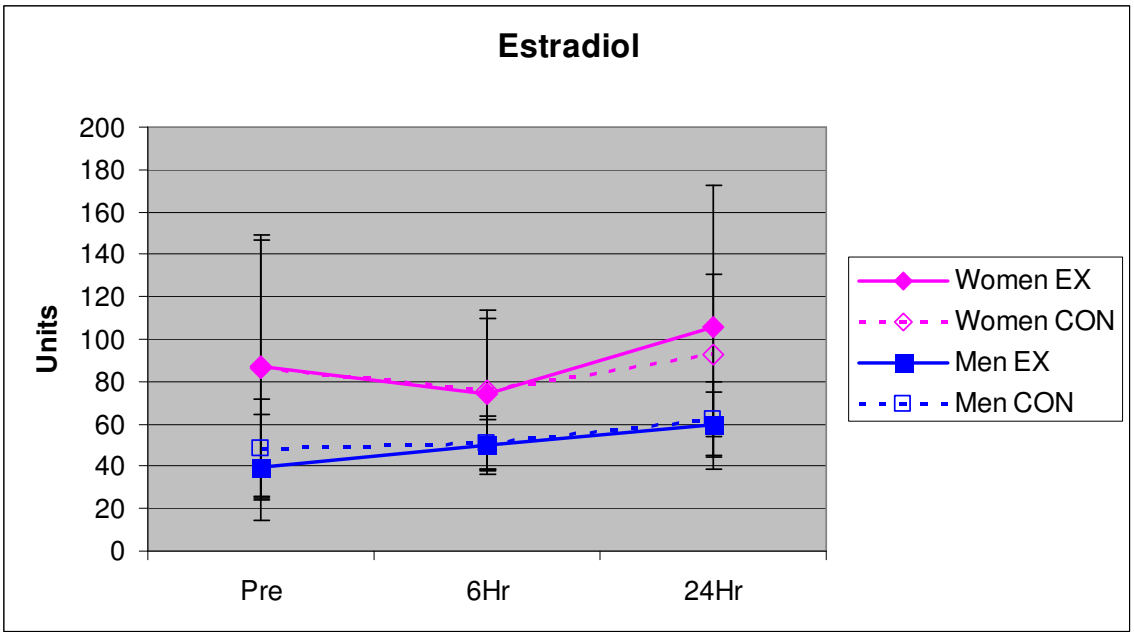



**Figure 4.** No significant gender differences of estradiol at basal levels or after exercise.

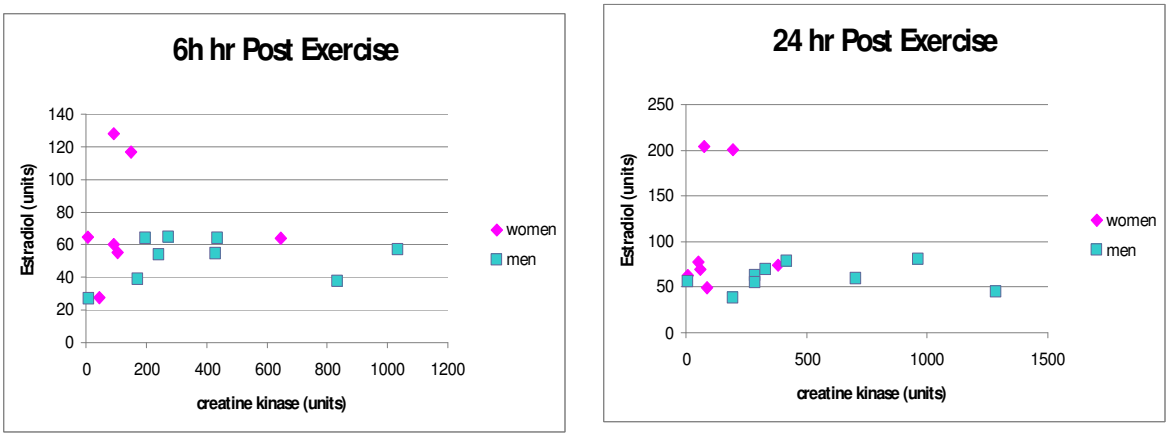



**Figure 5.** No significant correlations were found between Estradiol and CK in response to exercise at either +6HR or +24HR.

## *Estrogen Receptor β Expression on Granulocytes*

No significant differences were shown between men and women in the expression of estrogen receptor β on granulocytes in the blood (Figure 6). Furthermore, exercise had no effect on the expression of estrogen receptor β on granulocytes. Gating through the software was used to differentiate granulocytes (Figure 7).

