The Effect of Estradiol on IL-6 Concentration in Response to an Acute Bout of Exercise

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ABSTRACT

KRISTEN J. KOLTUN: The Effect of Estradiol on IL-6 Concentration in Response to an Acute Bout of Exercise
(Under the direction of Anthony C. Hackney, Ph.D., D.Sc.)

This study attempted to find the effect of differing estradiol (E$_2$) concentrations on the IL-6 response to an acute bout of exercise. Ten eumenorrheic women (n=10) who were not using oral contraceptives completed two 60 minute running sessions at 61% VO$_2$ peak, once during the follicular phase (low E$_2$) and once during the luteal phase (high E$_2$) of the menstrual cycle. Resting, immediately post exercise, and 30 minute recovery blood samples were assessed for IL-6. Resting E$_2$ concentrations confirmed the appropriate hormonal status for each experimental session. Neither the absolute concentration of IL-6 nor the percent change in IL-6 from rest was significantly different between phases (main effect and interaction effect; responses approached significance; p=0.07). In conclusion, there is no relationship between E$_2$ and IL-6 in response to exercise; although IL-6 trends towards being lower with high E$_2$ concentration. Based on this, it cannot be suggested that female athletes should alter training levels based on hormonal status, however, additional research is necessary as this study was underpowered ($\beta$=0.73).
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<tbody>
<tr>
<td>ACSM</td>
<td>American College of Sports Medicine</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>FFA</td>
<td>Free fatty acids</td>
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<td>Hb</td>
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<td>Hct</td>
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<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>Km/h</td>
<td>Kilometers per hour</td>
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<td>L</td>
<td>Liter</td>
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<td>µg</td>
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<td>Nanogram</td>
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<td>pg</td>
<td>Picograms</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory Exchange Ratio</td>
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<tr>
<td>RM</td>
<td>Repetition Maximum</td>
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<tr>
<td>Symbol</td>
<td>Description</td>
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<tr>
<td>--------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>RPE</td>
<td>Rating of Perceived Exertion</td>
</tr>
<tr>
<td>VO₂⁻max</td>
<td>Maximal oxygen consumption</td>
</tr>
<tr>
<td>VO₂⁻peak</td>
<td>Peak oxygen consumption</td>
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*"x"*
CHAPTER I

Introduction

The inflammatory response is a localized tissue response to injury, which consists of a series of non-specific events. This local response to infection or injury involves the production of cytokines, which are released at the site of inflammation. Cytokines then act as intracellular signals, facilitating the arrival of other immune cells, such as lymphocytes, neutrophils, and monocytes, which work to clear any antigens and facilitate the healing process.

Cytokines are released in two phases. Those released early in the process include Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1). These are pro-inflammatory cytokines which initiate and progress the inflammatory response. The second phase of cytokine release consists primarily of Interleukin-10 (IL-10) and Interleukin-1 Receptor Antagonist (IL-1ra), which are anti-inflammatory and lessen inflammation. Interleukin-6 (IL-6), which is released throughout the inflammatory response is an interesting cytokine because it is produced in larger amounts than any other cytokine and, at different points in the inflammation response, IL-6 may have different functions. Early in the inflammatory response, IL-6 concentration is correlated with TNF-α and IL-1 and acts as a pro-inflammatory cytokine. However, later in the response, IL-6 can upregulate IL-1ra and function as an anti-inflammatory cytokine.

Exercise has successfully been shown to result in local inflammation. Numerous studies have demonstrated that IL-6 values increase in response to physical activity. Estrogen concentrations are also raised in response to acute exercise bouts. Figure 1 demonstrates the timeline and interaction of the pro- and anti-inflammatory cytokines.
Figure 1. The cytokine response during inflammation. TNF-α and IL-1 are pro-inflammatory cytokines released early in the cascade. Later, the anti-inflammatory cytokines are released. These include IL-10 and IL-1ra. IL-6 peaks immediately post-exercise and has both pro- and anti-inflammatory effects.

Figure 2. Estrogen levels are low early in the cycle during the follicular phase, but rise just prior to ovulation. They then decline and plateau during the luteal phase at a higher concentration than during menses. At the end of the luteal phase, levels decline until menses begins.
Estrogens are the end product of the hypothalamus-pituitary-gonadal axis. Estrogen levels fluctuate in a regular pattern across the menstrual cycle, with most cycles ranging from 24-36 days and lasting 28 days on average. The follicular phase is the time prior to ovulation, which occurs at day 14, and the luteal phase is the second half of the cycle. The mid-follicular phase is characterized by low estrogen levels, and the mid-luteal has higher estrogen levels. Figure two demonstrates the cyclical pattern of estrogens during the menstrual cycle.

The three estrogens that circulate in females are estradiol, estrone, and estriol. The most prevalent and biologically active of these is estradiol. Estrogens have many functions in the body, including a link to the inflammatory response. Previous research suggests that estrogen, particularly estradiol, may have immune effects and be able to alter the inflammatory response, acting in an anti-inflammatory capacity.

Due to the immune and potentially anti-inflammatory effects of estrogen, the interaction between estrogen and IL-6 has been studied. In vitro and murine studies have consistently shown that estrogen inhibits IL-6 production and the inflammatory response. Puder et al. demonstrated that estrogen supplementation attenuates the IL-6 response in post-menopausal women. In studies conducted in humans in response to exercise, there has been conflicting data. Estradiol supplementation in men did not result in a decreased IL-6 response after exercise, but it is thought that there are differences in the estrogen receptor or the inflammation pathway between the genders or that the exposure time to estradiol was insufficient. Timmons et al. conducted a similar study, involving eleven men, six women on oral contraceptives, and six women not using oral contraceptives. This experiment found no significant differences in post-exercise IL-6 levels between the groups. However, although there were no significant differences, the IL-6 concentration was greater in each group when estradiol was at a lower concentration. It is possible that the sample size for this study was not large enough to find any significance between groups. Most recently Ives et al. found no significant differences in post-exercise IL-6 values across the menstrual cycle. But,
these women were on oral contraceptives and there were no significant differences in estrogen concentration across the two sessions. Oral contraception minimizes the fluctuation in estrogen concentration across the menstrual cycle, which would limit the effects on IL-6. Also, if the resting estrogen concentration was not significantly different between the two experimental trials and the exercise bouts were the same duration and intensity, it would not be expected that the change in IL-6 concentration would differ between the trials. Based on these results, there is mixed information on whether estrogen has an effect on IL-6. There is a need for a study examining the effect of an acute bout of exercise on local IL-6 concentration in women not using oral contraceptives at points during the menstrual cycle when the estrogen concentration is significantly different.

