THE EFFECT OF ESTROGEN ON INFLAMMATORY MARKERS FOLLOWING
PROLONGED AEROBIC EXERCISE IN EUMENORRHEIC WOMEN

Elizabeth A. Walz

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in
partial fulfillment of the requirement for the degree of Masters of Arts in the Department
of Exercise and Sports Science (Exercise Physiology).

Chapel Hill
Spring 2014

Approved by:

Anthony C. Hackney

Abbie Smith-Ryan

Alain Aguilar

ABSTRACT

Elizabeth A. Walz: The Effect of Estrogen on Inflammatory Markers Following
Prolonged Aerobic Exercise in Eumenorrheic Women
(Under the direction of Anthony C. Hackney)

The study purpose was to determine if estrogen (E_2) concentrations attenuate inflammation after exercise-induced muscle damage. Blood responses of pro-inflammatory cytokine biomarker TNF-α, and pro- and anti-inflammatory cytokine biomarker IL-6 were measured. Ten, eumenorrheic, endurance-trained women (Mean±SD; 21±1 years, 24.1±2.8 body fat%) were studied. They completed a 60 minute running protocol at ~60-65% of their oxygen uptake (VO$_{2peak}$ 53.5±4.7ml/kg/min) during two hormonal conditions (low E_2 and high E_2). Inflammation was assessed at rest, immediately post exercise, 30 minutes post exercise, and 24 hours post exercise. There was not a significant interaction effect for TNF-α (p=0.60). There was a significant interaction effect for IL-6 (p=0.001). The response at 30 minutes post exercise was significantly elevated from rest and significantly reduced in high E_2. Results suggest high E_2 conditions attenuate the IL-6 response. Due to the pro- and anti-inflammatory influence of IL-6, it is unclear whether this attenuation is positive or negative.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER I

INTRODUCTION

Basis For Study:

There has been debate surrounding the influence of 17β-estradiol (E_2) on skeletal muscle, and whether E_2 has a protective influence on skeletal muscle damage, inflammation, and repair after prolonged aerobic exercise. This area of research is limited. There are multiple studies indicating that high levels of E_2 attenuate circulating cytokines, and as a result attenuate inflammation during non-exercise induced inflammatory responses (Pfeilschifter et al., 2002; Pottratz et al., 1994; Puder et al., 2001; Schwarz et al., 2000). This indicates that there may be a similar phenomenon occurring during exercise induced inflammatory responses. A clear relationship between E_2 and inflammation after exercise induced muscle damage, in women, is not well defined in existing literature. Some studies suggest there is an estrogenic effect on circulating cytokines, while other do not (Dieli-Conwright et al., 2009; Ives et al., 2011; Timmons et al., 2005; Timmons et al., 2006). These conflicting results may be due to study limitations, such as small sample sizes, lack of a treatment condition, or inadequate exercise stimuli (Ives et al., 2011). The intent of the current study was to overcome these limitations, with sufficient power, a treatment condition, and adequate exercise stimulus. Unfortunately, due to the difficulty of recruiting subjects, the study was underpowered for TNF-α (β= 0.65). Evaluating the influence of E_2 fluctuations across the menstrual cycle, and how this impacts skeletal muscle, will add to this body of knowledge, and is an

1

important consideration for women designing training programs to improve performance and minimize risk.

E_2 is a steroid hormone-molecule that plays an important role in maintaining and regulating sexual and reproductive function in females. Additionally, E_2 exerts an influence on other physiological systems in females, such as the cardiovascular, musculoskeletal, immune and central nervous systems. The form of E_2 that has the largest effect on these systems is 17β-estradiol (Enns & Tiidus, 2010). There are three mechanisms by which E_2 might exert a protective effect on skeletal muscle damage, inflammation, and repair. E_2 may act as an antioxidant, membrane stabilizer, and gene regulator (Perskey et al., 1999). Similar to other antioxidants with similar structures, it has been suggested that the phenolic hydroxyl group on E_2 donates a hydrogen atom which disrupts free radical damaging cascades, minimizes lipid peroxidation and thus limits cell membrane damage (Perksy et al., 1999; Sugioka et al., 1987). E_2 may also stabilize membranes by intercalating with membrane phospholipids, similar to cholesterol (Perksy et al., 1999). Finally E_2 may also exert protective effects through gene regulation, affecting cytokine and cell-adhesion activity, as well as activation of satellite cells (Smith et al., 2000; Enns et al., 2008; Enns & Tiidus, 2008). With these protective properties, E_2 may attenuate exercise-induced muscle damage, and inflammation while also facilitating repair in skeletal muscle.

While exercise-induced oxidative stress and membrane damage are important signals for the skeletal muscle tissue to adapt, there are potential negative side-effects, such as reduced muscle strength, muscle soreness, and increased creatine kinase, as well as increased β-glucoronidase activity and histologic damage of the cell (Clarkson et al.,

2001; Komulainen et al., 1999). E_2 may play a role in decreasing the severity of these side-effects during high intensity exercise (Bar et al., 1988; Roth et al., 2000, Komulainen et al., 1999). After exercise induced muscle damage, inflammatory processes are activated to clear and repair the damaged cell. These processes include an infiltration of fluid, plasma proteins and circulating leukocytes, which is mediated by a variety of cytokines (St. Pierre Schneider et al. 1999; Tiidus et al. 2001). The cytokines of interest in this study are tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6). TNF-α is considered to be a pro-inflammatory cytokine, produced to recruit more leukocytes to the inflamed tissues. IL-6 has been shown to be both a pro- and anti-inflammatory cytokine. Until recently, researchers considered IL-6 to be a pro-inflammatory cytokine. For example, MacIntyre et al. (2001) reported that neutrophil and IL-6 increased up to 6 h post-exercise, and there was a significant relationship between IL-6 and DOMS, suggesting that IL-6 is a pro-inflammatory cytokine that initiates inflammation. It is thought that anti-inflammatory IL-6 may be released locally in the muscle after strenuous exercise and the amount released is dependent on the type of physical activity, as well as the duration of exercise (Hamer & Karageorghis 2007). Currently, the literature is unclear as to the exact role this cytokine plays in the inflammatory response (Pedersen et al., 2004; Smith et al., 2000).

Some researchers have suggested that E_2 may positively influence inflammation by attenuating pro-inflammatory cytokine TNF-α, reducing leukocyte activity and collateral damage to healthy cells, as well as initiating satellite cell activity (Tiidus et al., 2001). Satellite cells activation initiates cell growth and repair. An estrogenic effect on satellite cell activation was shown by Roth et al. (2001) where resistance trained women

displayed a greater increase in satellite cell number when compared to men. Additionally, Enns et al. (2008) demonstrated that satellite cell activity increased in E_2 supplemented, exercised rats compared to other controls. The Enns study also suggests that E_2 may increase satellite cell activation through receptor-mediated mechanisms (Enns et al., 2008). As evidenced here, there are strong reasons to believe that E_2 exerts a protective effect on exercise induced muscle damage, inflammation, and repair.

The present study focused on inflammatory mechanisms after exercise induced skeletal muscle damage; specifically the effect of E_2 on inflammatory markers, TNF-α and IL-6. Since IL-6 has been shown to have both anti- and pro-inflammatory properties, it is not primary variable of interest. It was included to add to investigations previously produced from this research group. This particular study focused on the effect of E_2 on TNF-α during the inflammatory response after an exercise induced muscle damage bout. Ostrowski et al. (1999) reported that strenuous exercise increased concentrations of TNF-α. Chao et al. (1995) found that TNF-α fluctuated with changes in estrogen concentrations. Additionally Schwarz et al. (2000) found that the release of TNF-α was diminished in pre-menopausal women during the luteal phase of the menstrual cycle, when compared to the follicular phase, which suggests an anti-inflammatory response. This author also found that TNF-α and IL-6 were inhibited in females, compared to male controls. These studies provide evidence that there may be a relationship between E_2 and inflammatory markers. Thus with this in mind, a key goal of the present study was to clarify the role of E_2 on TNF-α after exercise induced muscle damage. In this study, the primary outcome variable of interest was TNF-α, and the secondary variable of interest

was IL-6; each was assessed at four time points (at rest, immediately post-exercise, 30 minutes post-exercise, and 24 hours post exercise).

Purpose

The purpose of this study was to determine if E_2 levels influence pro-inflammatory marker TNF-α following a moderate intensity exercise protocol in eumenorrheic women.

A secondary purpose of this study was to determine if E_2 levels influence pro- or anti-inflammatory cytokine IL-6 following a moderate intensity exercise protocol in eumenorrheic women.

Research Hypothesis

If E_2 is related to pro-inflammatory cytokine TNF-α, then higher E_2 levels in eumenorrheic women will attenuate TNF-α concentrations after a moderate intensity exercise protocol.

A secondary research hypothesis is if E_2 is related to pro- or anti-inflammatory cytokine IL-6, then higher E_2 levels in eumenorrheic women will attenuate IL-6 concentrations after a moderate intensity exercise protocol.

Definition of terms

Cytokines

Cytokines are released in response to stress, infection, illness and inflammation, as part of the innate immune response of the human body. Cytokines mediate immune responses activated in order to repair and clear damaged cells. Cytokines can be pro- or anti-inflammatory (Corwin 2002; Pederson et al., 1998; Pederson et al., 2004).

Delayed Onset Muscle Soreness (DOMS)

Following high intensity exercise, muscle injury can cause myofiber damage, including sarcolemma disturbances, swelling or disturbances of the contractile proteins, cytoskeletal and extracellular matrix damage and disturbance (Kendall & Eston, 2002).

Estrogen (E_2)

18-carbon steroid molecule-hormone, secreted by the ovaries in women and (to a much lesser extent) in the testes in men. E_2 is important in the maintenance of normal sexual and reproductive function in females, but also plays a role in many cardiovascular, musculoskeletal, immune and central nervous system functions. E_2 exists in several forms, the most prominent is estradiol β-17, and is known to fluctuate across the menstrual cycle (Ruggerio & Likis 2002; Enns & Tiidus 2010).

Eumenorrhea

Normal menstrual cycle, typically seen in women ages 18-30 years, in which cycles establish an early follicular phase increase in follicle-stimulating hormone, a pre-ovulatory luteinizing hormone peak, a luteal phase of at least 11 days, and a progesterone peak greater than 10ng/mL (Sherman & Korenman, 1975). A typical menstrual cycle lasts 28 days.

Moderate intensity prolonged exercise bout

Exercise performed on a treadmill at 0% grade with a speed equal to ~60-65% of VO_2max for 60 minutes. Previous investigations from the same research group indicate this exercise to be a high enough intensity to elicit muscle damage (Hackney et al., 2012).

Inflammatory Response

The inflammation process and satellite cell activation and proliferation is initiated by local and systemic signals, such as cytokines, growth factors and leukocytes, released by the injured muscle tissues (Hamer & Karageorghis, 2007).

Leukocytes

During the inflammatory process after muscle damage leukocytes, such as neutrophils and macrophages, accomplish three tasks: breakdown damaged muscle tissue, remove the damaged muscle tissues, and restore function of muscle tissues (Kendall & Eston, 2002).

Delimitations

1. Participants were healthy females between the ages of 18-30 years.

2. Participants were eumenorrheic and not currently taking oral contraceptives or other hormone therapy six months prior to participation in this study.

3. Had not sustained an injury within the last six months that limited the ability to exercise or have a doctor's clearance.

4. Had not been taking anti-inflammatory medicines, such as ibuprofen, naproxen, or aspirin six months prior to participation in this study.

5. Were not pregnant or become pregnant during the study.

6. Become ill with an immune responding condition, such as a cold or respiratory infection during the study.

7. Had a current minimum training volume of 3-5 days a week, 45-120 minutes per session of aerobic activity, and a maximal oxygen consumption (VO_2 max) of at least 45 ml/kg/min.

8. Abstained from strenuous physical activity and maintained a diet similar in calories and carbohydrate content 24 hours prior to experimental protocol.

Limitations

1. Results may not be applicable to men and some women (amenorrheic, oligomenorrheic, or post-menopausal).

2. Subjects may not comply with specific pre-test instructions.

3. Cytokine concentrations will be measured in blood, there will not be another method to verify local cytokine changes (such as muscle messenger RNA [mRNA]).

Significance of study

Understanding the influence of E_2 on the inflammatory response following muscle damage is an important consideration for both women and researchers. Women who experience amenorrhea or are post-menopause may be missing the potential protective influence of E_2 related to muscle damage, inflammation, and repair. Additionally women who are eumenorrheic would benefit from knowing the potential protective properties of estrogen. This information could be used to design training programs that optimize performance and minimize risk, such as periodization of training based on hormonal condition. While there are many important training considerations for understanding estrogenic influence, it is especially crucial to account for estrogenic influence when it comes to designing research studies involving women. Failing to account for hormonal fluctuations throughout the menstrual cycle may affect the results of these studies. Lastly, research in this area is limited and results are contradictory. More research needs to be done in this area to clarify the role of E_2 on the inflammatory response after exercise-induced muscle damage.

CHAPTER II

REVIEW OF LITERATURE

In this review, the influence of E_2 on skeletal muscle damage, inflammation, and repair will be examined. There are three potential mechanisms by which E_2 exerts a protective effect on skeletal muscle. E_2 may act as an antioxidant, membrane stabilizer, and gene regulator (Kendall & Eston, 2002; Enns and Tiidus, 2010). It is a well-documented phenomenon that muscle damage occurs after strenuous, unaccustomed exercise (Clarkson et al., 2001). Following exercise-induced skeletal muscle damage, pro- and anti-inflammatory cytokines and other chemo-attractants facilitate inflammation and repair. This response involves the recruitment of leukocytes, such as neutrophils and macrophages, and the activation and proliferation of satellite cells (Belcastro et al., 1998). This review will discuss how E_2 exerts protective mechanisms on skeletal muscle, the effect of E_2 on pro-inflammatory cytokine TNF-α, and the pro- or anti-inflammatory cytokine IL-6 after exercise-induced muscle damage.

Antioxidant, membrane stabilizing and gene regulating properties of E_2:

Antioxidant properties of E_2

It is well known that during strenuous exercise oxygen consumption increases to meet metabolic demand. With this increase in oxygen consumption, there is a similar increase in free radical production. It is estimated that with every 25 oxygen molecules

9

reduced by oxidative metabolism, there is one free radical produced (Kanter 1998). Thus oxygen free radicals may rapidly accumulate during strenuous exercise and result in oxidative damage, production of reactive oxygen species (ROS), and lipid peroxidation, all of which alter membrane fluidity and cell membrane stability (Sen 1995). In addition to free radicals produced during oxidative metabolism, free radicals can be produced as a result of enzyme activity with the recruitment of neutrophils in the inflammatory response (Fantone et al., 1982). There is a link between excess free radicals, due to either over-production or a decrease in the effectiveness of antioxidants, and developing diseases, such as cancer, atherosclerosis, and Alzheimer's (Persky et al., 1999).