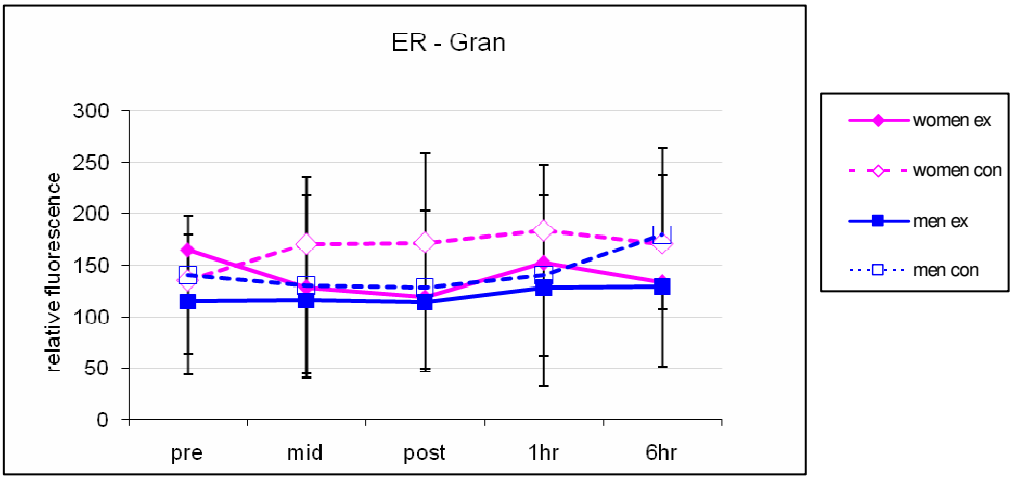



**Figure 6.** No significant changes in estradiol receptor expression in response to exercise were found up to 6 hours post exercise.

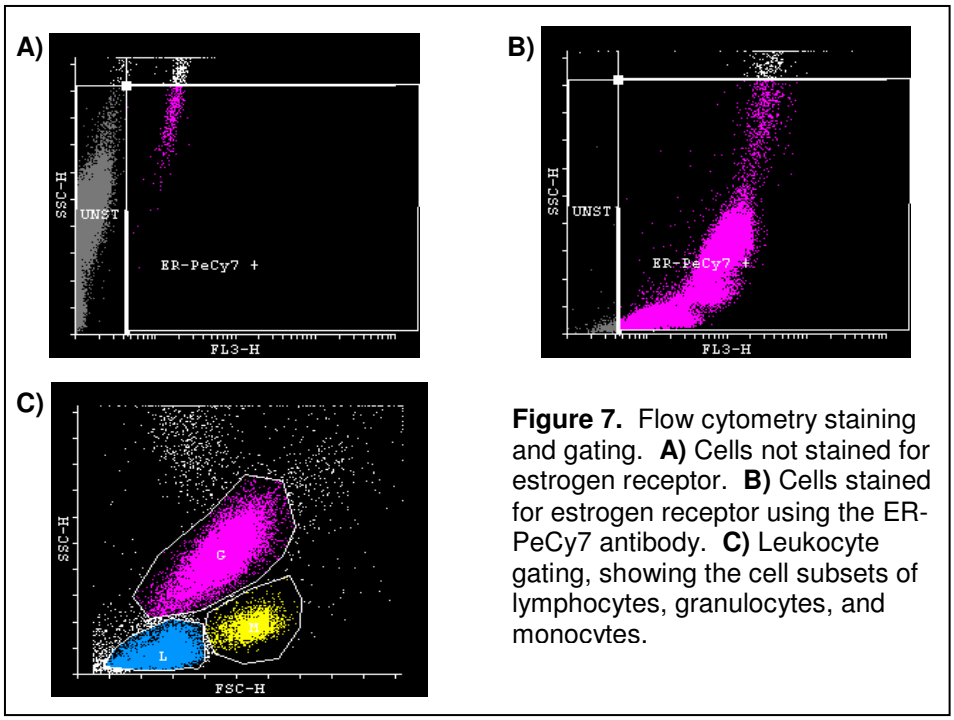



**Figure 7.** Flow cytometry staining and gating. **A)** Cells not stained for estrogen receptor. **B)** Cells stained for estrogen receptor using the ER-PeCy7 antibody. **C)** Leukocyte gating, showing the cell subsets of lymphocytes, granulocytes, and monocvtes.

# CHAPTER 5

# DISCUSSION

When muscle is damaged by exercise, the muscle fiber membrane is disrupted. This damage to the sarcolemma could result in a release of proteins and enzymes into the interstitial fluid, including CK, lactate dehydrogenase (LDH), aspartate aminotransferase, and myoglobin (Clarkson & Sayers, 1999). Therefore, creatine kinase is a useful and less-invasive measure of membrane disruption and further muscle damage.