Statement of the Problem

The purpose of this experiment was to determine the effect of estradiol concentration on IL-6 concentration in response to an acute bout of exercise in females who are not using oral contraceptives.

Research Questions

1. What is the acute effect of exercise on plasma IL-6 concentration?

2. What is the effect of exercise and 30 minutes of recovery on plasma IL-6 concentration?

3. Is there a significant difference in plasma estradiol values at rest between the follicular and luteal phases?

4. Is there a significantly different %Δ in IL-6 concentration from rest to immediately post-exercise between the follicular and luteal phases?

5. Is there a significantly different %Δ in IL-6 concentration from rest to 30 minutes of recovery between the follicular and luteal phases?
Research Hypotheses

Hypothesis 1: Plasma IL-6 concentration will be significantly increased from rest immediately post-exercise.

Hypothesis 2: Plasma IL-6 concentration will be significantly increased from rest after 30 minutes of recovery from exercise.

Hypothesis 3: Pre-exercise plasma estradiol will be significantly higher during the luteal trials compared to follicular trials.

Hypothesis 4: The %Δ in plasma IL-6 concentration will be significantly lower during luteal trials compared to follicular trials from rest to immediately post-exercise.

Hypothesis 5: The %Δ in plasma IL-6 concentration will be significantly lower during luteal trials compared to follicular trials from rest to 30 minutes of recovery.

Definition of Terms

Inflammation – Acute inflammation is an early post-injury response characterized by a rapid increase in local blood flow and vascular permeability, and an influx of lymphocytes, such as neutrophils, and cytokines.4

Cytokines – Intracellular signals that are released at the site of inflammation and attract immune cells.4

Pro-inflammatory Cytokine – These are cytokines that enhance the inflammatory immune response, such as TNF-α and IL-1.18

Anti-inflammatory Cytokine – These are cytokines that restrict the magnitude and duration of the inflammatory response, and include IL-1ra and IL-10.18
**Interleukin-6 (IL-6)** – This cytokine is released in large amounts in response to exercise and has both pro- and anti-inflammatory effects.\(^{18}\) IL-6 peaks immediately post-exercise and is primarily pro-inflammatory at this time.\(^3\)

**Estradiol** – The primary and most biologically active estrogen, which has a specific biological rhythm based on the menstrual cycle.\(^5\) It is believed to have a strong anti-inflammatory effect.\(^8\)

**Eumenorrhea** – Having a regular menstrual period for at least the previous six months.

**Follicular Phase** – The first phase of the menstrual cycle, which lasts from menses until ovulation at approximately day 14. Estrogen levels remain low during this phase until there is a peak just prior to ovulation.\(^1\)

**Luteal Phase** – Lasts from ovulation until menses begins. It is characterized by estrogen levels that plateau, approximately between days 20-25, at a higher concentration than the follicular phase.\(^1\)

**Assumptions**

1. Subjects adhered to all diet and physical activity recommendations prior to the experimental trials.

**Limitations**

1. A pre-testing diet was recommended, but the subjects will be left to follow the guidelines on their own.

**Delimitations**

1. Only healthy, recreationally active, college aged, eumenorrheic females who are not on birth control were recruited for this study.

2. Diet was monitored, but not strictly controlled.

3. Sixty minutes of exercise at 65\% VO\(_2\) peak was the exercise duration and intensity.
4. Menstrual cycle phase was determined by the forward counting method and confirmed with urinary ovulatory kits.

**Significance**

This study attempted to determine the relationship between inflammation and the menstrual cycle, particularly how estradiol affects IL-6 levels in response to an acute bout of exercise. Inflammation produces local swelling and pain, which can be uncomfortable and hinder future activities. If inflammation is more severe, characterized by higher IL-6 levels, at different times in the menstrual cycle it may be a recommendation to decrease training levels or supplement with an anti-inflammatory drug during that phase. If inflammation can be controlled, this may allow for a high training load to be maintained, which could result in a higher performance capability.
CHAPTER II

Review of Literature

Introduction

This review of literature covers a variety of topics. First, the basic inflammatory response is discussed, with a particular focus on the release of cytokines. Following that, the role of IL-6 and its response to exercise is examined. Next, the fluctuation in estrogen resulting from the menstrual cycle is introduced. Estrogen is then further reviewed with respect to its roles in the body as well as how it is affected by an acute bout of exercise. Lastly, the interaction between IL-6 and estrogen is reviewed. This last portion begins with in vitro studies, then animal models and moves toward human based models ending with how this relationship is affected by exercise.

Cytokine Response

An acute bout of exercise can act as a physical stress on the body, often leading to inflammation. The inflammatory response is a localized tissue response to injury. This response consists of a series of non-specific events and local inflammation can lead to the systemic acute phase response. The local response to infection or injury involves the production of cytokines, which are then released at the site of inflammation. These cytokines act as intracellular signals, facilitating the arrival of other immune cells, such as lymphocytes, neutrophils, and monocytes, which work to clear any antigens and facilitate the healing process.

In response to exercise, or any other inflammation inducing event, there is a well-documented cascade of cytokines. Cytokines that are released early include TNF-α and IL-1. They are referred to
as pro-inflammatory because if they are injected, most, if not all, of the acute phase response will be produced. IL-6 alone will not induce inflammation and can be classified as an inflammation responsive cytokine. IL-6 has both pro- and anti-inflammatory effects, but immediately post-exercise its role is primarily pro-inflammatory and it is highly correlated with TNF-α and IL-1. IL-1ra and IL-10 are released into recovery from exercise and are anti-inflammatory in nature. All of these cytokines work in conjunction with each other in order to balance the inflammatory response, thereby allowing for recovery and healing of the damaged tissue.

**IL-6 Response to Exercise**

Numerous studies have been conducted in order to determine the relationship between IL-6 and an acute exercise bout. The exercise has ranged from a various types of aerobic exercise, to eccentric leg contractions, and a later purpose was to determine what type of mode and intensity is necessary to induce an IL-6 response to exercise.