Endogenous production of antioxidants, such as E_2, serves to protect skeletal cell membranes from free radical damage. More specifically, the phenolic hydroxyl group on estrogen donates a hydrogen atom to disrupt free radical damaging cascades, minimize lipid peroxidation and thus limit cell membrane damage (Perksy et al., 1999; Sugioka et al., 1987). Both E_2 status in females and intensity of exercise affect lipid peroxidation. Ayers et al (1998) evaluated the difference between eumenorrheic (measured during high E_2) and amenorrheic athletes' responses to exercise-induced oxidative stress. These authors found that there was a greater potential for lipid peroxidation after 15 minutes of maximal treadmill exercise for amenorrheic athletes when compared to eumenorrheic athletes. Additionally, Feng et al. (2004) found that physiological levels of E_2 could increase membrane stability, reduce consumption of glutathione (GSH) and Vitamin E (both of which are antioxidants), and maintain overall antioxidant capability of the strained muscles in female rats. Additionally the results showed that there was decreased muscle injury and increased muscle regeneration after an acute strain injury in female rats.

These studies highlight how skeletal muscle cells might be susceptible to free radical damage and how E_2 may exert protective effects through direct antioxidant actions.

Membrane stabilizing properties of E_2

E_2 may also be able to exert protective effects through membrane stabilizing mechanisms. The ability of fat-soluble E_2 to interact with phospholipids also contributes to the membrane stabilizing properties of E_2, similar to cholesterol (Whiting et al., 2000). Whiting et al. (2000) studied the effect of testosterone, progesterone, and E_2 on various liposomes, plasma membranes, and sarcoplasmic reticulum membranes. This group suggests that E_2 has the ability to intercalate with phospholipids, alter the fluidity of phospholipids, and increase protein mobility in membrane bilayers, all of which may affect protein function. Although the authors in this study found E_2 to increase membrane fluidity, they demonstrated a mechanism by which steroid hormones influence these actions. Multiple researchers have suggested the opposite, that E_2 decreases membrane fluidity and stabilizes membrane phospholipids due to its structure and antioxidant ability (Kendall & Eston 2002; Persky et al., 1999). Nonetheless, E_2 may play an important role in the stabilization of skeletal cell membranes and the conflicting findings show the need for further research in this area.

Gene regulating properties of E_2

Lastly, E_2 may potentially exert protective effects on skeletal muscle through gene regulation. Gene regulation by E_2 may affect cytokine and cell adhesion activity, as well as the activation of satellite cells. Research involving another known antioxidant, tocopherol, has shown that tocopherol regulates nuclear factor kappa B, which is a regulator of cytokines and cell adhesion molecules. Yoshikawa and Yoshida (2000)

suggested that tocopherol prevents signal transduction of leukocyte-endothelial cell adhesion. Cytokine production and leukocyte-endothelial cell adhesion are important factors regulating leukocyte infiltration. In another study Enns et al. (2008) demonstrated that E_2 may increase muscle satellite cell numbers through E_2 receptor mediated mechanisms, indicating upstream gene regulation of satellite cell activation. Furthermore these authors suggested that the attenuation of exercise-induced muscle damage and leukocyte infiltration via estrogenic effects was not mediated by E_2 receptor mechanisms (Enns et al., 2008; Enns & Tiidus, 2008). This supports the findings by Yoshikawa and Yoshida, who suggested that the attenuation of exercise-induced muscle damage and leukocyte infiltration may be attenuated via estrogenic regulation of endothelial cell adhesion and leukocyte infiltration. The ability of E_2 to decrease leukocyte infiltration and increase satellite cell activation may be due to the gene regulating properties of E_2.

Estrogenic effect on exercise-induced skeletal muscle damage, inflammation and repair

Skeletal muscle damage

Skeletal muscle damage caused by strenuous, unaccustomed exercise can be measured directly or indirectly. It can be directly measured via muscle biopsies, or indirectly measured through muscle strength loss, muscle soreness, and increased muscle proteins, such as creatine kinase (Clarkson et al., 2001). Additionally, the protein β-glucoronidase activity can reflect the histopathological state of the cell, which indicates if muscle damage results from the inflammatory response (Komulainen et al., 1999).

As previously mentioned, E_2 may have the ability to attenuate skeletal muscle damage by acting as an antioxidant and membrane stabilizer. As a result of exercise induced oxidative stress and membrane damage, more creatine kinase is able to permeate the membrane and elicit a larger inflammatory response (Bar et al., 198; Sewright, 2008, Roth et al., 2000). Bar et al. (1988) suggested that E_2 may have a protective effect on skeletal muscle damage by showing reduced creatine kinase values after exercise induced stress. In a more recent study, Sewright (2008) hypothesized that there would be similar responses between men and women regarding indirect skeletal muscle damage markers, but there would be sex differences in the variability and distribution of indirect skeletal muscle damage markers. These authors found that women experienced greater immediate strength loss, while men showed greater creatine kinase activity. These results indicate sex differences in fatigue and muscle damage after intense exercise. Sex differences in fatigue may be due to metabolism, blood flow or intracellular calcium, while sex differences in muscle damage may be due to estrogenic protective mechanisms. Additionally, Roth et al. (2000) suggests that E_2 levels in women influences the degree of muscle damage after heavy-resistance strength training three times a week for nine weeks. Muscle damage increased significantly in older women, from 5 to 17% of muscle fibers damaged, compared to younger women, from 2 to 5% of muscle fibers damaged.

Taking a different approach to evaluating skeletal muscle damage differences between males and females, Komulainen et al. (1999) evaluated β-glucoronidase activity, histological assessment of muscle samples for inflammation, and immunohistochemistry of structural proteins of muscle fibers, such as actin, desmin, and dystrophin, and extracellular matrix proteins, such as fibronectin, in rats after eccentrically biased

downhill running exercise. β-glucoronidase activity was smaller and histological changes were slower and less prominent in female rats compared to male rats. Additionally, immunohistochemical changes in structural and extracellular matrix proteins were unchanged in female rats when compared to male rats.

Both rodent and human studies suggest that the skeletal muscle cell membranes in females compared to males, are stronger and better able to resist exercise-induced skeletal cell membrane damage. This may be due to a protective estrogenic effect on skeletal muscle cells following damaging exercise.

Acute inflammatory response

To clear and repair skeletal muscle tissue after an acute bout of high intensity exercise there is an acute inflammatory response in which there is an increase in fluid, plasma proteins and circulating leukocytes. Vascular endothelial cells, tissue-resident leukocytes, and circulating leukocytes produce a variety of cytokines that mediate the inflammatory response. For example, the up-regulation of pro-inflammatory TNF-α is associated with resident macrophages in the damaged muscle tissue (Smith et al., 2000). Interestingly IL-6 can be either pro- or anti-inflammatory, depending on how much of it is released or what is being released along with it. Thus pro-inflammatory IL-6 may be associated with resident macrophages, while anti-inflammatory IL-6 may be associated with the exercising muscle tissue, specifically substrate mobilization (Pederson et al., 2004). There are multiple families of cytokines that play a role in regulating the acute inflammatory response, including; interleukins (IL), tumor necrosis factors (TNF), interferons, growth factors, colony stimulating factors (CSFs), and cell adhesion molecules (CAM) (Smith et al., 2000). While TNF-α and IL-6 are the main cytokines of

interest in this study, it is important to understand there are many cytokines that play a crucial role in inflammation.

The infiltration of leukocytes, primarily neutrophils and macrophages, remove damaged muscle tissues and stimulate repair processes. St. Pierre Schneider et al. (1999) induced skeletal muscle injury using an in vivo lengthening contraction model in 50 sexually mature mice. Leukocyte infiltration was assessed after 1, 3, 5, and 7 days of recovery. While leukocytes invaded muscle fibers in both sexes after 1 day, there were differences in the subsets of leukocytes between the sexes. The authors concluded that leukocyte activity associated with the inflammation may be prevented or delayed in female mice after exercise induced injury. Both neutrophils and macrophages play a crucial role in removing and repairing damaged muscle tissues. While this activity is necessary, excessive infiltration of leukocytes can cause an increase in muscle membrane and oxidative damage. It is still unknown whether E_2 enhances or hinders the inflammation process. E_2 may positively influence inflammation by reducing leukocyte activity, and thus reducing oxidative damage and collateral damage to healthy cells. However, estrogenic influence that reduces inflammation may result in a diminished ability to repair damaged muscle tissue (Tiidus et al., 1999, Tiidus et al., 2001).

With exercise induced muscle damage there are disruptions to the sarcomere Z line and sarcoplasmic reticulum, resulting in changes in calcium concentrations and the rate of protein degradation. Calpain is a non-lysosomal cysteine protease that degrades the damaged cytoskeletal and myofibrillar proteins. Protein degradation, induced by Calpain activity, produces peptide fragments that act as chemo-attractants to neutrophils (Belcastro et al., 1998). E_2 may reduce neutrophil infiltration, acting as an antioxidant

15

and membrane stabilizer, by reducing sarcomere disruption, and calcium disturbances (Tiidus et al., 2001). Neutrophil activity contributes to further oxidative damage in muscle tissues via the production of ROS from NADPH oxidase and the production of hypochlorous acid from hydrogen peroxidase from the myeloperioxidase (MPO) reaction (Suzuki et al., 1999; Tiidus et al., 1999). MPO activity is known as an indicator of neutrophil activity in damaged muscle tissue. Tiidus et al., 1999 found there were significant elevations in muscle MPO activity 24 hours post exercise in male rats compared to female rats and estrogen supplemented male rats. The authors suggest that while MPO represents neutrophil activity, infiltration of macrophages at 24 hours post exercise may have begun, thus MPO activity at 24 hours post exercise is indicative of overall leukocyte activity and inflammation. Increasing MPO activity plays an important role in clearing damaged muscle tissue, but can be damaging to healthy muscle tissue. Further clarification in the literature will be needed in order to elucidate whether an E_2 mediated reduction in inflammation is advantageous or not.

Skeletal muscle repair and regeneration

After skeletal muscle cell damage, satellite cells are activated to proliferate and provide the necessary materials to initiate muscle growth and repair. There may be an estrogenic effect on the activation and proliferation of satellite cells. For example, resistance trained women displayed a greater increase in satellite cells when compared to men (Roth et al., 2001). In another study, histochemical analysis was used to show that the greatest number of muscle fibers containing total, activated and proliferating satellite cells were in the exercised, E_2 supplemented group of female rats (Enns et al., 2007), compared to unexercised and no estrogen rats, unexercised and E_2 supplemented rats, and

finally exercised and no E_2 rats. E_2 might increase satellite cell activation through receptor-mediated mechanisms. For example, when E_2 receptors are blocked or there is an E_2 receptor antagonist, exercise and E_2-mediated increases in satellite cells are inhibited (Enns et al., 2008).

Another important promoter of satellite cell propagation might be the infiltration of leukocytes. Even though it is hypothesized that E_2 decreases the leukocyte response following muscle damage, new research proposes that E_2 increases IL-6 levels and nitric oxide, which are known activators of satellite cells. This means that E_2 can both increase satellite cell production and proliferation and still attenuate the leukocyte response (Enns & Tiidus, 2010, Tiidus 2003). The effect of E_2 on the inflammatory response requires further research, in order to clarify E_2's role in activation of satellite cells and leukocyte recruitment. As IL-6 is an important player in these mechanisms, the effect of E_2 on IL-6 levels is an important step in identifying these mechanisms.

Interaction of E_2 and progesterone

There has been limited research investigating the effects of progesterone and E_2 on exercise induced damage, inflammation and repair. Studies involving female ovariectomized rats treated with E_2 showed less neutrophil and macrophage infiltration in skeletal muscle following eccentric exercise, compared to rats not treated with E_2 (Enns et al., 2008; Iqbal et al., 2008) The reduced inflammatory response in rats treated with E_2 may be related to reduced muscle damage as shown by reduced skeletal muscle damage markers, such as β-glucuronidase and creatine kinase (Enns et al., 2008). Reduced skeletal muscle damage may be a result of the estrogenic muscle membrane stabilizing properties (Tiidus et al., 2001). In most tissues there are both E_2 receptors and

17

progesterone receptors present and there is the possibility that there are interactive effects in response to exercise induced muscle damage (Iqbal et al., 2008). To investigate these interactive effects between E_2 and progesterone, Iqbal et al., (2008), compared the concentrations of each hormone at 24 hours post exercise. The female rats were divided into 4 exercise and 4 control groups (sham, E_2, progesterone, and a combination of E_2 plus progesterone) following 8 days of hormone replacement. They confirmed that E_2 attenuated leukocyte infiltration following exercise induced muscle damage. They also found that progesterone also attenuated leukocyte infiltration, but to a smaller extent than E_2. In rats supplemented with both progesterone and E_2, leukocyte infiltration was not significantly different from the E_2 only group, suggesting that progesterone does not affect estrogenic influence.

Role of E_2 and cytokines in exercise induced muscle damage, inflammation and repair

The acute inflammatory response and satellite cell activation and proliferation are initiated by local and systemic signals, such as cytokines, growth factors and leukocytes, released by the injured muscle tissues. In response to moderate to high intensity exercise, pro-inflammatory cytokines TNF-α and anti-inflammatory cytokine IL-6 are produced. Pro-inflammatory cytokines up-regulate leukocytes, calpains, and nitric oxide in order to initiate the inflammatory response at the site of tissue damage. Anti-inflammatory cytokines are thought to limit the inflammatory response to exercise and inhibit pro-inflammatory cytokines (Pedersen et al., 2003). In the inflammatory response, anti-inflammatory IL-6 is one of the first cytokines present after high intensity exercise, and it

is the produced in large amounts.   As muscle damage occurs, resident leukocytes produce pro-inflammatory cytokine TNF-α to initiate inflammation and repair mechanisms. While the inflammatory response is critical to muscle damage repair and regeneration, the large response affects both damaged and healthy tissues, resulting in more inflammation than might be needed to repair the tissue. It is hypothesized that if E_2 plays a role in limiting the inflammatory response, less muscle damage will occur (Kendall & Eston, 2002).   TNF-α and IL-6 are major inflammatory markers during exercise induced muscle damage, inflammation and repair.   If these inflammatory markers are attenuated as a result of E_2 in the human body, then it could be inferred that E_2 has a protective role in limiting muscle damage and inflammation.