The major finding in this study supports the current literature in that women have an attenuated creatine kinase response to strenuous exercise, especially eccentric exercise. Previous research has led to a dispute in the significance of gender differences in CK response, and its feasibility to accurately depict the amount of muscle damage that occurs due to exercise (Stupka et al., 2000).

To our knowledge, few studies have attempted to characterize gender difference in humans in CK response after resistance exercise (Sewright, Hubal, Kearns, Holbrook, & Clarkson, 2008; Stupka, Tarnopolsky, Yardley, & Phillips, 2001). Stupka, et al. reported that women had lower concentrations of CK at all time points than men after eccentric leg press and leg extension exercises (Stupka et al., 2001). A review by Clarkson and Hubal (2001) illustrates studies that have further shown an attenuation in creatine kinase activity in women in response to aerobic exercise, downhill running, and high-force eccentric exercise (Clarkson & Hubal, 2001).

Creatine kinase, however, has high inter-subject variability in response to strenuous exercise ((Stupka et al., 2000), an observation that was reproduced in our study. This high variability may have had an affect on significance between genders, time points, and conditions, and its correlation with estradiol.

The purpose of this study was to describe the cause of gender difference in creatine kinase activity in response to an acute resistance exercise protocol. Although we did not find significant correlations in creatine kinase activity and 17-β-estradiol concentration, several studies and reviews have described that increased estradiol results in an attenuated CK response following muscle-damaging exercise (Carter et al., 2001; Clarkson & Sayers, 1999; Clarkson & Hubal, 2001; Roth et al., 2001; Tiidus, 1999; Tiidus, 2000; Tiidus, 2003). Furthermore, rats supplemented with estrogen showed a decreased creatine kinase leakage following eccentric exercise (Clarkson & Sayers, 1999; Tiidus, 2000). A high inter-subject variability and a low n size might have contributed to this result. On the other hand, this lack of correlation may be due to the intensity of the resistance exercise protocol. A more aggressive exercise, such as the one investigated in this study, may overwhelm the initial protective effects of estrogen (Tiidus, 2000).

Estrogen may have a protective effect on muscle tissue following strenuous exercise. Thus, muscle damage may be attenuated and creatine kinase concentrations lowered because of this interaction. Estrogen has been hypothesized to protect muscle through its antioxidant properties (Carter et al., 2001; Stupka et al., 2000; Tiidus, 1999;

Tiidus, 2000; Tiidus, 2003) and its ability to stabilize plasma membrane fluidity through direct interaction with phospholipids (Tiidus, 1999; Tiidus, 2003) or estrogen receptors(Tiidus, 1999; Tiidus, 2001).

Exercise produces an increase in reactive oxygen species in the area of muscle damage. Further inflammatory processes, including neutrophil infiltration into the muscle, exacerbates this already hostile environment, causing further muscle damage following by a muscle regeneration process. Estrogen may act as an antioxidant by donating a hydrogen atom from the hydroxyl group located on its phenyl group (Carter et al., 2001), thus attenuating further muscle damage and release of proteins and enzymes such as CK.

Another protective effect of estrogen may be its ability to attenuate an inflammatory response following strenuous exercise. The inflammatory response is used for removal of debris and stimulation of muscle regeneration (Clarkson & Hubal, 2001) and is characterized by infiltration by neutrophils and macrophages (Tiidus, 2000). Tiidus, et al. (1999) reported that rats treated with estrogen for two weeks had a decreased post-exercise myeloperoxidase (MPO) activity, which indicates the infiltration of neutrophils (Tiidus & Bombardier, 1999). Therefore, estrogen caused a decrease in the inflammatory response through its interaction with neutrophils.

Because of this proposed system, we looked at estrogen receptors (ER) on granulocytes. However, we did not find significant changes between estradiol expression

before and after exercise. These results may be due to a non-specific gating of granulocytes. Future studies should examine anti-neutrophil antibodies and anti-ER-β antibodies to specifically test the significance of estrogen receptor expression specifically on neutrophils.

This study provided evidence for gender difference in muscle damage in response to an acute, strenuous resistance exercise protocol. Although no correlation between creatine kinase concentration and estrogen were observed, the study may be used as a starting point for the structure of subsequent studies using a resistance exercise protocol. Women should be analyzed at different phases of the menstrual cycle, as determined by composite hormonal concentrations, to test during significantly different estrogen concentrations between males and females. Creatine kinase concentration should be analyzed up until 48 hours post exercise, as CK concentration continue to increase after 24 hours post exercise. Furthermore, neutrophils should be specifically selected for in conjunction with estradiol receptors during flow cytometry analysis. Muscle fibers should also be analyzed for estradiol receptor expression. Although the observation that creatine kinase concentrations are significantly different between genders has been well-established, further investigation is needed to determine the mechanism that surrounds this effect of strenuous exercise.

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# APPENDIX A