The earlier study conducted by Ostrowski et al. had sixteen males complete the Copenhagen marathon. Blood was drawn one week prior to the race, immediately post-exercise, and two hours post-exercise and was used to measure plasma IL-6 and the mRNA for IL-6. Plasma IL-6 increased from 1.5 ± 0.7 pg/mL to 94.4 ± 12.6 pg/mL immediately after the race. This study also found that mRNA for IL-6 was detectable after the marathon. Ostrowski et al. followed up these findings with a similar study. Similar testing was conducted on ten male runners in the next year’s Copenhagen marathon. This study found similar results to the previous one and reported that IL-6 peaked immediately post-exercise, where it presented a 128 fold increase compared to pre-race values.

Later studies also used aerobic exercise to measure the IL-6 response to exercise, however they were conducted in a laboratory setting with a different duration and intensity. Timmons et al. found a significant increase in IL-6 concentration after having eleven recreationally active men cycle for ninety minutes at 65% of their VO₂ max. Ives et al. required ten active females who used oral
contraceptives to run on a treadmill for one hour at 65% of their VO$_2$ max and also found a significant increase above baseline values. This shows that IL-6 will increase with sufficient exercise in both genders.

Other studies utilized resistance exercise as the stimulus for increased IL-6. One particular protocol significantly increased IL-6 in twelve males after completing 300 eccentric leg contractions. Steensberg et al. required subjects do five hours of knee extensions at 40% maximal workload. This study not only further confirms that IL-6 will increase in response to exercise, but it also helps to target the source. Arterial plasma concentration of IL-6 increased from 0.74 ng/L at rest to 14.13 ng/L after five hours of one-legged knee exercise. By drawing blood from the femoral artery and vein, it was possible to measure the IL-6 produced by the active muscle. During this exercise bout net skeletal muscle IL-6 production increased significantly and this demonstrates that IL-6 can be produced by working muscle.

Mendham et al. examined the effect of mode and intensity of exercise on the acute IL-6 response. Four different exercise protocols were used. Two were resistance exercises at either 60% or 80% of their 1 repetition maximum (RM). The other two involved cycling at either 30% or 50% of their maximal aerobic workload. All four protocols lasted 40 minutes to control for time. When examining mode, there were no significant differences between the moderate-vigorous intensity exercise protocols. The other main result from this study was that the acute increase in IL-6 was significantly larger in both moderate-vigorous intensity protocols compared to the low-intensity protocols. In response to moderate-vigorous aerobic exercise the relative increase in IL-6 was $70.9 \pm 4.5\%$. Overall, this study suggests that when matched for time, moderate-vigorous resistance exercise and aerobic activities will result in similar IL-6 increases post-exercise. All of these studies support the concept that IL-6 is significantly increased from baseline values after an acute bout of exercise in both men and women. Also, the magnitude of the rise in IL-6 can vary depending on the duration and intensity of exercise.
Menstrual Cycle Phases

The sex hormone levels in females of reproductive age fluctuate in a cyclical pattern across the menstrual cycle. A typical cycle ranges from 24-36 days. Menses occurs approximately the first five days of the cycle and ovulation around day 14. The early days of the menstrual cycle, prior to ovulation, are referred to as the follicular phase and the days after ovulation are the luteal phase. By knowing where an individual is during the menstrual cycle it is possible to predict whether estrogen levels will be high or low.

Early in the follicular phase, estrogen levels are low until around day 8, when the levels begin to rise. The baseline estradiol concentration during the follicular phase is 97.3 ± 19.0 pg/mL. Estrogen levels peak at about day 12, just prior to ovulation. After ovulation estrogen levels decline, but reach a plateau that is of a higher concentration than during the follicular phase at approximately days 20-25. During the luteal phase, baseline estradiol levels are 196.3 ± 20.0 pg/mL. After that, estrogen levels are lowered even further until menses when the cycle begins again.

Estrogens and Estradiol

Estrogens are the end product of the hypothalamus-pituitary-gonadal axis. Gonadotropin releasing hormone (GnRH) is released from the hypothalamus, which then stimulates the release of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the anterior pituitary. These hormones affect the follicles, which then alter the estrogen levels.

There are three estrogens that circulate in females. These include estradiol (17β-estradiol), estrone, and estriol. The most abundant of these and the one that has the largest effects on target tissues is estradiol. These estrogens have multiple functions throughout the body, which include stimulating bone and muscle growth, forming secondary sex characteristics, altering central nervous system activity, and maintaining the function of reproductive glands and organs as well as altering the endometrium.
What makes estrogen so relevant to the inflammatory process is that some research has indicated that this hormone, particularly estradiol, may have immune and anti-inflammatory effects. Physiological levels of estradiol have been observed to boost the immune response in young adults during the reproductive years. Similarly, cytokines can activate the hypothalamic-pituitary-adrenal axis and gonadal steroids can also affect this pathway, thereby altering the response to inflammatory stimuli. These findings suggest that estrogen may have a significant effect on the inflammatory pathway and this interaction should be studied further.

**Estrogen Response to Exercise**

Previous studies have been conducted in order to examine the effect of exercise on estrogen and estradiol. These experiments first sought to find if estrogen levels would be altered in response to the physical stress of exercise. A later aim was to determine what type of stimulus would be necessary to induce those changes as well as examining phase differences.

Jurkowski *et al.* studied the effect of exercise on ovarian and gonadotropic hormones, as well as the effect of intensity and menstrual phase on these hormones. The design of this study required nine healthy females with regular menstrual cycles, who were not on oral contraceptives, to exercise at two different time points during the menstrual cycle. These time points were in the follicular (six to nine days after menses), and in the luteal (six to nine days after ovulation) phases. The exercise bout consisted of twenty minutes of cycling at 30-35% of their max power output, twenty minutes at 60-66% and then to exhaustion at 85-95% of maximal power output. This study determined that estradiol will increase with exercise. When comparing changes in concentration between the two testing dates, estradiol was only significantly higher at exhaustion during the follicular phase, but during the luteal phase estradiol increased with each rise in intensity. When examining the effects of intensity, this study suggests that estradiol will increase with greater intensity.
exercise. Light exercise did not significantly increase estradiol levels from rest, but it was significantly higher in both the heavy and exhaustive exercise.