The cytokine TNF-α plays a major role in regulating the influx of leukocytes in clearing damaged muscle tissue and stimulating repair. Ostrowski et al., (1999) reported that strenuous exercise resulted in increases in pro-inflammatory cytokine TNF-α. In addition this group suggested that anti-inflammatory cytokines, such as IL-6, may restrict the potency and duration of the inflammatory response after exercise. E_2 has been shown to alter the concentration of TNF-α. For example Chao et al., (1995) found that TNF-α fluctuates with changes in the E_2 and progesterone. While this is an endotoxin model, not an exercise model, these results suggest a relationship between E_2 and TNF-α.   In another endotoxin model, E_2 was shown to attenuate TNF-α and IL-6 in post-menopausal women receiving E_2 replacement. Bacterial endotoxin studies can serve as a model to study TNF-α and other cytokines in the acute phase response because endotoxin stimulates the production of cytokines and leukocytes, similar to the response seen during exercise induced muscle damage (Puder et al., 2001).

IL-6 is an interesting cytokine because it has been shown to have both anti- and pro-inflammatory properties. It is thought that anti-inflammatory IL-6 is released locally in the muscle after strenuous exercise and the amount released is dependent on the type of physical activity, as well as the duration of exercise (Hamer & Karageorghis, 2007). After strenuous exercise, IL-6 is markedly increased more so than any other cytokine. Pederson et al., (2004) suggests that IL-6 is produced first and plays an important role in the inflammatory response. Previous studies suggest that muscle damage was related to IL-6 production, while later studies showed that high intensity training elevated creatine kinase levels, but failed to increase IL-6 levels (Pederson et al., 2004). This suggests that IL-6 response may be independent of muscle damage; Although, this view is not held by all immunology researchers. Furthermore a different study, comparing the levels of IL-6 production in the hind legs of rodents, found that there was no difference in IL-6 production in concentric and eccentric contractions (Jonsdottier et al., 2000). While muscle damage may not be directly dependent on IL-6 levels, it has been shown that IL-6 produced locally from the exercising muscles exerts an anti-inflammatory effect on the immune response. Until recently, researchers considered IL-6 to be a pro-inflammatory cytokine. For example, MacIntyre et al., (2001) reported that neutrophil and IL-6 increased up to 6 h post-exercise, and there was a significant relationship between IL-6 and DOMS, suggesting that IL-6 as a pro-inflammatory cytokine initiates inflammation. Further research needs to be completed in order to clarify the roles of pro- and anti-inflammatory IL-6. Due to the uncertainty of the role of IL-6, it is not the primary variable of interest in this study.

In the present study, the effects of E_2 and TNF-α on muscle damage, inflammation and repair are being evaluated. IL-6 is included as a secondary variable in this study to investigate how IL-6 affects TNF-α and if there is a relationship between E_2, IL-6 and inflammation. Schwarz et al (2000) found that the release of TNF-α was diminished in pre-menopausal females during the luteal phase of the menstrual cycle when compared to the follicular phase of the menstrual cycle, as well as the inhibited release of both TNF-α and IL-6 in females during the luteal phase when compared to male controls. In a study by Pottratz et al (1994), E_2 was shown to inhibit the expression of the IL-6 gene through an E_2 receptor mediated effect on the transcription of the gene's promoter region. Due to the unclear pro- or anti-inflammatory cytokine properties, IL-6 is hard cytokine to measure and accurately describe its effect on physiological systems related to muscle damage and inflammation. Thus TNF-α is the primary inflammatory marker measured in this study, because it is a known pro-inflammatory cytokine marker and its effect on physiological systems related to exercise induced skeletal muscle damage, inflammation and repair can be accurately described.

Figure 1: Theoretical model illustrating the potential protective role of estrogen in skeletal muscle damage, inflammation, and repair.
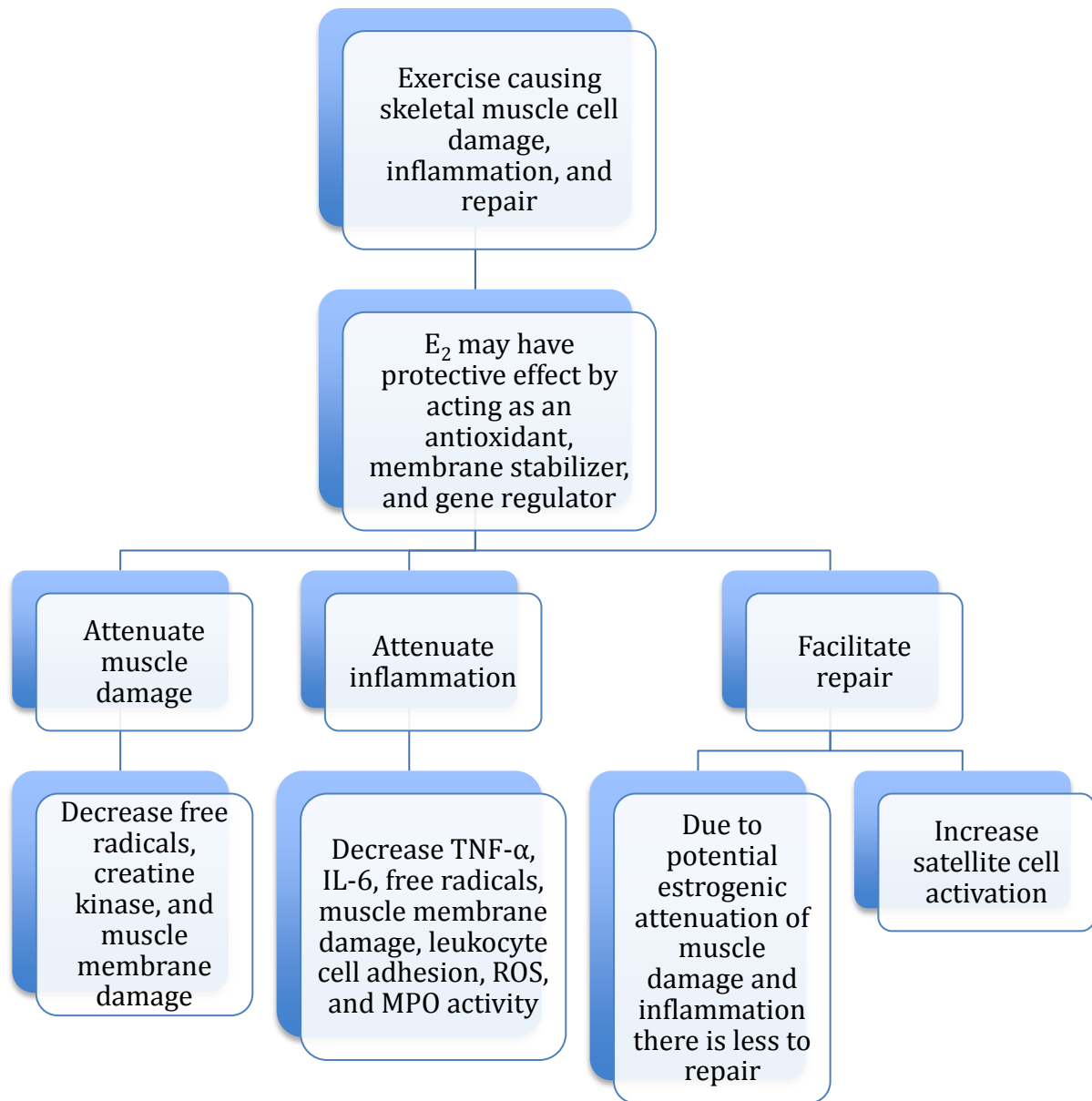
In summary, clarifying the role of E_2 during exercise induced muscle damage, inflammation, and repair is beneficial towards expanding this area of the research literature, as well as providing women with more information that may be potentially useful when designing training programs. As demonstrated in this review, E_2 plays a role in many physiological systems in addition to sexual and reproductive function. Understanding E_2's role in these systems, especially these systems involving exercise induced muscle damage, inflammation, and repair is especially important. It has been suggested in this review that E_2 may attenuate the inflammatory response, so that after exercise induced muscle damage enough cytokines are produced and leukocytes recruited to clear the damaged muscle tissues and limit excessive inflammation that may damage healthy muscle tissues. The results of this study will contribute to this body of knowledge and provide insight as to the role of estrogen on inflammatory markers TNF-α and IL-6 after prolonged aerobic exercise.

CHAPTER III

METHODOLOGY

The recruited participants in this study made five visits to the Applied Physiology Laboratory at the University of North Carolina, Chapel Hill. The first visit was an orientation visit, where informed consent was obtained, subjects were determined eligible for the study, descriptive characteristics were acquired, menstrual histories were recorded, and maximal oxygen consumption (VO_2max) tests were completed. The menstrual cycle was used to create two hormonal conditions, low E_2 (LE) and high E_2 (HE). Participants reported to the investigator the first day of menses, which was denoted as day 1. The LE phase occurs early in the menstrual cycle, roughly between days 3-7, when E_2 is lower, while the HE phase occurs later, approximately between days 20-24, when E_2 is much higher. Session two and four was an experimental protocol where subjects performed 60 minutes of treadmill running at 65% of their predetermined VO_2max in each hormonal condition, LE and HE. The variables TNF-α, and IL-6 were measured at baseline, immediately post-exercise, 30 minutes post-exercise. E_2 was measured at baseline. During session three and five, the days after the exercise protocol, the 24-hour post-exercise blood draw was taken in each hormonal condition.

Participants

Healthy, highly trained, pre-menopausal women between the ages of 18-30 years were recruited for this study. Samples size was estimated from previous research in the literature to ensure adequate power (Appendix A). To be considered for this study,

participants were eumenorrheic for the past six months, had not taken oral contraceptives or other hormone therapy six months prior to participation, and were not currently taking anti-inflammatory medications for chronically diagnosed conditions, such as ibuprofen, naproxen, or aspirin. Additionally participants had a current minimum training volume of 3-5 days a week, 45-120 minutes per session of aerobic activity, a VO_2max of 45 ml/kg/min, and no major injuries that limited the ability to engage in exercise. Participants that had sustained an injury in the past 6 months were fully recovered with a physician's clearance for exercise before participating in the study. Participants that became ill with a immune responding condition were dropped from the study. Once cleared, participants signed informed consent after being thoroughly informed of the experimental protocol and any risks or rewards related to the study. Additionally, participants agreed to abstain from strenuous physical activity and maintain a diet similar in calories and carbohydrate content for the 24 hours prior to the two experimental trials. Compliance was assessed through the use of a 24-hour food log and compliance questionnaire. Participants were asked to replicate their diet prior to each prolonged treadmill bout.

Instrumentation

Height was determined using a portable stadiometer (Perspectives Enterprises, Portage, MI USA). Body mass was measured using a mechanical scale (Detecto, Webb City, MO USA). Skinfolds were measured with a Lange skin caliper (Beta Technology, Inc., Santa Cruz, CA USA). Maximal oxygen consumption was determined during a continuous, incremental treadmill test on a Quinton Q65 treadmill (Bothell, WA USA). Respiratory gases were obtained for the orientation session, as well as the two

experimental sessions using the Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT USA). Heart rate was monitored continuously using the Polar telemetry system (Polar Electro, Inc., Lake Success, NY USA). Ratings of perceived exertion were determined using Borg's 20 point scale. Urine specific gravity was assessed using CLINITEK Atlas Automated Urine Chemistry Analyzer (Bayer, Erlangen, Germany). Hematocrit was determined using an Adams MHCT II microhematocrit centrifuge (Becton Dickinson, Franklin Lakes, NJ USA) and an International Microcapillary Reader (International Equipment Company, Needham Heights, MA USA). Hemoglobin was determined from a Stanbio Lab Hemopoint H^2 analyzer (Boerne, TX USA). Whole blood samples were placed in an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA USA) and the resultant separated plasma was stored and frozen at -80˚C. Plasma TNF-α and IL-6 were measured using ABNOVA ELISA kits (Taipei, Taiwan). Plasma E_2 was measured using the radioactive (^{125}I) immunoassay technique, (Siemens Healthcare Technologies, Los Angeles, CA USA).

Protocol

Pre-Screening

Women interested in participating in this study emailed the investigator. During this initial communication the investigator determined if they met the inclusion criteria. Women accepted into the study were scheduled for an orientation session.

Orientation Session I

Participants arrived at the Applied Physiology Laboratory at The University of North Carolina, Chapel Hill and were informed of the experimental protocol, made aware

of the possible risks and rewards associated with the protocol, and signed an informed consent form (Appendix B). The Office of Human Research Ethics, Institutional Review Board, of The University of North Carolina, Chapel Hill approved the informed consent form. After giving informed consent, participants underwent a physical screening and a 12 lead electrocardiogram. Then participants filled out the Exercise and Sports Science medical history questionnaire and passed the medical screening (Appendix C). Descriptive characteristics, such as height, weight, age and percent body fat were obtained. Percent body fat was measured via measurements at the triceps, thigh, and suprailliac skinfolds, using the 3-site Jackson, Pollock, and Ward equation (Jackson et al., 1980).

Next, participants completed a VO_2max test using a continuous, incremental treadmill test. They were allowed a five minute warm up consisting of running on the treadmill at their preferred pace, followed by light stretching. Following the warm up, resting oxygen consumption VO_2 was recorded for at least three minutes. Participants then began the graded exercise test, as determined by the Bruce treadmill protocol (ACSM Guidelines, 2010). Table 1 lists the Bruce protocol treadmill test. Throughout the test, heart rate (HR), 20 point scale ratings of perceived exertion (RPE), and respiratory gases were collected. At the conclusion of the test, participants recovered (actively or passively) and were permitted to leave the laboratory when HR dropped below 100 beats per minute (bpm). In order to confirm that the VO_2 test was a maximal test (rather than a peak test) participants showed three of the four following criteria: a plateau or decrease in VO_2 with increases in workload, respiratory exchange ratio (RER) greater than or equal to 1.1, RPE $\geq$ 18, and a HR within 10% of predicted heart rate max

(220-age, [ACSM Guidelines, 2010]). ACSM guidelines were used to estimate exercise intensity corresponding to 65% of VO_2max, as shown in Appendix D (ACSM Guidelines, 2010).

Table 1: Speed (miles per hour; mph) and grade for each three minute stage of the Bruce protocol incremental exercise test.