Bonen et al. further investigated the effects of an acute bout of exercise on the concentration of estradiol as well as on LH, FSH, and progesterone. This study consisted of ten healthy females participating in thirty minutes of intense cycling with a mean intensity of 75% VO\textsubscript{2} max. Based on records of their menstrual histories their phase was determined. These participants were mostly in the luteal phase and during menses, but some had cycles that were too irregular to determine. Estradiol was measured pre- and post-exercise and the percent change was calculated. Overall, estradiol increased from 163.3 ± 45.7 pg/mL pre-exercise to 185.7 ± 50.5 pg/mL post-exercise for a percent change of 13.5 ± 7.5%. This study also suggests that there is relationship between the rise in estradiol and both the intensity and duration of exercise. With respect to intensity, estradiol concentrations increased when exercise was at intensity greater than 60% VO\textsubscript{2} max. Duration of thirty minutes was a long enough stimulus to bring about these changes as well.

More recent research has been conducted, showing similar rises in estradiol in response to acute bouts of exercise. Forty minutes of cycling at 75% of maximal heart rate has produced a significantly greater change in estradiol compared to control. One hour of treadmill exercise at 65% of VO\textsubscript{2} peak has also caused increases in estradiol concentration. Estradiol increased significantly with exercise, and there was no difference in concentration between thirty minutes and sixty minutes. These studies add more evidence to the concept that estradiol concentrations will increase in response to an acute bout of exercise.

Interaction of IL-6 and Estrogen

Many studies have been conducted in order to determine the effects of estrogen on the inflammatory response, particularly on IL-6 levels. These experiments have used in vitro cell
cultures, animal models, and human models and many different types of stress were utilized to induce inflammation.

One of the first studies on this topic used IL-1 and TNF-α to induce IL-6 production in human bone derived cells, as well as rat and murine osteoblasts. Girasole et al.\textsuperscript{15} measured the inhibition of estradiol on IL-6 production. In this study, under basal conditions, cells produced very little IL-6. Cells treated with IL-1 and TNF-α had much higher levels of IL-6 demonstrating that these are effective ways of inducing an IL-6 response. The addition of estradiol to these cells did not affect the basal production of IL-6 but it did significantly inhibit IL-6 when it was produced in response to either IL-1, TNF-α, or both. Inhibition was in a dose-dependent fashion. This study further examined if the inhibitory effect of estradiol on IL-6 was at the level of mRNA. It was determined that pretreatment with estradiol caused a 49% decrease in IL-6 mRNA after exposure to both IL-1 and TNF-α. These findings suggest that estrogens, such as estradiol, can have an inhibitory effect on IL-6.

Pottraz et al.\textsuperscript{16} examined the molecular basis of how estradiol inhibits IL-6 production using a human cell model (HeLa) as well as a mouse cell line (MBA 13.2). These cells were treated with 17α-estradiol, 17β-estradiol, progesterone, testosterone and diethylstilbestrol (DES; synthetic estrogen). Some cells were transfected with an estrogen receptor plasmid and some were given a control. In human HeLa cells that were both pretreated with 17β-estradiol and had the estrogen receptor, the stressor of phorbol 12-myristate 13-acetate (PMA) did not stimulate the promoter region. The mean value of the reporting factor in cells given both the IL-6 plasmid and the estrogen receptor plasmid and which were treated with estradiol was significantly less compared to cells treated with only estradiol or those without the receptor. In murine bone marrow cells that were treated similarly, cytokine stimulated production of IL-6 was also inhibited by estradiol. Overall, this study observed that IL-6 production by bone marrow and bone-derived cells from rodents and humans is inhibited by...
estradiol. The hormone requires an estrogen receptor to be present in order to have an effect and the inhibition of IL-6 production by estradiol is through a receptor-mediated action.

Rather than just using a particular human or mouse cell line, mice as a whole have also been used as a model to study the interaction between estradiol and IL-6. Kovacs et al.\textsuperscript{13} used burning/scalding of mice as a physical stress. Young mice that were injured showed a larger increase in IL-6 levels compared to animals that were not burned. Part of this study required the mice to be pretreated with estrogen. In the injured mouse group, estrogen attenuated IL-6 production. In the presence of estrogen, IL-6 levels were lowered to nearly half of the value compared to when they were injured but not treated with estrogen. The IL-6 value in mice that were injured and treated with estrogen was not significantly greater than the value in mice that were not injured. This experiment used a mouse model and burning as a stressor to induce an IL-6 response and it further demonstrates that treatment with estrogen can lower the IL-6 response.

On a larger scale, IL-6 and estradiol were examined in humans. Puder et al.\textsuperscript{14} used an endotoxin to stimulate IL-6 production. Endotoxin is known to stimulate the synthesis and release of inflammatory cytokines from peripheral monocytes and macrophages. This study consisted of six females between the ages of 42-68 who were menopausal or had undergone an oophorectomy. All of these women had low estrogen levels and they were treated with a transdermal estradiol patch for one month and injected with endotoxin. Endotoxin successfully increased IL-6 concentrations in both the presence and absence of estradiol. However, in the estradiol treated group, mean IL-6 values peaked at $341 \pm 94$ pg/mL compared with $936 \pm 620$ pg/mL in the control. The area under the curve (AUC) of this response was also calculated. The AUC for IL-6 calculated for a five hour period after endotoxin injection was 31.3\% lower in the group treated with estradiol compared to the untreated group. Although the mechanism is still unclear, this is another example of how the IL-6 response was attenuated in the presence of estradiol.
More recent studies have focused on this interaction in response to exercise. Timmons et al.\(^8\) used eleven healthy, recreationally active men and had them cycle at 65% VO\(_2\) max for 90 minutes. Each group was treated with estradiol for eight days and was left untreated for eight days. Both trials resulted in an increase in IL-6, however no effect of estradiol supplementation was found. A potential reason for there being no differences between groups could be based on gender differences in the estrogen receptor or that eight days was not a sufficient exposure time.

Timmons et al.\(^{17}\) did further research on this topic. This time the subjects consisted of twelve women, six of which were on oral contraceptives, and eleven men. They underwent the same exercise protocol consisting of 90 minutes of cycling at 65% VO\(_2\) max. Women were tested during the follicular (day 8 ± 3) and luteal (day 20 ± 2) phases. Resting levels of IL-6 were not significantly different across any group or menstrual phase. Estradiol concentrations were lower in oral contraceptive users compared to non-users, and they did not fluctuate significantly across the menstrual cycle. The estradiol concentration in men was 134 ± 70 pmol/L. Estradiol in non-contraceptive users in the follicular phase was 195 ± 188 pmol/L and in the luteal phase was 361 ± 263 pmol/L. In oral contraceptive users, estradiol was 42 ± 41 pmol/L in the follicular phase and 14 ± 11 in the luteal phase. Post exercise IL-6 values were also different, but not significantly. Men had a post exercise IL-6 concentration of 6.7 ± 2.8 pg/mL. Non oral contraceptive users in the follicular phase had values of 7.7 ± 2.4 pg/mL and in the luteal phase had values of 6.3 ± 0.8 pg/mL. Oral contraceptive users in the follicular phase had concentrations of 5.5 ± 2.1 pg/mL and in the luteal phase it was 6.8 ± 1.0 pg/mL. Although there were no significant differences, the IL-6 concentration was greater in each group when estradiol was at a lower concentration.

Most recently, Ives et al.\(^5\) studied ten women on oral contraceptives and required them to run on a treadmill for one hour at 65% VO\(_2\) max. This study also suggests that estradiol and IL-6 were not significantly related across phase or time. However, in this study there were no significant differences in the estradiol concentration of the subjects across the two time points.
Many studies have examined the interaction between IL-6 and estrogen, particularly estradiol, in response to a physical stress. Early in vitro and murine studies have suggested that there is a relationship between these two variables, and that estradiol will attenuate the IL-6 response. Some human based studies have also supported this finding. More recent experiments have used exercise as the stressor and measured estradiol and IL-6 values. In these examples, there has been no significant effect of estradiol on IL-6 levels.

Summary

This review of literature has provided information on numerous topics. First, the basic inflammatory response to a stress was introduced. Next, the IL-6 response to the physical stress of exercise was discussed. Then, the fluctuations in hormone levels across the menstrual cycle were presented. Next, the physiology of estrogen release was mentioned and how it relates to the immune response was introduced. After that, the estrogen response to exercise was discussed. Finally, the interaction between IL-6 and estrogen was detailed.
CHAPTER III

Methodology

This study consisted of three visitations for each participant. The first session included an orientation and maximal oxygen uptake (VO₂ max) test. The next two sessions were the testing days, which occurred during the midfollicular and midluteal phases of the menstrual cycle. During the orientation session the protocols were explained, participant characteristics were obtained, menstrual history was recorded, and VO₂ max was measured. Sessions I and II were the experimental tests which required participants to perform 60 minutes of treadmill running at 65% of their predetermined VO₂ max. Baseline, immediately post-exercise, and 30 minutes into recovery blood samples were taken to measure estradiol and IL-6 concentrations.

Participants

Ten healthy, recreationally active, pre-menopausal women were recruited for this study. Sample size was estimated from previous research in this laboratory to have sufficient power based on ten subjects. To be included in this study, participants had to be eumenorrheic for the past six months and have not used oral contraceptives or any other hormone therapy in the six months prior to participation. Also, subjects could not currently be taking any anti-inflammatory medications, such as ibuprofen, naproxen, or aspirin. Each participant was informed of the experimental protocol and any risks and rewards. They signed an informed consent form in order to participate. All participants refrained from strenuous physical activity and were asked to eat a diet similar in calories and carbohydrates 24 hours prior to both of the experimental trials.
Instruments

Height was determined using a portable stadiometer (Perspectives Enterprises, Portage, MI). Body mass was measured using a mechanical scale (Detecto, Webb City, MO). Skinfolds were measured with a Lange skin caliper (Beta Technology, Inc., Santa Cruz, CA). Maximal oxygen consumption was determined during a continuous, incremental treadmill test on a Quinton Q65 treadmill (Bothell, WA). 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). Heart rate was monitored continuously using the Polar telemetry system (Polar Electro, Inc., Lake Success, NY). Ratings of perceived exertion were determined using Borg’s 20 point scale. Ovulation kits (Baby Hopes, Morrisville, NC) were used to determine menstrual cycle phase. Hematocrit was determined using an Adams MHCT II microhematocrit centrifuge (Becton Dickinson, Franklin Lakes, NJ) and an International Microcapillary Reader (International Equipment Company, Needham Heights, MA). Hemoglobin was determined from a Stanbio Lab Hemopoint H2 analyzer (Boerne, TX). Whole blood samples were placed in an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA) and the resultant plasma was stored. Plasma IL-6 was measured using BioLegend Legend Max™ ELISA kits (San Diego, CA). Plasma estradiol was measured using the radioactive (125I) immunoassay technique, (Siemens Healthcare Technologies, Los Angeles, CA). Lactate was measuring using a NOVA Biomedical Lactate+ Analyzer (Waltham, MA).

Protocol

Pre-Screening

Potential subjects emailed and made contact with the investigator. During that correspondence, it was determined if they fit all inclusion and exclusion criteria. Upon being accepted to the study their orientation and experimental sessions were scheduled.
Orientation Session

The participant arrived at the Applied Physiology Laboratory and was informed of the experimental protocol, made aware of possible risks and rewards associated with the protocol, and signed an informed consent form (Appendix A). After consenting to participate in the experiment, the participant underwent a physical screening and a 12 lead electrocardiogram. Once the participant filled out the medical history questionnaire (Appendix B) and passed the medical screening, physical characteristics including height, weight, and age were obtained. Percent body fat was determined from skinfold thicknesses, gluteal circumference and age using the Jackson, Pollock and Ward method.19

Following this, VO₂ max was determined using a continuous, incremental treadmill test. The participant began with a short warm up of her preferred pace followed by some light stretching. After the warm up, resting oxygen consumption (VO₂) was recorded for three minutes. The participant then began the graded exercise test. A Bruce treadmill protocol was used.20 Figure 3 lists the three minute stages of the Bruce protocol treadmill test. Heart rate (HR), rating of perceived exertion (RPE), and respiratory gases were monitored constantly throughout the test (Appendix E). After the test was completed, the participant recovered actively or passively and was able to leave the laboratory once their heart rate returned to less than 100 beats per minute (bpm). For the VO₂ max test to be valid, a participant must have three of the following four criteria: a plateau in VO₂ with increases in workload, respiratory exchange ratio (RER) greater than or equal to 1.1, RPE ≥ 18, and HR within 10% of predicted max HR. Exercise intensity estimated to be 65% of VO₂ max was calculated mathematically using ACSM guidelines.20
Figure 3. The speed (mph; miles per hour) and grade for each three minute stage of the Bruce protocol incremental exercise test.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Speed (mph)</th>
<th>Grade (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>18</td>
</tr>
<tr>
<td>6</td>
<td>5.5</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>6.0</td>
<td>22</td>
</tr>
</tbody>
</table>