Stage	Speed (mph)	Grade (%)
1	1.7	10
2	2.5	12
3	3.4	14
4	4.2	16
5	5.0	18
6	5.5	20
7	6.0	22

Experimental Sessions II and IV

Participants arrived at the Applied Physiology Laboratory once during the mid-follicular (LE) phase of the menstrual cycle and once during the mid-luteal (HE) phase. The forward counting method (Chavanne & Gallup 1998) was used to determine and schedule sessions during the respective phases of the menstrual cycle. Participants reported to the investigator the first day of menses, this was denoted as day 1. The LE phase occurs early in the menstrual cycle, roughly between days 3-7, when E_2 is lower, while the HE phase occurs later, approximately between days 20-24, when E_2 is much higher. The LE and HE phases of the menstrual cycle were used for the two experimental sessions to find the largest differences between naturally fluctuating E_2 levels and maximize effect size. These date ranges are approximations, thus exact days in each cycle

varied as a result of the actual length of a subjects' menstrual cycle and when they were scheduled for a testing session. A menstrual cycle questionnaire was used to determine each participant's respective phases of the menstrual cycle (Appendix E). The two experimental sessions were counterbalanced to prevent order effects.

Subjects were asked to refrain from intense physical activity and replicated their diet 24 hours prior to each experimental session. Subjects were asked if they followed all guidelines prior to the testing sessions, and a food diary was used to ensure nutrient intake was replicated between trials, as well as to make sure they consumed adequate calories and carbohydrate (Appendix F). To ensure adequate hydration, a urine sample was requested to assess urine specific gravity. If subjects were compliant, the experimental trial was continued. Subjects rested supine in a relaxed, quiet environment for 10 minutes. After the resting period, blood was obtained (3mL) via individual blood draws. The blood sample was placed in a sterile K_2–EDTA (purple top) Vacutainer™ tube and immediately put on ice. Subjects then completed a five-minute warm-up consisting of cycling and stretching, followed by 60 minutes of running at their previously determined workload of 65% of VO_2 max. This intensity and time frame was chosen to make sure E_2, IL-6 and TNF-α were elevated as a result of exercise (Bonen et al., 1979; Mendham et al., 2011; Pederson 2000). During both treadmill bouts heart rate and VO_2 was assessed at rest, while heart rate, ratings of perceived exertion, and VO_2 was assessed from 6-10 minutes, 26-30 minutes, and 56-60 minutes. This is shown in figure 2. During the first treadmill bout, at 10 and 30 minutes, VO_2 was checked to ensure participants reached this intensity of 65%. If participants were below 65%, the running speed was increased. This was not done in the second treadmill bout, because the running

trials were exactly replicated. Immediately post exercise and 30 minutes into recovery, blood samples were taken (3mL) and promptly put on ice. Plasma was separated from blood samples and stored until later analysis for E_2, TNF-α, and IL-6. Blood samples are stored for three years.

Follow Up Blood Draws Session III and V

Participants returned to the APL 24 hours post exercise for additional blood draws. Upon entering the APL, participants laid supine, in a quiet environment, for 10 minutes. Blood samples were obtained to measure E_2, TNF-α and IL-6 using the same procedures specified above. All data collection forms used are included in Appendix G.

Figure 2: A diagram showing the protocol of the experimental sessions.
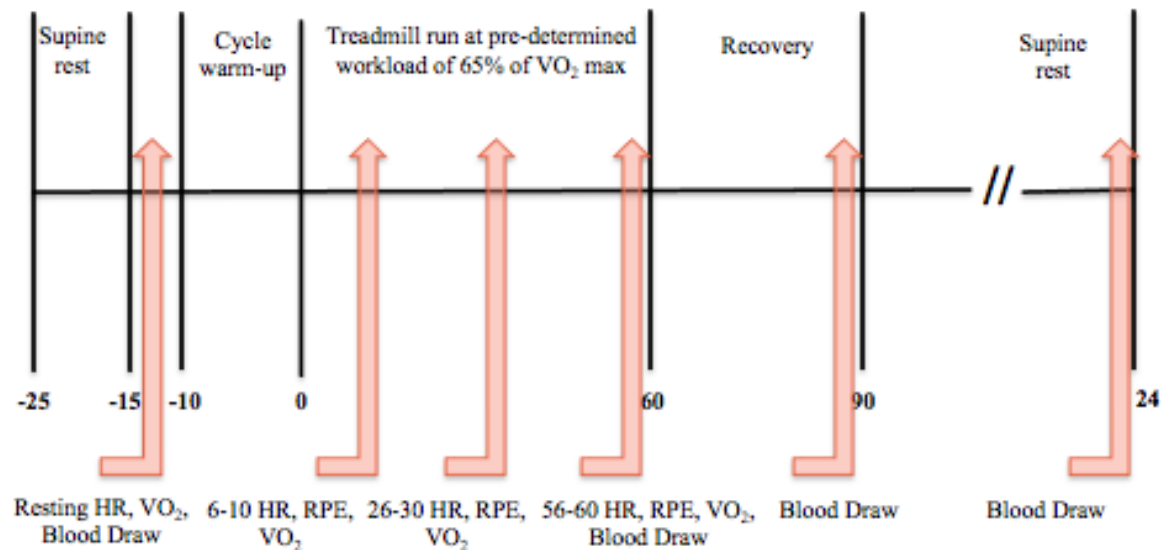
Blood Procedures

Hematocrit

Immediately post each exercise test, resting and post-exercise Hematocrit (Hct) values were determined in triplicate from whole blood samples. Whole blood was put

into 75mm Allied Corporation microcapillary tubes (Fisher Scientific, Pittsburgh, PA) and sealed using Critoseal (Krackeler Scientific, Albany, NY). Capillary tubes were spun in a microhematocrit centrifuge for three minutes at 10,000 RPM and then placed on a hematocrit wheel to determine the hematocrit values of each sample. A mean was calculated from three samples and used in data analysis.

Hemoglobin

Resting, immediately post-exercise, and recovery hemoglobin (Hb) values from each experimental session were measured in duplicate from the whole blood samples using the Stanbiolab Hemopoint H^2 analyzer (Boerne, TX). These values were determined immediately after completion of the exercise tests. A mean was calculated from three samples and used in data analysis.

Plasma Volume Shift

Hb and Ht values were used to calculate exercise induced plasma volume shifts according to the Dill and Costill equation (Dill & Costill 1974).

Cytokines (TNF-α and IL-6), E_2

To separate plasma from whole blood, the blood samples were centrifuged at 3,000 x g for 10 minutes. The separated plasma was transferred to storage tubes and stored until analyses are conducted. Radioactive (125I) immunoassay technique (Siemens Healthcare Technologies, Los Angeles, CA USA) with solid-phase antibody procedures was used to measure plasma E_2 concentrations. The assay manufacturer reports a minimum detectable concentration of 2.0 pg/mL. High-sensitivity enzyme-linked immunosorbent assay kits (Abnova, Taipei, Taiwan) were used to measure both TNF-α and IL-6. The assay manufacturer reports a minimum detectable concentration of

5.0 pg/ml for TNF-α and 0.92 pg/mL for IL-6. All blood assays were performed in duplicate while standards were done in triplicate. See Appendix H for assay sheets.

Data Analysis

Statistica® statistical software was used to analyze the data in this study (version 6.3 Tulsa, OK USA). Significance for all data was set at $\alpha < 0.05$.   Descriptive statistics were shown as means $\pm$ standard deviations (SD). Sample size of fifteen participants was estimated from previous research in the literature to ensure adequate power ($\beta=0.80$). Effect size was calculated for all significant measures to determine if statistically significance effects had practical meaning.

Separate 2 x 4 (estradiol level x time) totally within, repeated measures ANOVAs and where appropriate, Bonferoni post hoc test, was used to assess the effects of estradiol on blood TNF-α and IL-6 concentrations.

At dependent t-test analysis was used to evaluate statistical significance between resting levels of estradiol-β-17 (E$_2$) at LE and HE phase of each experimental session.

CHAPTER IV

RESULTS

Due to the stringency of the inclusion criteria and study protocol, only 10 of the 15 initially recruited subjects completed all aspects the study. Three subjects completed a VO_2 max test, but did not meet the 45 ml/kg/min criteria. One subject dropped out for personal health reasons. One subject was not able to complete the treadmill bouts due to scheduling constraints. The remaining 10 subjects met and maintained all of the inclusion criteria. However, due to medical reasons, one subject was unable to complete the mid-luteal prolonged running bout. In order to not lose this subject's data within the statistical analysis, mean substitution was used to approximate their mid-luteal values. Additionally, several subject's HR or RPE data were accidentally missed during data collection and this data was also approximated using mean substitution.

Subject characteristics

As noted, ten eumenorrheic, aerobically trained females completed this study. These subjects met all the inclusion criteria: healthy females between the ages of 18-30 years, eumenorrheic, not currently taking or have taken oral contraceptives or other hormone therapy 6 months prior to participation, have not had an injury in the previous six months, not currently taking anti-inflammatory medication, and have a current minimum training volume of 3-5 days per week, 45-120 minutes per session of aerobic activity and a VO_2 max of at least 45 mL/kg/min. Participant physical characteristics are reported in Table 2.

Table 2: Descriptive data for subjects.

Measure	Mean ± SD
Age (yr)	21±1
Height (cm)	164.5±5.7
Body Mass (kg)	61.3±8.3
Estimated Body Fat (%)	24.1±2.8

VO_2 peak testing

All the criteria for a maximal oxygen consumption test was not achieved by all subjects, thus all maximal oxygen consumption tests are referred to as VO_2 peak tests. Average relative VO_2 peak was 53.5 ± 4.7 ml/kg/min, while the average peak RPE obtained was 18 ± 1 Borg units, and the average peak HR was 191 ± 7 bpm. The average calculated 65% of VO_2 peak to use during the submaximal 60 minute prolonged runs was 34.8 ± 3.0 ml/kg/min.

Hormonal condition determination

Average menstrual cycle length of subjects was 28 ± 1 days. Menstrual cycle condition was determined using the protocol detailed in the Methodology chapter. With the onset of menses denoted day 1, subjects were tested on 7 ± 2 days during the mid-follicular (low E_2; LE) phase, while subjects were tested on 23 ± 3 days for the mid-luteal (high E_2; HE) phase. Analysis of resting blood samples for E_2 indicated appropriate hormonal condition was achieved. The LE concentration was 39.3 ± 18.3 pg/mL and the HE concentration was 148.1 ± 35.2 pg/mL. These concentrations were significantly different from one another (p=0.003). The significant difference between hormonal conditions confirms the desired treatment effect was achieved.

Prolonged treadmill running bout

Before each prolonged treadmill running bout, subjects submitted a 24 hour food log, and answered a questionnaire regarding compliance of pre-testing guidelines. Between the two testing sessions, all subjects complied with all guidelines (no strenuous exercise or consumption of anti-inflammatory medication, replication of dietary intake, and adequate consumption of fluids within 24 hours prior to the testing session). Three subjects participated in light exercise within the 24 hour period prior to the first testing session, and this exercise was replicated exactly prior to the second prolonged running session. The resting urine specific gravity was well below 1.030 cc^3 for all subjects for both LE and HE prolonged running sessions, indicating adequate hydration prior to exercise. Mean body mass prior to exercise for LE was 61.4 ± 8.6 kg and for HE was 61.1 ± 8.3 kg; these values did not differ significantly (p>0.05).

Each prolonged running bout was performed for 60 minutes at the calculated running speed to elicit 65% of the individual's VO$_2$ peak. All subjects were able to complete each of the 60 minute running bouts. Actual treadmill running speed was replicated for each prolonged running bout, which was equal to 14.7 ± 1.3 km/hr with a corresponding VO$_2$ of 61.7 ± 5.0% during LE and 59.7 ± 2.8% during HE. The prolonged running sessions were counterbalanced in order to prevent order balances, six subjects completed LE then HE, while four subjects completed HE before LE. The mean VO$_2$, HR and RPE were nearly identical for each of the 60 minute running sessions. Descriptive data at each measurement time is shown in Table 3 below.

Table 3: Descriptive data (mean ± SD) for VO$_2$, HR and RPE for each prolonged running bouts in each hormonal condition (LE and HE).

Hormonal Condition	Measure	Time (min)			
		Rest	10	30	60
LE	VO$_2$ (mL/kg/min)	4.9±0.6	30.4±4.2	33.2±3.2	35.2±2.9
	HR (bpm)	63±7	150±15	157±13	165±13
	RPE (Borg units)	-	11±1	12±1	14±1
HE	VO$_2$ (mL/kg/min)	4.9±0.4	30.0±3.2	32.7±3.3	33.1±4.5
	HR (bpm)	61±4	149±7	154±6	164±6
	RPE (Borg units)	-	11±1	12±1	14±2

Using the Dill and Costill method of determining plasma volume shifts, plasma volume decreased over the prolonged running bouts were calculated. These plasma volume shifts are reported in Table 4.

Table 4: Mean (± SD) plasma volume shifts from rest to immediately post exercise (R-IP), rest to 30 minutes post exercise (R-30P) and rest to 24 hours post exercise (R-24P).

Hormonal Condition	Plasma Volume Shift (%)		
	R-IP	R-30P	R-24P
LE	-8.5±6.9	-3.1±8.0	6.6±16.1
HE	-8.1±5.9	-8.6±7.6	-2.6±6.8

Blood responses to prolonged exercise

Tumor necrosis factor-α (TNF-α)

TNF-α responses are reported in Table 5. The main effect for hormonal condition was not significant (p=0.48). The main effect for time was significant (p=0.001), with post-hoc tests indicating there was a significant increase from rest to 30 minutes post exercise (p=0.001), from rest to 24 hours post exercise (p=0.001), and from immediately post exercise to 24 hours post exercise (p=0.03). There was not a significant interaction effect for hormonal condition and time (p=0.60).

Interleukin-6 (IL-6)

Interleukin-6 responses are reported in Table 6. The main effect for hormonal condition was significant (p = 0.022), with post hoc revealing IL-6 response was greater in LE than HE. The main effect for time was also significant (p=0.001), with IL-6 elevated from rest to immediately post exercise, and from rest to 30 minutes post exercise. There was a significant interaction effect between hormonal condition and time for IL-6 (p=0.001). Post hoc revealed that IL-6 was significantly increased from rest to immediately post exercise, and from rest to 30 minutes post exercise in both hormonal conditions. The response at immediately post exercise did not differ between LE and HE, however, the response at 30 minutes post exercise was significantly elevated in LE when compared to HE (p=0.002).