Sessions I and II

Participants arrived at the Applied Physiology Laboratory at one point during the mid-follicular phase of the menstrual cycle and at one point during the mid-luteal phase. Menstrual cycle phase was determined and scheduled based on the forward counting method\textsuperscript{21} and confirmed by urinary ovulation kits (Appendix C). The two experimental trials were counterbalanced to prevent any order effects. The menstrual cycle is being used to manipulate the estrogen levels between the two trials. Early in the menstrual cycle, during the mid-follicular phase (approximately between days 3-7), estrogen levels are low. Later in the cycle, during the mid-luteal phase (approximately days 20-25), estrogen levels are significantly higher. Based on this natural fluctuation, the effect of differing estrogen levels can be examined. The two sessions were scheduled during these two phases in order to utilize the natural variation in estrogen concentrations; however, the exact days varied depending on the actual length of the subject’s menstrual cycle. Twenty four hours prior to the experimental trials participants were told to refrain from strenuous physical activity and completed a food diary so that nutrient intake was relatively constant between the two trials. Both experimental trials were done at similar times in the day in order to minimize any effects of circadian rhythms on hormone concentrations.
Participants were asked if they complied with all guidelines and their responses were recorded. Dietary logs were collected to ensure that participants were consuming adequate calories and carbohydrates. After compliance was given, participants rested in the supine position for ten minutes. After ten minutes resting, blood was obtained (3 mL) using the standard venipuncture technique. The blood sample was placed in a sterile K$_2$-EDTA (purple top) Vacutainer$^\text{TM}$ tube and immediately put on ice. Next, participants completed five minutes of a warm up and stretching. The participants then ran for 60 minutes at the previously determined workload of 65% of VO$_2$ max (Appendix F). Sixty minutes at 65% intensity was chosen in order to ensure that both estradiol and IL-6 would be elevated in response to the exercise.$^6,9,18$ After completion of the 60 minutes of exercise, a blood sample (3 mL) was obtained and immediately put on ice. A third and final blood sample draw (3 mL) was taken at 30 minutes into recovery. Plasma was separated from blood samples and stored at -80° C until later analysis for estradiol, IL-6, and lactate.

Blood Procedures

Hematocrit

Resting and post-exercise Hematocrit (Hct) values for each of the experimental trials were determined in triplicate from whole blood samples immediately at the time of exercise tests. Whole blood was drawn into 75 mm Allied Corporation microcapillary tubes (Fisher Scientific, Pittsburgh, PA) and sealed using Critoseal (Krackeler Scientific, Albany, NY). Capillary tubes were then spun in a microhematocrit centrifuge for three minutes at 10,000 RPM. The capillary tubes were then placed on a hematocrit wheel to determine the hematocrit values of each sample. A mean of the three samples was calculated and used in data analysis.
**Hemoglobin**

Resting, immediately post-exercise, and recovery hemoglobin (Hb) values were determined in duplicate from the whole blood samples of the experimental trials using the Stanbio Lab Hemopoint H² analyzer (Boerne, TX) immediately at the time of exercise tests.

**Plasma Volume Shift**

Exercise induced plasma volume shifts were calculated from Hb and Hct values according to the Dill and Costill method. Plasma volume shift was used as a potential explanation for changes in cytokine and estradiol concentrations.

**Cytokine (IL-6), Estradiol and Lactate Assays**

Whole blood was centrifuged at 3,000 x g for 10 minutes to separate the plasma. The resultant plasma was transferred to storage tubes and stored at -80°C until cytokine and estradiol analyses were conducted. Plasma estradiol was measured using the radioactive (¹²⁵I) immunoassay technique (Siemens Healthcare Technologies, Los Angeles, CA) involving solid-phase antibody procedures. The assay manufacturer reports a minimal detectable level of 2.0 pg/mL. Plasma levels of IL-6 were measured using high-sensitivity enzyme-linked immunosorbent assay kits (BioLegend, San Diego, CA). The assay manufacturer reports a minimum detectable concentration of 1.6 pg/mL (Appendix D). Lactate was measured using a NOVA Biomedical Lactate+ Analyzer (Waltham, MA) with a reported minimum-detectable concentration of 0.5 mM/L. All blood assays (unknowns) were performed in duplicate while standards were in triplicate (For details, see Appendix).

**Data Analysis**

All data were analyzed using SPSS statistical software (version 18.0, Chicago, IL). Significance was set at α<0.05. Descriptive statistics are presented as means ± standard deviations.
(SD). In addition, effect size was calculated for all significant measures. This later calculation was used to determine practical vs. significant findings.

A 2x3 (estradiol level x time) totally within subjects repeated measures ANOVA with a Bonferroni post-hoc test was used to evaluate the effects of estradiol on IL-6 concentration. A 2x2 (estradiol level vs. time) ANOVA was used to evaluate the percent change (%Δ) in IL-6 values between pre- and post-exercise and recovery during each menstrual cycle phase (estradiol level). Percent change was calculated using the equation:

\[ \% \Delta = \left[ \frac{(Post\text{-}exercise\text{-}Baseline)}{Baseline} \right] \times 100 \]

Resting levels of estradiol at midfollicular and midluteal phase experimental sessions were statistically tested for differences using a dependent t-test analysis.
CHAPTER IV

Results

Subject Characteristics

Twelve active female subjects were recruited for this study. Ten of the twelve subjects recruited fully completed all aspects of the study protocol. Two subjects were forced to withdraw due to personal reasons. All subjects recruited met the inclusion criteria of being eumennorheic and not using oral contraceptives for the previous six months. Ovulation kits confirmed normal menstrual function for all subjects. Participant physical characteristics are reported in Table 1.

Table 1: Physical characteristics of the participants (n=10). Values are Means (± SD).