Table 5: Mean (± SD) for TNF-α at rest, immediately post exercise (IP), 30 minutes post exercise (30P), and 24 hours post exercise (24P). An * indicates significant increase from rest, while an ^ indicates significant increase from IP.

TNF-α (pg/mL)

Hormonal Condition	Rest	IP	IP Change (%)	30P	30P Change (%)	24P	24P Change (%)
LE	5.5±1.5	6.5±1.9	21.5±19.7	8.1±2.4*	64.8±96.8	8.1±2.6*^	67.8±81.3
HE	5.5±1.4	6.7±1.6	26.2± 24.4	8.4±3.0*	57.8±52.5	9.3±2.0*^	75.6±40.4

Table 6: Mean (± SD) for IL-6 at rest, immediately post exercise (IP), 30 minutes post exercise (30P), and 24 hours post exercise (24P). An * indicates significance from rest, an ^ indicates significantly lower IL-6 compared to LE, while a # indicates an interaction effect between hormonal condition and time.

Hormonal Condition	IL-6 (pg/mL)						
	Rest	IP	IP Change (%)	30P	30P Change (%)	24P	24P Change (%)
LE	3.0±1.5	6.7±2.0*	167.8±118.0	13.7±4.7*	496.6±417.7	3.7±2.4	24.2±43.6
HE	2.5±1.2	6.9±1.5*	241.0±167.4	9.5± 2.3*^#	375.5±283.6	3.1±18.3	33.4±69.0

CHAPTER V

DISCUSSION

The purpose of this study was to determine if circulating E_2 levels influenced the pro-inflammatory cytokine biomarker TNF-α responses in the blood, following a moderate intensity prolonged exercise session in eumenorrheic women. A secondary purpose of this study was to determine if E_2 levels influenced the cytokine biomarker IL-6 (both a pro- or anti-inflammatory) response in the blood, following a moderate intensity prolonged exercise session in eumenorrheic women. It was hypothesized that higher E_2 levels would attenuate both TNF-α and IL-6 responses after the exercise bout. To test these hypotheses, ten aerobically trained, eumenorrheic women completed 60 minute treadmill bouts in a LE condition and again in a HE condition, with blood collection occurring at rest, immediately post exercise, 30 minutes post exercise, and 24 hours post exercise.

Hormonal condition determination

In this study, there was a significant treatment condition between the LE and HE menstrual cycle conditions (p=0.003), with an approximately four times greater increase in E_2 from LE to HE (39.3 $\pm$ 18.3 pg/ml to 148.1 $\pm$ 35.2 pg/ml, respectively). These results fell within expected values determined by the ELISA kit used in analysis; as mid-follicular (LE) levels were reported to range from 30-100 pg/ml and mid-luteal (HE) levels were reported to range from 60-400 pg/ml (Human E_2, Abnova, Walnut, CA). The

existence of a significant treatment condition is in alignment with other studies investigating the effect of E_2 on inflammatory markers (Chiu et al., 2000; Hackney et al., 1991; Northoff et al., 2008).

Prolonged aerobic exercise session

The physiological responses (results in Table 3) to the 60 minute the treadmill running bouts confirm the experimental sessions were closely replicated. That is, the results show that VO_2, HR, and RPE responses were similar between bouts. Furthermore, actual running speed in each session was replicated exactly and corresponded to an average VO_2 of $61.7 \pm 5.0\%$ during LE and $59.7 \pm 2.8\%$ during HE. While this elicited VO_2 was below the prescribed 65% of VO_2peak desired, this intensity has been shown in previous research done by this laboratory to elicit suitable muscle damage to initiate inflammation (Hackney et al., 2012). With the physiological markers confirming that there was very little difference between 60 minute testing sessions, it is concluded that any differences in blood measured cytokine responses to the exercise resulted primarily from fluctuating E_2 across the menstrual cycle.

Plasma volume values indicate that plasma fluid left the vascular bed from rest to immediately post exercise and from rest to 30 minutes post exercise during LE ($-8.5 \pm 6.9\%$ and $-3.1 \pm 8.0\%$, respectively) and during HE ($-8.1 \pm 5.9\%$ and $-8.6 \pm 7.6\%$, respectively). This hemoconcentration of the blood is most likely indicative of slight exercise induced dehydration (Dill & Costill, 1974). From rest to 24 hours post exercise, the plasma volume shift was positive during LE ($+6.6 \pm 16.1\%$) and slightly negative during HE ($-2.6 \pm 6.8\%$) indicating the subjects may have rehydrated well and plasma volume was closer to normal values, respectively. The percent changes in the blood

measured responses of TNF-α and IL-6 were much larger than the plasma volume shifts, indicating that the changes in these outcome variables were indicative of increased production level and thus an inflammatory response and not hemoconcentration or hemodilution induced changes.

Blood responses

Tumor Necrosis Factor (TNF-α)

There was a significant main effect for time with post hoc tests indicating that there was a significant increase from rest to 30 minutes post exercise, from rest to 24 hours post exercise and from immediately post exercise to 24 hours post exercise. These results are in line with other previous research, suggesting that there is an acute inflammatory response, mediated by the pro-inflammatory cytokine TNF-α, after exercise induced muscle damage (Ostrowski et al., 1999; Pederson et al., 2000: Smith et al., 2000). The concentrations of TNF-α reported in this study are in line with the concentrations reported in one other study (Ostrowski et al., 1999). Specifically, Ostrowski et al. reported TNF-α values significantly increase in males after a marathon, and these values stayed elevated for up to 1.5 hours post exercise. The significant increase in TNF-α complements the current study, which suggests that TNF-α followed the same response in women after a much shorter exercise intervention.

In the current study there was not an interaction effect for TNF-α for hormonal condition by time. The comparative literature is limited in the number of investigations, which have studied the effects of E_2 on TNF-α, hence this is why the present study was conducted. Those available studies have shown an effect of fluctuating E_2 on TNF-α at rest (Schwarz et al., 1990), and in endotoxin models (Chao et al., 1995; Puder et al.,

2001). Schwarz et al., found that TNF-α was attenuated during HE when compared to LE. Puder et al., suggests that high concentrations of E_2 can limit the inflammatory response, with the reduction of circulating TNF-α. Lastly, Chao et al., proposes that E_2 greatly regulates the release of TNF-α, with very high and low physiological levels of E_2 attenuating TNF-α and mid-physiological concentrations of E_2 increasing TNF-α. While it has been shown in these few select studies that E_2 does affect TNF-α, there may not be as large of an effect following an exercise intervention to be physiologically detectable; which this study supports. Additionally, the sample size was not adequately powered to see significance for TNF-α ($\beta=0.68$; i.e., underpowered). However it is un-likely significance would have occurred with 15 subjects, because the largest mean difference (TNF-α response at 24 hours post exercise) would have required a sample size of 55 subjects to reach significance.

Interleukin-6 (IL-6)

There was a main effect for both hormonal condition and time, and an interaction effect for hormonal condition by time. Post hoc testing revealed that IL-6 was significantly increased from rest to immediately post exercise, and from rest to 30 minutes post exercise in both hormonal conditions. The response immediately post exercise did not differ between LE and HE; however, the response at 30 minutes post exercise was significantly greater in LE condition when compared to HE. The practical significance of these findings, as shown by the effect size, was large (Cohen d statistic = 1.135). This suggests that there is a strong relationship between E_2 and IL-6 and the results are meaningful.

The results of this study are in line with the results of previous research produced in our laboratory where E_2 was found to attenuate IL-6 immediately post exercise and 30 minutes post exercise (Hackney et al., 2012). These results are also in line with studies investigating the effect of E_2 on IL-6 at rest (Angstwurm et al., 1997; Chiu et al., 2000; Schwarz et al., 2000). Interestingly, and somewhat in contradiction to the current study, Northoff et al., found HE facilitates up-regulation of TNF-α genes and down-regulation of IL-6 genes, suggesting higher E_2 levels influences a pro-inflammatory environment. This study was set up very similarly to the current study, with the measurement of TNF-α and IL-6 occurring after a high intensity (~93% of anaerobic threshold) exercise bout. Other studies, however, indicate that there is a weak or non-existent relationship between E_2 and IL-6. Ives et al., found E_2 to be unrelated to IL-6 at rest or in response to exercise. These results of Ives et al. should be interpreted with caution, however, because there was not a treatment effect (LE was not significantly different from HE). Considering the differing results regarding the estrogenic effect on inflammation, it is hard to know for certain how or if E_2 exerts a strong influence on IL-6. The current results suggest there is an effect; nonetheless, more research is warranted

It is well documented that IL-6 increases dramatically with exercise (Brenner et al., 1999; Edwards et al., 2006; Ives et al., 2011; Ostrowski et al., 1998). IL-6 is a unique cytokine because of its both pro- and anti-inflammatory effects. Recent investigations suggest IL-6 is released locally from the skeletal muscle after an acute exercise bout, with the amount released dependent on the type of physical activity and the duration of the exercise (Hamer & Karageorghis. 2007). This acute IL-6 released is thought to have primarily anti-inflammatory cytokine effects, such as the inhibition of pro-inflammatory

TNF-α (Febbraio et al., 2002; Pederson et al., 2003; Schwarz et al., 2000). The acute IL-6 response is also thought to have myokine effects such as the stimulation of lipolysis and fat oxidation to aid in the maintenance of glucose metabolism (Ives et al., 2011). In the context of this study, the attenuation of IL-6 may be considered negative because there is less IL-6 exerting anti-inflammatory and glucose sparing effects or it may be considered positive because there is overall less inflammation due to the protective effects of E_2. Further work is necessary to address this paradox.

The mechanism by which E_2 exerts a protective effect on skeletal muscle and inflammation is not well understood, but thought to be a result of E_2 acting as an antioxidant, membrane stabilizer and gene regulator (Perksy et al., 1999). While it can be interpreted that there is less IL-6 exerting anti-inflammatory and glucose sparing effects during HE, a bulk of the research literature suggests that the attenuation of IL-6 is positive because it means there is less overall inflammation following damaging exercise (Angstwurm et al., 1997; Chiu et al., 2000; Schwarz et al., 2000). Additionally previous research in this laboratory, found HE concentrations to promote glucose-sparing mechanisms and increase fat utilization by increasing the activity of lipoprotein lipase, circulating growth hormone, and decreasing circulating insulin levels (Hackney et al., 1999). This supports the theory that HE levels promote fat utilization and the attenuation of IL-6 may result from less muscle damage and inflammation following the exercise bout and less of a need for IL-6 as enhancer of lipolysis since HE may be exerting some of these actions directly. Additionally, previous research has shown that there is less muscle damage following an exercise intervention during HE concentrations (Carter et al., 2001). This also suggests that the attenuation of IL-6 is positive, indicating that there is

less potential overall inflammation following the exercise bout, less collateral damage to healthy skeletal muscle tissue and less muscle soreness. While there is uncertainty regarding the exact mechanistic influence of IL-6, future research is needed to fully elucidate the role of E_2 as it fluctuates across the menstrual cycle and inflammation after a moderate intensity aerobic exercise bout.

The inability to determine whether pro- and anti-inflammatory IL-6 exerts a positive or negative influence on skeletal muscle after exercise-induced muscle damage was a major limitation of this study. Other limitations include a small sample size. While the sample size was adequate to see a meaningful significance between hormonal condition and IL-6, the sample size was not adequately powered to see significance for TNF-α (β=0.65; i.e., underpowered). In order to see significance, additional subjects needed to be recruited. Additionally, results are only applicable to eumenorrheic women because post-menopausal, amenorrheic, oligomenorrheic, and oral contraceptives users have different hormonal profiles, which alters their inflammatory response to exercise.

Due to the pro- and anti-inflammatory actions of IL-6, and the uncertainty in the literature as to how IL-6 exerts its influence with respect to E_2 after muscle damaging exercise, there is great need to conduct more research in this area. Recommendations for future research include clarifying the role of E_2 and its effect on inflammation after exercise induced muscle damage, as well as clarifying the role of IL-6. In order to determine a more comprehensive understanding of the effect of E_2 on the immune response, additional cytokines needed to be investigated, both pro- and anti-inflammatory, in order to elucidate whether there is an estrogenic effect after exercise. Future research should also investigate whether there is a possibility of interaction between E_2 and

progesterone on the immune response following exercise, since both of these hormones fluctuate across the menstrual cycle.

In conclusion, there was not a significant interaction effect between E_2 and the cytokine TNF-α, indicating that HE does not attenuate pro-inflammatory TNF-α responses. However, there was a significant interaction effect between E_2 and IL-6, the significance of this finding is unclear due to the pro- and anti-inflammatory effects of IL-6. Other known pro-inflammatory markers should also be looked at to determine if there is in fact a protective influence of E_2 on muscle damage, inflammation, and repair. Information regarding the estrogenic effect on inflammation across the menstrual cycle would be useful for eumenorrheic women not on birth control, when designing exercise programs, as well as considerations in study design when investigating the inflammatory response in women.

SAMPLE SIZE CALCUATIONS

Sample Size Calculations			
TNF-α	(-) E2	(+) E2	Difference
	448.0	157.0	291.0
	52.0	20.0	32.0
	27.0	20.0	7.0
	125.0	30.0	95.0
	150.0	73.0	77.0
	519.0	307.0	212.0
Average	220.2	101.2	119.0
Standard Deviation			110.2
Sample Size	14.0		

Estrogen	(-) E2	(+) E2	Difference
	7.3	102.0	94.7
	9.5	113.0	103.5
	9.6	29.1	19.5
	125.0	255.0	130.0
	54.0	392.0	338.0
	12.3	139.0	126.7
Average	36.3	171.7	135.4
Standard Deviation			107.0
Sample Size	10.0		

INFORMED CONSENT

University of North Carolina at Chapel Hill
Consent to Participate in a Research Study
Adult Participants

Consent Form Version Date: __2013-2014_____
IRB Study # 10-2109
Title of Study: Influence of Estrogen on Cytokine Response to Prolonged Treadmill Running
Principal Investigator: Anthony Hackney
Principal Investigator Department: Exercise And Sport Science
Principal Investigator Phone number: (919) 962-0334
Principal Investigator Email Address: ach@email.unc.edu
_____

What are some general things you should know about research studies?
You are being asked to take part in a research study. To join the study is voluntary.
You may refuse to join, or you may withdraw your consent to be in the study, for any reason, without penalty.

Research studies are designed to obtain new knowledge. This new information may help people in the future.   You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies. Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study.