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>20 ± 2</td>
<td>18-25</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.1 ± 5.5</td>
<td>155.9-176.9</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>58.7 ± 7.9</td>
<td>43.1-68.5</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>20.0 ± 5.9</td>
<td>11.1-28.5</td>
</tr>
</tbody>
</table>

VO₂ Peak Testing

Only eight of the ten participants who completed the study met the criteria for a valid VO₂ max test during the incremental exercise test (see Methods chapter). Because of this, all maximal oxygen uptake results will be referred to as VO₂ peak tests.

The absolute VO₂ peak (mean ± SD) obtained was 2.96 ± 0.57 L/min (range 2.00-4.04 L/min) and the relative VO₂ peak obtained was 50.4 ± 8.2 mL/kg/min (range 38.4-64.6 mL/kg/min). The peak HR obtained was 195 ± 9 bpm (range 184-209 bpm) and the peak RPE obtained was 18 ± 1 Borg units (range 15-20 Borg units). The duration of the exercise test was 11.57 ± 1.24 minutes.
(range 9.49-13.15 minutes) ending with an average stage of 4.25 ± .45 of the Bruce protocol (range 4-5) corresponding to a speed of 11.4 ± 1.0 km/h (range 10.9km/h-13.0 km/h) and an incline of 17 ± 1% (range 16-18%). These responses suggest the subjects were at a high level of cardiovascular fitness based upon the ACSM guidelines.18

Hormonal Status Determination

Menstrual cycle length for the subjects who completed all aspects of the protocol (n=10) was 30 ± 3 days (range 25-35 days). Individual menstrual cycle phases were estimated using the forward counting method21 and confirmed by urinary ovulation kits (see Methods chapter). The day on which subjects performed the running exercise during the midfollicular phase was 5 ± 3 days (range 2-9 days) after the onset of menses. The day on which subjects performed the running exercise during the midluteal phase was 24 ± 4 days (range 18-29 days) after the onset of menses.

Hormonal analysis of resting blood samples for estradiol confirmed the appropriate menstrual phase and hormonal condition of the subjects. The midfollicular estradiol concentration was 27.04 ± 6.12 pg/mL and the midluteal estradiol concentration was 67.64 ± 21.70 pg/ml. The difference in estradiol concentration between the two phases was significantly different (p<.001).

Extended Running Session

Each participant completed two sixty minute running trials, one of which was during the midfollicular phase of the menstrual cycle and the other after ovulation during the midluteal phase. All subjects complied with all pre-session guidelines (see Methods chapter) before each of the 60 minute trials. Each running trial was to consist of 60 minutes of running at 65% VO₂ peak. The overall mean absolute running VO₂ was 1.81 ± 0.46 L/min and the relative running VO₂ was 31.2 ± 7.8 mL/kg/min. This corresponded to an actual running intensity of 61.3 ± 7% (range 49.9-73.1%) for both trials.
No significant difference in the relative running VO$_2$ (p=.103) or absolute VO$_2$ (p=.066) between the two phases was found. Although there was no difference in VO$_2$ across hormonal conditions, relative VO$_2$ did demonstrate an increase in effort over time. Analysis showed a higher VO$_2$ at time points 30 and 60 minutes compared to minute 10 (p=.035, p=.016, respectively); i.e., main effect for time. However, there was no significant difference in VO$_2$ between 30 minutes and 60 minutes (p=.187). Also, no interaction effect was found between phase and time point for VO$_2$.

No significant main effect for menstrual cycle phase or interaction effect was found for HR. However, ANOVA results did indicate a significant main effect for time during exercise (p<.001). *Post Hoc* analysis indicates that HR at all time points was significantly higher than rest (p<.001) and that HR was significantly higher at 60 minutes compared to 30 minutes (p=.049). Mean HR was not significantly higher from minute 10 compared to minute 30 (p=.631) or minute 60 (p=.096).

Similarly to HR, there was no significant main effect for menstrual cycle phase with regards to RPE (p=.111) although there was a significant main effect for time. *Post Hoc* analysis indicates that at minute 30 and minute 60, RPE was significantly higher than at minute 10 (p=.019, p=.007, respectively) and RPE was also significantly higher at minute 60 compared to minute 30 (p=.005).

Lactate immediately post-exercise was not significantly different between the two phases (p=.160). The lactate concentration during the midfollicular phase was 2.95 ± 1.80 mM/L. During the midluteal phase it was 2.41 ± 1.29 mM/L.

Plasma volume increased by +0.7 ± 23.8% over the sixty minute running bout during the midfollicular phase, whereas it decreased by -5.3 ± 10.7% during the midluteal phase. These plasma volume shifts, however, were not significantly different between the hormonal conditions (p=.182).
Table 2: Mean (± SD) VO\textsubscript{2}, HR, and RPE for prolonged running bouts for the midfollicular and midluteal menstrual phases.

<table>
<thead>
<tr>
<th>Menstrual Cycle Phase</th>
<th>Measure (mL/kg/min)</th>
<th>Time</th>
<th>10 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VO\textsubscript{2}</td>
<td>REST</td>
<td>4.51 ± 0.65\textsuperscript{*}</td>
<td>30.73 ± 8.11\textsuperscript{€}</td>
<td>32.02 ± 7.78</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td></td>
<td>75 ± 13\textsuperscript{¥}</td>
<td>153 ± 25</td>
<td>161 ± 19</td>
</tr>
<tr>
<td>Midfollicular</td>
<td>RPE</td>
<td></td>
<td>10 ± 2\textsuperscript{£}</td>
<td>11 ± 2\textsuperscript{£}</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Midluteal</td>
<td>VO\textsubscript{2}</td>
<td>REST</td>
<td>4.89 ± 0.59\textsuperscript{*}</td>
<td>29.74 ± 8.07\textsuperscript{€}</td>
<td>30.91 ± 8.02</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td></td>
<td>66 ± 12\textsuperscript{¥}</td>
<td>155 ± 18</td>
<td>164 ± 18</td>
</tr>
<tr>
<td></td>
<td>RPE</td>
<td></td>
<td>10 ± 2\textsuperscript{£}</td>
<td>12 ± 3\textsuperscript{£}</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

A “” indicates no value was recorded for that particular time point. * Indicates a significantly lower VO\textsubscript{2} compared to 10, 30 and 60 minutes (p<.001). € Indicates a significantly lower VO\textsubscript{2} compared to 30 and 60 minutes (p<.05). ¥ Indicates a significantly lower HR compared to 10, 30, and 60 minutes (p<.001). £ Indicates a significantly lower RPE compared to 30 and 60 minutes (p<.05).