You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?
Recent work has shown a negative relationship between concentration of estrogens and the inflammatory response, specifically cytokines, at rest. Females in the midluteal phase of the menstrual cycle, high estrogen concentration, exhibited significantly lower circulating cytokines (and creatine kinase) compared to females in the midfollicular phase, low estrogen concentration. These demonstrated fluctuations at rest begs the question - Is there an altered cytokine response during exercise at different points within the menstrual cycle as estrogen changes?

To date, few exercise studies on human female subjects with respect to estrogen concentration and cytokines exist. The studies that do exist present divergent results. The studies performed have limitations; primarily small sample sizes, potential inaccurate menstrual phase determination, or dosage of exercise was not enough to provoke a response.

The purpose of this research study is to learn about the influence estrogen has on the cytokine response to prolonged treadmill running. The results of this study will add to the limited body of knowledge available on the influence of estrogen on cytokine expression in response to exercise within women, potentially providing insight as to how training regimens might be altered for optimal performance and minimal risk.

The main aim of the study is to determine if there is a significant difference in cytokine response at rest,

immediately post exercise, 30 minutes post exercise, and 24 hours post exercise between two phases of the menstrual cycle, midluteal and midfollicular. On separate days, you will perform an exercise test on a treadmill to determine your maximal aerobic capacity (VO_2max), two 60-minute running bouts at approximately 65-70% VO_2max, and have blood drawn before, immediately post, 30 minutes post, and 24-hours post the 60-minute running bout. Blood samples will be assessed for estrogen and cytokine concentration and creating kinase levels.

You are being asked to be in the study because you are a healthy, highly trained woman between the ages of 18 and 30 with a normal menstrual cycle for at least 6 months. You have not used oral contraceptives for at least six months prior. Have no major injuries within the last six months that limit ability to engage in exercise or if have sustained an injury are completely recovered and cleared by a physician to partake in exercise. Your current VO_2max is at least 45 ml/kg/min. Your current minimum training volume is 3-5 days a week, 45-120 minutes per session of aerobic activity.

Are there any reasons you should not be in this study?
You should not be in this study if you are knowingly pregnant or become pregnant during the study, if you have an irregular or absent menstrual cycle, are currently taking or have taken within the six months prior oral contraceptives, you have sustained an injury within the last six months that has limited your ability to exercise, use substances known to alter immune response (e.g. NSAIDS) the week before each 60-minute exercise session, or you become ill with immune responding conditions (i.e., colds, respiratory infections…etc.).

How many people will take part in this study?
There will be approximately 25 people in this research study.

How long will your part in this study last?
You will be enrolled in the study for approximately 6 weeks. Within the 6 weeks, 3 visits are made to the Applied Physiology Laboratory at the University of North Carolina at Chapel Hill.

- Visit 1: Orientation Session, duration is approximately 90 minutes
- Visit 2 (approximately 1-6 weeks after visit 1): Prolonged treadmill running bout (60 min) with a before and immediately, and 30 minutes after exercise blood draws performed by a certified phlebotomist (NCPT 56147) visit, duration is approximately 2 hours
- Visit 3: 24-hours after prolonged running bout blood draw, duration is approximately 30 minutes

Visits 4-5 will be a repeat of visits 2-3 approximately 2-6 weeks after visit 2. Blood specimens will be stored for 3 years following the completion of the study.

What will happen if you take part in the study?
Orientation/Familiarization Session, duration approximately 90 minutes (visit 1):

- The study protocol, schedule, inherent benefits, and potential risks will be explained to you, followed by signing the informed consent.
- You will go through a physical screening that includes completing the Department of Exercise & Sport Science (EXSS) medical history questionnaire and undergoing a physical examination and 12-lead electrocardiogram in the Applied Physiology Laboratory. The physical examination includes auscultation of blood pressure, review of heart sounds, and pulmonary assessment. The physical examination and 12-lead electrocardiogram will be conducted and assessed by approved personnel.

- Height and mass will be obtained and you will be fitted for a heart rate monitor and then asked to rest lying down for 10 minutes. After obtaining your resting heart rate, you will be fitted for a mouthpiece that will be used to collect expired air.

- You will then perform a modified Bruce Protocol to volitional fatigue to determine VO_2max. The protocol consists of 3-minute stages with progressive speed at 0% inclined to near max, then grade will be introduced to maximal exertion (e.g. 3 minutes: 6.0, 0%, 3 minutes: 7.5, 0%, 3 minutes: 9.0, 0%, 3 minutes, 9.0, 2.5%). Heart rate (HR), rating of perceived exertion (RPE), and expired air will be monitored throughout the test. VO_2max attained will be used to determine running speed for the specific menstrual phase prolonged running bout for each individual subject.

Menstrual Phase Determination:

You will need to inform the principal investigator at the start of menses (the first menses following visit 1 to the lab), which will be denoted as day one. Scheduling of the prolonged treadmill running bout will correspond to a specific menstrual phase.

Prolonged treadmill running bout 1, duration approximately 2 hours (Visit 2 and 4):

The time lapse between visit 1 and visit 2 is not determined by investigators, rather it is determined based upon your menstrual cycle. The start of your menstrual cycle (e.g. menses, day 1) and the approximate length of your menstrual cycle (day 1 of menses to the start of the next menses) will determine when you are scheduled for the prolonged running bouts so as to ensure you are in the appropriate menstrual cycle phase. The approximate time between visit 1 and visit 2 can be 1-6 weeks. Between visit 1 and 24-hours before visit 2 you can partake in normal daily activities and exercise training with no restrictions. Twenty-four hours prior to the prolonged running bout you will be asked to refrain from exercise, drink plenty of fluids, and eat a diet rich in carbohydrates.

- Upon arrival, you will be asked to urinate into a sterile specimen container. The urine will be assessed for hydration status. If the urine analysis comes back as dehydrated you will not participate in the prolonged running bout, you will be encouraged to consume plenty of fluids, and you will be rescheduled.
- If the urine analysis comes back normal you will be weighed, fitted with a HR monitor, and asked to rest lying down for 10 minutes. After, a resting HR will be recorded and a 1-teaspoon blood sample will be drawn from your arm by a certified phlebotomist (NCPT 56147), placed into a K_3-EDTA blood collection tube and immediately put on ice. The blood sample will be used to confirm menstrual cycle status and resting IL-6, and estrogen levels.
- You will then be transferred to the treadmill and fitted for a mouthpiece. You will be asked to sit quietly for 4 minutes as your expired air is collected to determine resting VO_2.
- You will then have 10 minutes to warm-up: 5 minutes will be dedicated to easy walking on the treadmill followed by 5 minutes of stretching appropriate muscles used in the upcoming prolonged running bout (e.g. calf stretch, hamstring stretch, quadriceps stretch, and hip flexor stretch). During the walking, you will practice going on and off the mouthpiece as you are moving.
- Following warm-up, you will run on a treadmill at a 0% incline and a speed to elicit 65-70% VO_2max. At 6 minutes, 26 minutes, and 56 minutes you will be asked to return to the mouthpiece and expired air will be recorded for four minutes. This is to ensure appropriate intensity and make adjustments in running speed if necessary. Heart rate and RPE will be recorded during the last 10 seconds of minutes 9, 29, and 59 of the running bout.
- Throughout the running bout you will have a fan to keep you cool, can drink water <u>at your</u> convenience, and listen to music.
- At the completion of exercise, another 1-teaspoon blood sample will be drawn following the same procedure, placed in K_3-EDTA blood collection tube, and immediately placed on ice. This blood sample will be analyzed for IL-6 and estrogen concentrations. After blood collection you will cool down. The cool down will consist of walking on the treadmill at an easy pace for 5 minutes, stretching muscles used during the prolonged running bout (e.g. calf stretch, hamstring stretch, quadriceps stretch, and hip flexor stretch), and sitting quietly in a chair.
- 30 minute post blood draw
- Once your heart rate has returned to 100 bpm you are free to leave the laboratory.

Follow-up Blood Draws, duration approximately 30 minutes:

At 24 hours after the running bout you will report to the laboratory for additional blood draws. You will be asked to rest lying down for 10 minutes. Blood samples will be obtained following the same blood draw procedures as explained above. These blood samples will be analyzed for IL-6 and estrogen concentrations. During the 24 hours of recovery from the exercise you are asked to refrain from performing any physical activity other than that of daily routine living.

Prolonged treadmill running bout, duration approximately 2 hours:

You will be asked to repeat the aforementioned protocol during two different phases of your menstrual cycle. The time frame between the prolonged running bouts is approximately 2 to 6 weeks. Between visit 4 and 24-hours before visit 5 you can partake in normal daily activities and exercise training with no restrictions.

Blood Analysis:

The blood samples will be separated by centrifuging and frozen until later analysis. The blood plasma will be analyzed for estrogen levels, creatine kinase and immune markers.

What are the possible benefits from being in this study?
Research is designed to benefit society by gaining new knowledge. There is little chance you will benefit from being in this research study. The benefits to you from being in this study may be the obtaining of your Vo2max (aerobic capacity) which you can use in setting up a specific exercise training program.

What are the possible risks or discomforts involved from being in this study?
The potential risks to you from participating in this study may be related to exercise or the blood draw process.

Potential risks associated with exercise are outlined by American College of Sports Medicine as: sudden cardiac death, musculoskeletal injury, and falling.

- The risk of sudden cardiac death is low in healthy individuals; however, to minimize risk a health history questionnaire and physical examination will occur prior to testing.
- To minimize risk of musculoskeletal injury a proper warm-up will be completed prior to all testing.
- Given the prolonged nature of the exercise bout dehydration is a potential risk. To minimize this risk your hydration status will be determined before testing begins ensuring you are in normal hydrated state; if dehydrated the testing session will be cancelled and rescheduled. You will be asked to consume plenty of fluids 24 hours before testing and encouraged to drink water throughout and after the running bout.
- The universal sign for stopping an exercise session will be explained to you prior to all testing sessions. Research technicians will closely monitor your status during the exercise sessions for signs and symptoms of fatigue or a cardiac event to reduce risk of injury or falling.

Furthermore, the potential risk of exercise for you will be minimal because you have performed similar exercise intensities and durations within previous training programs.

Risks associated with blood draws include infection, bruising of the area around the needle insertion, and dizziness/fainting.

- To minimize infection, cleaning of the puncture area and sterile equipment will be used.

- Proper needle gauge and firm pressure applied to the puncture following the blood draw will help minimize risk of bruising.
- Following the blood draw, to minimize the risk of syncope you will be asked to move from a supine position to sitting and eventually standing slowly. Research technicians will monitor complexion and skin temperature for adverse signs.

A certified phlebotomist will perform all blood draws. First aid procedures and universal precautions will be followed during blood draws and handling of blood samples.

There may be uncommon or previously unknown risks. You should report any problems to the researcher.

What if we learn about new findings or information during the study?
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

How will information about you be protected?
Following initial screening, an identification number will be assigned to you for future identification. A hard copy of records will be stored in a locked cabinet in the Applied Physiology Laboratory. Electronic records will be maintained on a secured, password-protected computer. All identifiable hard-copy files will be shredded and disposed of using UNC-CH mechanisms and procedures. Blood samples will be stored in a secured ultra-freezer behind a access code protected door within a laboratory involving only electric ID card access. These specimens will be encoded and labeled so that no personal identifying information will be revealed. The identification number will consist of a unique number along with phase and the sample time (e.g. 00913, 009 is the subject ID, 1 is indicative of menstrual phase, 3 is time sample). Study data and specimens will only contain the identification number. These numbers will be indiscernible unless access to the master list which will be locked in a file cabinet in the Applied Physiology Laboratory. Only the principal investigator will have access to the records.

Participants will not be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies (for example, the FDA) for purposes such as quality control or safety.

What will happen if you are injured by this research?
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. You do not give up any of your legal rights by signing this form.

What if you want to stop before your part in the study is complete?
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped.

Will you receive anything for being in this study?
You will not receive anything for taking part in this study.

Will it cost you anything to be in this study?
It will not cost you anything to be in this study.

<u>What if you are a UNC student?</u>
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

<u>What if you have questions about this study?</u>
You have the right to ask, and have answered, any questions you may have about this research. If you have questions about the study (including payments), complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

<u>What if you have questions about your rights as a research participant?</u>
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

Participant's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

_____ _____
Signature of Research Participant   Date

_____
Printed Name of Research Participant

_____ _____
Signature of Research Team Member Obtaining Consent   Date

_____
Printed Name of Research Team Member Obtaining Consent

APPENDIX C

MEDICAL SREENING FORMS

Physical Examination Screening

Examination status: Approved   Disapproved

Department of Exercise and Sport Science
Physical Examination Screening

Name: _____ Age: _____ Gender: _____

Please respond to each of the following in writing.

Pulse rate and regularity: _____ ECG Interpretation: _____

Blood Pressure:
Supine: _____ Sitting: _____ Standing (Left side): _____
Squat: _____ Standing (Right side): _____

Marfan Syndrome evaluation: (Δ BP, Physical Char.) _____

Palpation of Pulses: Carotid: _____ Radial: _____ Pedal: _____

Auscultation of the Lungs:
Back: Lower: _____ Middle: _____ Upper: _____
Front: Middle: _____ Upper: _____

Auscultation of Heart Sounds (Supine, Standing, Squatting)
Non-Specific HS: _____/_____/_____
Murmur: ____ Gallop: ____ Click: ____ Rub: ____ Click w/ Murmur: ____

Bruits: Carotid: _____ Abdominal: _____

Edema: Abdominal: _____ Calf: _____ Pedal: _____

Tenderness: Abdominal: _____ Other: _____

Xanthoma or xanthelasm: _____

Medical/Family History:
High Blood Pressure: _____ Diabetes: _____ CHD/CAD: _____

Last examination w/ physician: _____
Medications (prescription/ counter): _____

Examiner: _____ Date: _____

Medical History Questionnaire

Department of Exercise and Sport Science
Medical History

Subject:_____ ID:_____ Telephone:_____

Address:_____

Occupation:_____ Age:_____

<div style="text-align: right">YES   NO</div>

Patient History

1. How would you describe your general health at present?
    Excellent_____ Good_____ Fair_____ Poor_____
2. Do you have any health problems at the present time?   _____ _____
3. If yes, please describe:_____

---

4. Have you ever been told you have heart trouble?   _____ _____
5. If yes, please describe:_____

---

6. Do you ever get pain in your chest?   _____ _____
7. Do you ever feel light-headed or have you ever fainted?   _____ _____
8. If yes, please describe:_____

---

9. Have you ever been told that your blood pressure has been elevated? _____ _____
10. If yes, please describe:_____

---

11. Have you ever had difficulty breathing either at rest or with exertion? _____ _____
12. If yes, please describe:_____

---

13. Are you now, or have you been in the past 5 years, under a doctor's care for any
    reason?   _____ _____
14. If yes for what reason?_____

---

15. Have you been in the hospital in the past 5 years?   _____ _____
16. If yes, for what reason?_____

---

17. Have you ever experienced an epileptic seizure or been informed that you have
    epilepsy?   _____ _____
18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or
    another infectious disease during the past year?   _____ _____
19. If yes, name the disease:_____
20. Have you ever been treated for or told you might have diabetes? _____ _____
21. Have you ever been treated for or told you might or low blood sugar? _____ _____
22. Do you have any known allergies to drugs?   _____ _____
23. If so, what?_____

---

24. Have you ever been "knocked-out" or experienced a concussion? ____ ____
25. If yes, have you been "knocked-out" more than once? ____ ____
26. Have you ever experienced heat stroke or heat exhaustion? ____ ____
27. If yes, when?_____

28. Have you ever had any additional illnesses or operations? (Other than childhood diseases) ____ ____
29. If yes, please indicate specific illness or operations:_____

30. Are you now taking any pills or medications? ____ ____
31. If yes, please list:_____

32. Have you had any recent (within 1 year) difficulties with your:
   a. Feet ____ ____
   b. Legs ____ ____
   c. Back ____ ____

Family History
33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
   a. Sudden death ____ ____
   b. Cardiac disease ____ ____
   c. Marfan's syndrome ____ ____

Mental History
34. Have you ever experienced depression? ____ ____
35. If yes, did you seek the advice of a doctor? ____ ____
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? ____ ____
37. If yes, please list condition and if you are currently taking any medication.
Condition   Medication
_____
_____
_____

Bone and Joint History
34. Have you ever been treated for Osgood-Schlatter's disease? ____ ____
35. Have you ever had any injury to your neck involving nerves or vertebrae? ____ ____
36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? ____ ____
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? ____ ____
38. Have you ever experienced any injury to your arms, elbows, or wrists?____ ____

39. If yes, indicate location and type of injury:_____
_____

40. Do you experience pain in your back? _____ _____
41. Have you ever had an injury to your back? _____ _____
42. If yes, did you seek the advice of a doctor? _____ _____
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____ _____
44. Do you think you have a trick knee? _____ _____
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint surgery that presently limits your physical capacity? _____ _____
46. If yes, indicate where:_____
_____

47. Have you ever had a bone graft or spinal fusion? _____ _____

Activity History
48. During your early childhood (to age 12) would you say you were:
    Very active_____ Quite active_____ Moderately active_____ Seldom active_____
49. During your adolescent years (age 13-18) would you say you were:
    Very active_____ Quite active_____ Moderately active_____ Seldom active_____
50. Did you participate in:
    a. Intramural school sports? _____ _____
    b. Community sponsored sports? _____ _____
    c. Varsity school sports? _____ _____
    d. Active family recreation? _____ _____
51. Since leaving high school, how active have you been?
    Very active_____ Quite active_____ Active_____ Inactive_____
52. Do you participate in any vigorous activity at present? _____ _____
53. If yes, please list:

Activity	Frequency	Duration	Intensity

54. How would you describe your present state of fitness?
Excellent_____ Good_____ Fair_____ Poor_____
55. Please list the type(s) of work you have been doing for the previous ten years:

Year	Work	Indoor/Outdoor	Location (city/state)

56. Whom shall we notify in case of emergency?
    Name:_____
    Phone: (Home)_____ (Work)_____
    Address:_____

57. Name and address of personal physician:_____

_____

_____

All of the above questions have been answered completely and truthfully to the best of
my knowledge.

Signature:_____ Date:_____

ACSM METABOLIC EQUATION FOR DETERMINING EXERCISE INTENSITY

Running

Running:	$VO_2 =$	H	+	V		+	R

Running: $VO_2 = 0.2$ (speed) $+ 0.9$ (speed)(fractional grade) $+ 3.5$

$0.2 \ ml \ x \ kg^{-1} \ x \ min^{-1} = O_2 \ cost \ of \ horizontal \ motion$

$0.9 \ ml \ x \ kg^{-1} \ x \ min^{-1} = O_2 \ cost \ of \ vertical \ ascent \ cost$

MENSTRUAL CYCLE QUESTIONNAIRE

Menstrual Cycle Questionnaire

Date: _____   Time: _____

Subject Name: _____   Age: _____   DOB: _____

(Evaluator, where appropriate, circle the answer)

1. At what age did you have your first period? _____

  - *If you think back over the last 6 months ......*

2. On average, how long is your monthly cycle (from menses to menses)? _____
     (Evaluator, they may answer by giving you a range of days)

3. What was the date of your last period (start)? _____

4. Are your monthly cycles regular (approximate same length) every time?   YES   NO

5. How long does your period typically last? _____

6. Do you have painful periods?   YES   NO

7. Does exercising affect your perception of how difficult your period is?
         MAKES IT WORSE   MAKES IT BETTER   NO EFFECT

8. Do you ever get any nausea or vomiting before, during or after your period?   YES   NO

9. Do you ever experience any mood changes before, during or after your period?   YES   NO

10. Do you take any form of birth control pills (oral contraceptive)?   YES   NO

  - If yes, what type? _____

  - if yes, for how long? _____

FOOD LOG

24 Hour Diet Recall

Name: _____ Date: _____
Day of the week: Monday Tuesday Wednesday Thursday Friday Saturday Sunday
Does this day represent your typical eating habits? Yes No
Please be as specific and honest as possible for review.
Day 1

Time	FOOD/BEVERAGES	Method of Preparation - (baked, fried, boiled, canned etc.) Brand Name	Amount/Serving Size

APPENDIX G

DATA COLLECTIONS FORM

Orientation Session

Subject ID: _____   Date: _____   Time: _____

Height: _____   Weight: _____   Resting HR: _____

Skf Triceps: _____ , _____ , _____   avg: _____   Skf Abd: _____ , _____ , _____   avg: _____

SKf Suprailium: _____ , _____ , _____   avg: _____   Skf Thigh: _____ , _____ , _____   avg: _____

Age: _____   Glut Circ: _____

Calc %BF: _____

Each stage is 3 minutes long. Please record average HR for the last 10 seconds of each minute within each stage. During the last 10 seconds for the stage record both HR and RPE. Regardless of if the stage is complete or not, get a final HR and RPE and hit STOP on the timer at the end of the test.

TREADMILL STAGE 1

Speed: __1.7__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __10__   Minute 3   HR: _____   RPE: _____

TREADMILL STAGE 2

Speed: __2.5__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __12__   Minute 3   HR: _____   RPE: _____

TREADMILL STAGE 3

Speed: __3.4__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __14__   Minute 3   HR: _____   RPE: _____

TREADMILL STAGE 4

Speed: __4.2__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __16__   Minute 3   HR: _____   RPE: _____

TREADMILL STAGE 5

Speed: __5.0__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __18__   Minute 3   HR: _____   RPE: _____

TREADMILL STAGE 6

Speed: __5.5__   Minute 1   HR: _____

    Minute 2   HR: _____

Grade: __20__   Minute 3   HR: _____   RPE: _____

The subject will give a 1 minute warning when the end of the test is nearing. I will count down that last minute. I will tell the subject to grab the handrails and walk the treadmill to a stop. Once they grab the handrails, hit "STOP" on the treadmill. When the treadmill stops, the subject will straddle the belt, I'll remove the headgear, and then hit "START" on the treadmill so the subject can cool down. Subject will walk on the treadmill for a few minutes, then be transferred to the table, where they will lie down until HR is below 100.

64

Experimental Session

ID:_____   Date:_____   Time:_____   Session:_____   Phase:_____

Calculated Ex. Intensity:_____mL/kg/min   Speed:_____mph   Supine RHR_____bpm

Exercise Session (HR and RPE Recording)

Minutes 6-10: VO$_2$ measurement		
10 minutes	HR	Notes:
	RPE	
Minutes 26-30: VO$_2$ measurement		
30 minutes	HR	Notes:
	RPE	
Minutes 56-60: VO$_2$ measurement		
60 minutes	HR	Notes:
	RPE	

Hematocrit and Hemoglobin

Hematocrit & Hemoglobin

Pre

Hematocrit

_____ _____ _____ Average _____

Hemoglobin

_____ _____ _____ Average _____

Immediately Post

Hematocrit

_____ _____ _____ Average _____

Hemoglobin

_____ _____ _____ Average _____

30 Minute Post

Hematocrit

_____ _____ _____ Average _____

Hemoglobin

_____ _____ _____ Average _____

24 Hour Post

Hematocrit

_____ _____ _____ Average _____

Hemoglobin

_____ _____ _____ Average _____

Subject Compliance

Have you had anything to eat or drink, besides water, within the last two hours?

Yes   No

Have you had any anti-inflammatory drugs (Advil, Tylenol, etc) within the last 24 hours?

Yes   No

Have you participated in any strenuous exercise within the last 24 hours?

Yes   No

Have you had any caffeine or alcohol within the last 24 hours?

Yes   No

Do you have your diet log? (make a copy and give back to subject)

Yes   No

APPENDIX H

ASSAY SHEETS

Estrogen Assay Information

REAGENT PREPARATION

1. All reagents should be brought to room temperature (18-25℃) before use.
2. Samples with expected Estradiol concentrations over 1000 ng/ml may be quantitated by dilution with diluent available from Abnova, Inc.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 25 µl of standards, specimens and controls into appropriate wells.
3. Dispense 100 µl of Estradiol-HRP Conjugate Reagent into each well.
4. Dispense 50 µl of rabbit anti-Estradiol(E2) reagent to each well.
5. Thoroughly mix for 30 seconds. It is very important to mix them completely.
6. Incubate at room temperature (18-25℃) for 90 minutes.
7. Rinse and flick the microwells 5 times with distilled or deionized water. (Please do not use tap water.)
8. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
9. Incubate at room temperature (18-25℃) for 20 minutes.
10. Stop the reaction by adding 100 µl of Stop Solution to each well.
11. Gently mix 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.
12. Read absorbance at 450 nm with a microtiter well reader within 15 minutes.

TNF-α Assay Information

<u>Sample Preparation</u>

Cell culture supernatant, serum and plasma (EDTA, citrate, heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human TNF-α. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently. Do not thaw samples in a 37 °C water bath. Do not vortex or sharply agitate samples.

<u>Assay Procedure</u>

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

2. Wash the microwell strips twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10-15 seconds before aspiration. Take care not to scratch the surface of the microwells.
   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes):
   Add 100 µl of Sample Diluent in duplicate to all standard wells. Pipette 100 µl of prepared standard (concentration – 3000 pg/ml) in duplicate into well A1 and A2 (see Plate Layout). Mix the contents of

wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 – 1500 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human TNF-α standard dilutions ranging from 1500.0 to 23 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7

Transfer 100 µl

S1   S2   S3   S4   -   S7

Reconstituted
Human TNF-α total
Standard

Sample Diluent
100 µl

Discard
100 µl
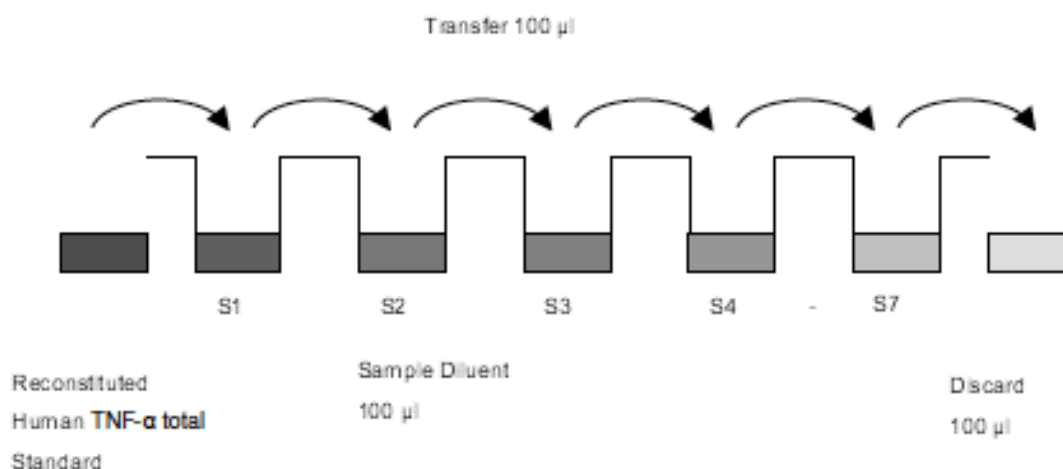
In case of an external standard dilution, pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Plate Layout.

4. Add 100 µl of Sample Diluent in duplicate to the blank wells.
5. Add 50 µl of Sample Diluent to the sample wells.
6. Add 50 µl of each sample in duplicate to the sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl of Biotin-Conjugate to all wells.
9. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on microplate shaker set at 400 rpm.
10. Prepare Streptavidin-HRP.
11. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point. 2. of the test protocol. Proceed immediately to the next step.
12. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.
13. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.
14. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
15. Pipette 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure

to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable.

Determination of the ideal time period for colour development has to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9-0.95.

17. Stop the enzyme reaction by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2-8°C in the dark.

18. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

IL-6 Assay Information

Sample Preparation

Cell culture supernatant, serum and plasma (EDTA, citrate, heparin) were tested with this assay. Other body fluids might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human IL-6. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Assay Procedure

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

2. Wash the microwell strips twice with approximately 400 μl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see External Standard Dilution): Add 100 μl of Assay Buffer (1x) in duplicate to all standard wells. Pipette 100 μl of prepared standard (concentration – 200.00 pg/ml), in duplicate, into well A1 and A2 (see Plate Layout). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1 S1 – 100.00 pg/ml), and transfer 100 μl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human IL-6 standard dilutions, ranging from 100.00 to 1.56 pg/ml. Discard 100 μl of the contents from the last microwells (G1, G2) used.

Figure 7

Transfer 100 µl

S1   S2   S3   S4   -   S7

Reconstituted
Human IL-6
Standard

Assay Buffer (1x)
100 µl

Discard
100 µl
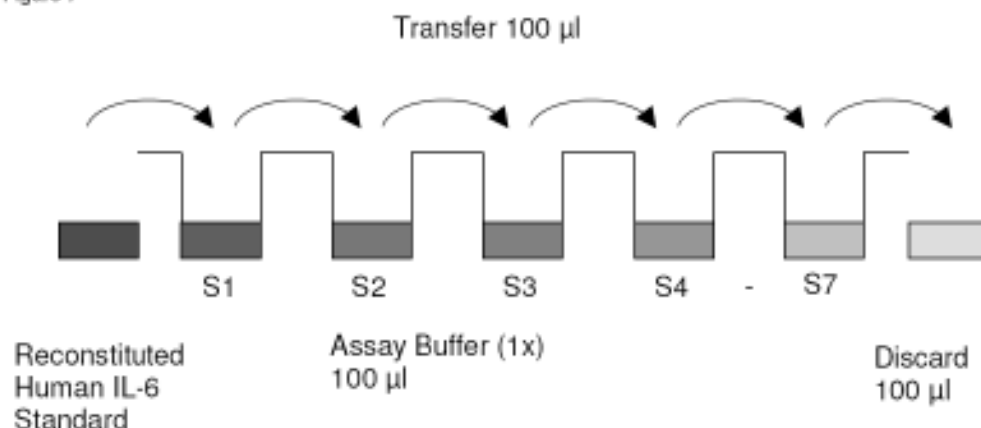
In case of an external standard dilution, pipette 100 µl of these standard dilutions (S1 to S7) in the standard wells according to plate layout.

4. Add 100 µl of Assay Buffer (1x) in duplicate to the blank wells.
5. Add 50 µl of Assay Buffer (1x) to the sample wells.
6. Add 50 µl of each sample in duplicate to the sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl of Biotin-Conjugate to all wells.
9. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 400 rpm.
10. Prepare Streptavidin-HRP.
11. Remove adhesive film and empty wells. Wash microwell strips 4 times according to point 2 of the test protocol. Proceed immediately to the next step.
12. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.
13. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.
14. Remove adhesive film and empty wells. Wash microwell strips 4 times according to point 2 of the test protocol. Proceed immediately to the next step.
15. Pipette 100 µl of TMB Substrate Solution to all wells
16. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells is no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

17. Stop the enzyme reaction by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

18. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

    Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

REFERENCES

Angstwurm, M., Gartner, R., & Loms Ziegler-Heitbrock, H. W. (1997). Cyclic plasma IL-6 levels during normal menstrual cycle. *Cytokine, 9*(5), 370-374.

Ayres, S., Baer, J., & Ravi Subbiah, M. T. (1998). Exercise-induced increase in lipid peroxidation parameters in amenorrheic female athletes. *Fertility and Sterility, 69*(1), 73-77.

Bar, P. R., Amelink, G. J., Oldenburg, B., & Blankenstein M.A. (1988). Prevention of exercise-induced muscle membrane damage by oestradiol. *Life Sciences, 42*, 2677-2681.

Belcastro, A., Shewchuk, L., & Raj, D. (1998). Exercise-induced muscle injury; A calpain hypothesis. *Molecular and Cellular Biochemistry, 179*, 135-145.

Bonen A, Ling W, MacIntyre K, Neil R, McGrail J, Belcastro A. (1979). Effects of exercise on the serum concentrations of FSH, LH, progesterone, and estradiol. *European Journal of Applied Physiology*. (42), 15-23.

Brenner, I. K. M., Natale, V. M., Vasiliou, P., Moldoveanu, A. I., Shek, P. N., & Shephard, R. J. (1999). Impact of three different types of exercise on components of the inflammatory response. *European Journal of Applied Physiology,* (80), 452-460.

Chavanne T, Gallup G. (1998). Variation in risk taking behavior among female college students as a function of the menstrual cycle. *Evolution and Human Behavior*. 19(1), 27-32.

Carter, A., Dobridge, J., & Hackney, A. C. (2001). Influence of estrogen on markers of muscle tissue damage following eccentric exercise. *Human Physiology, 27*(5), 626-630.

Chiu, K. M., Arnaud, C. D., Mayes, D., Bacchetti, P., Weitz, S., & Keller, E. T. (2000). Correlation of estradiol, parathyroid hormone, interleukin-6, and soluble interleukin-6 receptor during the normal menstrual cycle. *Bone, 26*(1), 79-85.

Clarkson, P., & Hubal, M. (2001). Are women less susceptible to exercise-induced muscle damage? *Current Opinion in Clinical Nutrition and Metabolic Care,* (4), 527-531.

Chao T, Van Alten P, Greager J, Walter R. (1995). Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cellular Immunology*. (160), 43-49.

Dieli-Conwright, C., Spektor, T., Rice, J., & Schroeder, E. T. (2009). Hormone therapy attenuates exercise-induced skeletal muscle damage in postmenopausal women. *Journal of Applied Physiology, 107*, 853-858.

Dill DB, Costill DL. (1974). Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *Journal of Applied Physiology*. (37), 247-248.

Edwards, K. M., Burns, V. E., Ring, C., & Carroll, D. (2006). Individual differences in the interleukin-6 response to maximal and submaximal exercise tasks. *Journal of Sports Science, 24*(8), 855-862.

Enns, D., Iqbal, S., & Tiidus, P. (2008). Oestrogen receptors mediate oestrogen-induced increases in post-exercise rat skeletal muscle satellite cells. *Acta Physiologica, 194*, 81-93.

Enns, D., & Tiidus, P. (2008). Estrogen influences satellite cell activation and proliferation following downhill running in rats. *Journal of Applied Physiology, 104*, 347-353.

Enns, D., & Tiidus, P. (2010). The influence of estrogen on skeletal muscle. *Sports Medicine, 40*(1), 41-58.

Fantone JC, Ward PA. (1982). Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *American Journal of Pathology*. 107(3), 395-418.

Febbraio, M. A., & Pedersen, B. K. (2002). Muscle-derived interleukin-6: Mechanisms for activation and possible biological roles. *The Journal of the Federation of American Societies for Experimental Biology, 16*, 1335-1347.

Feng, X., Li, G., & Wang, S. (2004). Effects of estrogen on gastrocnemius muscle strain injury and regeneration in female rats. *Acta Pharmacologica Sinica, 25*(11), 1489-1494.

Hackney, A. C. (1999). Influence of oestrogen on muscle glycogen utilization during exercise. *Acta Pharmacologica Scandinavia,* (167), 273-274.

Hackney, A. C., Curley, C. S., & Nicklas, B. J. (1991). Physiological response to submaximal exercise at the mid-follicular, ovulatory, and mid-luteal phases of the menstrual cycle. *Scandinavian Journal of Medicine Science and Sport,* (1), 94-98.

Hackney, A. C., Koltun, K., & Kallman-Meyer, A. L. (2012). Estrogen status and the IL-6 response to prolonged endurance exercise. *American Physiological Society-American College of Sports Medicine Joint Meeting, B*oulder, CO.

Hamer M, Karageorghis C. (2007). Psychobiological mechanisms of exercise dependence. *Sports Medicine*. 37(6), 477.

Iqbal, S., Thomas, A., Bunyan, K., & Tiidus, P. (2008). Progresterone and estrogen influence postexercise leukocyte infiltration in overiectomized female rats. *Applied Physiology, Nutrition, Metabolism, 33*, 1207-1212.

Ives, S., Blegen, M., Coughlin, M., Redmond, J., Matthews, T., & Paolone, V. (2011). Salivary estradiol, interleukin-6 production, and the relationship to substrate metabolism during exercise in females. *European Journal of Applied Physiology, 111*, 1649-1658.

Jackson, A., Pollock, M., Ward, A. (1980). Generalized equations for predicting body density of women. *Medicine and Science in Sports and Exercise.* 12(3), 175-182.

Jonsdottir, I. H., Schjerling, P., Ostrowski, K., Asp, S., Richter, E. A., & Pedersen, B. K. (2000). Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles. *Journal of Physiology, 528.1*, 157-163.

Kanter M. (1998). Free radicals, exercise and antioxidant supplementation. *Proceedings of the Nutrition Society.* 57, 9-13.

Kendall, B., & Eston, R. (2002). Exercise-induced muscle damage and the potential protective role of estrogen. *Sports Medicine, 32*(2), 103-123.

Komulainen, J., Koskinen, S. O. A., Kalliokoski, R., Takala, T. E. S., & Vihko, V. (1999). Gender differences in skeletal muscle fibre damage after eccentrically biased downhill running in rats. *Acta Pharmacologica Scandinavia, 165*, 57-63.

MacIntyre, D., Sorichter, S., Mair, J., Berg, A., & McKenzie, D. (2001). Markers of inflammation and myofibrillar proteins following eccentric exercise in humans. *European Journal of Applied Physiology, 84*, 180-186.

Malm, C., Sjoden, T. L. B., Sjoberg, B., Lenkei, R., Renstrom, P., Lundberg, I., & Ekbolm, B. (2004). Leukocytes, cytokines, growth factors, and hormones in human skeletal muscle and blood after uphill or downhill running. *Journal of Physiology, 556.3*, 983-1000.

Mendham, A., Donges, C., Liberts, E., & Duffield, R. (2011). Effects of mode and intensity on the acute exercise-induced IL-6 and CRP responses in sedentary, overweight population. *European Journal of Applied Physiology, 111*, 1035-1045.

Northoff, H., Symons, S., Zieker, D., Schaible, E. V., Schafer, K., Thoma, S., . . . Fehrenbach, E. (2008). Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. *Exercise Immunology Review,* (14), 86-103.

Ostrowski, K., Rohde, T., Zacho, M., Asp, S., & Pederson, B. K. (1998). Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *Journal of Physiology, 508.3*, 949-953.

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., & Pedersen, B. K. (1999). Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *Journal of Physiology, 515.1*, 287-291.

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, C., Keller, P., Plomgaard, P., . . . Febbraio, M. (2004). The metabolic role of IL-6 produced during exercise: Is IL-6 an exercise factor? *Proceedings of the Nutrition Society, 63*, 263-267.

Pedersen, B. K., Steensberg, A., Fischer, C., Keller, P., Plomgaard, P., Febbraio, M., & Saltin, B. (2003). Searching for the exercise factor: Is IL-6 a candidate? *Journal of Muscle Research and Cell Motility, 24*, 113-119.

Pedersen, B. (2000). Exercise and cytokines. *Immunology and Cell Biology,* (78), 532-535.

Persky, A., Green, P., Stubley, L., Howell, C., Zaulyanov, L., Brazeau, G., & Simpkins, J. (2000). Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. *Proceedings of the Society for Experimental Biology and Medicine, 223*

Pfeilschifter, J., Koditz, R., Pfohl, M., & Schatz, H. (2002). Changes in pro-inflammatory cytokine activity after menopause. *Endocrine Reviews, 23*(1), 90-119.

Pottratz, S., Bellido, T., Mocharia, H., Crabb, D., & Manolagas, S. (1994). 17β- estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism *The Journal of Clinical Investigation, 93*, 944-950.

Puder, J., Freda, P., Goland, R., & Wardlaw, S. (2001). Estrogen modulates the hypothalamic-pituitary-adrenal and inflammatory cytokine responses to endotoxin in women. *The Journal of Clinical Endocrinology & Metabolism, 86*(6), 2403-2408.

Roth, S. M., Martel, G. F., Ivey, F. M., Lemmer, J. T., Tracy, B. L., Metter, E. J., . . . Rogers, M. A. (2001). Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training. *Journal of Gerontology, 56A*(6), B240-B247.

Roth, S., Martel, G., Ivey, F., Lemmer, J., Metter, J., Hurley, B., & Rogers, M. (2000). High-volume, heavy-resistance strength training and muscle damage in young and older women. *Journal of Applied Physiology, 88*, 1112-1118.

Schwarz, E., Shafer, C., Bode, J., & Bode, C. (2000). Influence of the menstrual cycle on the LPS-induced cytokine response of monocytes. *Cytokine, 12*(4), 413-416.

Sewright, K., Hubal, M., Kearns, A., Hobrook, M., & Clarkson, P. (2008). Sex differences in response to maximal eccentric exercise. *Medicine and Science in Sports and Exercise. 40(2),* 242-251.

Smith, L. L., Anwar, A., Fragen, M., Rananto, C., Johnson, R., & Holbert, D. (2000). Cytokines and cell adhesion molecules associated with high-intenisty eccentric exercise. *European Journal of Applied Physiology, 82*, 61- 67.

St. Pierre Schneider, B., Correia, L., & Cannon, J. (1999). Sex differences in leukocyte invasion in injured murine skeletal muscle. *Research in Nursing & Health, 22*, 243-251.

Sugioka, K., Shimosegawa, Y., & Nakano, M. (1987). Estrogens as natural antioxidants of membrane phospholipid peroxidation. *Federation of European Biochemical Societies Letters. 210*(1), 37-39.

Suzuki, K., Totsuka, M., Nakaji, S., Yamada, M., Kudoh, S., Liu, Q., . . . Sato, K. (1999). Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *Journal of Applied Physiology, 87*, 1360-1367.

Thompson W, Gordon N, Pescatello L, eds. (2010). *ACSM's guidelines for exercise testing and prescriptions.* 8th Edition ed. Lippincott Williams and Wilkins.

Tiidus, P. M., & Bombardier, E. (1999). Oestrgen attenuates post-exercise myeloperoxidase activity in skeletal musce of male rats. *Acta Pharmacologica Scandinavia, 166*, 85-90.

Tiidus, P. (2001). Oestrogen and sex influence on muscle damage and inflammation: Evidence from animal models. *Current Opinion in Clinical Nutrition and Metabolic Care,* (4), 509-513.

Tiidus, P. (2003). Influence of estrogen on skeletal muscle damage, inflammation, and repair. *Exercise and Sports Science Reviews, 31*(1), 40-44.

Tiidus, P., Holden, D., Bombardier, E., Zajchowski, S., Enns, D., & Belcastro, A. (2001). Estrogen effect on post-exercise skeletal muscle neutrophil infiltration and calpain activity. *Canadian Journal of Applied Physiology, 79*, 400-406.

Timmons, B., Hamadeh, M., Devris, M., & Tarnopolsky, M. (2005). Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. *Journal of Applied Physiology, 99*, 979-985.

Timmons, B., Hamadeh, M., & Tarnopolsky, M. (2006). No effect of short-term 17β-estradiol supplementation in healthy men on systemic inflammatory responses to exercise. *American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology, 291*, R285-R290.

Whiting, K., Restall, C., & Brain, P. (2000). Steroid hormone-induced effects on membrane fluidity and their potential roles in non-genomic mechanisms. *Life Sciences, 67*, 743-757.

Yoshikawa T, Yoshida N. (2000). Vitamin E and leukocyte-endothelial cell interactions. *Antioxidants and Redox Signalling*. 2(4), 821-825