Cytokine (IL-6) Response to Running Trials

Resting, post exercise, and recovery IL-6 concentrations as well as the percentage change from rest are reported in Table 3. There was no significant main effect for phase (p=.076) or the interaction effect (p=.069) for the absolute concentration of IL-6. The only significant finding for absolute IL-6 concentration was a main effect for time (p=.003). Post Hoc analysis indicates that IL-6 was not significantly elevated from rest to immediately post exercise (p=.072), but that IL-6 concentration did increase significantly from rest to 30 minutes of recovery after exercise (p=.003). The effect size for this change was 0.963 (Cohen \textit{d}) which is between a moderate (0.6 Cohen \textit{d}) and large (1.2 Cohen \textit{d}) effect.
Since all IL-6 changes observed were substantially greater than the plasma volume shifts detected, no fluid shift corrections of IL-6 concentrations were conducted.

The percent change of IL-6 from rest to immediately post-exercise and from rest to 30 minutes of recovery was also examined. The main effect for phase was not significant (p=.072). When comparing the relative change from rest between immediately post-exercise and 30 minutes of recovery there was also no significant difference (p=.301) and no significant interaction effect between phase and time was found either (p=.768).

Table 3: Mean (± SD) IL-6 response and percent change (relative to rest) during the prolonged running bout for midfollicular and midluteal menstrual phase.

<table>
<thead>
<tr>
<th>Menstrual Cycle Phase</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest (pg/mL)</td>
</tr>
<tr>
<td>Midfollicular</td>
<td>6.25 ± 6.84</td>
</tr>
<tr>
<td>Midluteal</td>
<td>8.46 ± 9.65</td>
</tr>
</tbody>
</table>

Estradiol-IL-6 Relationship

As an exploratory analysis, correlations (Pearson) were calculated between the difference in estradiol concentration between phases and the difference in IL-6 concentration between phases for the immediately post-exercise and the 30 minutes into recovery samples. The correlation between estradiol and IL-6 immediately post was r=0.168 (p=.643) and the correlation between estradiol and IL-6 during recovery was r=0.108 (p=.766), neither of which were statistically significant.
CHAPTER V

Discussion

The purpose of this study was to determine the effect of estradiol fluctuations during the menstrual cycle on IL-6 concentration at rest and in response to an acute bout of exercise in females who are not using oral contraceptives. It was hypothesized that the IL-6 concentration after exercise would be significantly lower during the midluteal phase of the menstrual cycle when estradiol concentrations are elevated. This chapter will address this purpose and report on whether the study design was effective, describe the cytokine response to exercise and relate the findings to previously published literature, and explain possible reasons for these outcomes.

Study Design

Hormonal Condition

In order to use the natural fluctuation of estradiol in females, subjects were tested once during the midfollicular phase and once during the midluteal phase of the menstrual cycle. That is, after the onset of menses, during the midfollicular phase, estradiol levels are low, whereas during the midluteal phase, estradiol levels plateau at a higher concentration. All subjects were able to perform the prolonged running bout once during each phase. Because of the cyclical nature of female hormone levels, having a subject tested during a particular menstrual phase it did not ensure that the estradiol levels would change and be significantly different. For this study, however, statistical analysis confirmed that the estradiol concentration was significantly different between the two menstrual cycle phases and changed in the expected manner. Estradiol concentration during the midluteal phase was more than double that of the midfollicular phase (67.64 ± 21.70 vs. 27.04 ± 6.12 pg/mL,
respectively), thus all subjects demonstrated the correct hormonal changes between the two testing sessions with midluteal estradiol concentration being higher than the midfollicular estradiol concentration. These hormonal values fell within the physiologically accepted ranges for each phase and the fact that the estradiol concentration was approximately doubled from the midfollicular phase to the midluteal phase also agrees with numerous other studies.\textsuperscript{9,11,23} However, the absolute concentrations of estradiol were lower than that found in some studies.\textsuperscript{9,11,23,24} Jurkowski \textit{et al.}\textsuperscript{11} reported a midfollicular estradiol concentration of $103.8 \pm 16.0$ pg/mL and a midluteal concentration of $199.5 \pm 16$ pg/mL. Bonen \textit{et al.}\textsuperscript{9} had even higher values of $163.3 \pm 45.7$ pg/mL for the midfollicular phase and $266.1 \pm 87.8$ pg/mL for the midluteal phase. The most similar numbers to the current were found by Fu \textit{et al.}\textsuperscript{31} who reported follicular estradiol concentration as $34.1 \pm 12.3$ pg/mL and luteal estradiol concentration as $55.3 \pm 20.4$ pg/mL. The somewhat lower absolute concentrations in the present study could be a result of the high physical activity levels of the current subjects. That is, estrogen levels have been shown to decrease after aerobic training\textsuperscript{30} and all subjects in the present study were physically active individuals.

\textit{Prolonged Running Bout}

All participants successfully completed 60 minutes of treadmill running on the two separate occasions at an average intensity of 61\% of VO\textsubscript{2} peak. This intensity is slightly lower than the goal of 65\% of VO\textsubscript{2} peak intended, but research would suggest this is intense enough to illicit a rise in IL-6.\textsuperscript{6} For all subjects, the speed and grade of the treadmill during each run was identical between the two trials. In order to limit the effect of natural circadian rhythms the two trials for each individual were at approximately the same time of day. However, the time of day for all trials was not consistent for all subjects. Furthermore, due to time constraints of the study, participants were not evenly counterbalanced. Seven subjects performed the midfollicular trial first while three subjects performed the midluteal trial first. This potential order effect could lead to some degree of error in findings,
although there was no apparent differences in responses of the seven subjects tested in one order and the three tested in the opposite order. Also, the performance characteristics of the exercise were not different between sessions.

No difference in VO₂, HR, or RPE values was found between phases for the 60 minute exercise trials (main effect for phase). This is consistent with other literature that has examined exercise performance across the menstrual cycle.²³,²⁶,²⁷ The one study that did find a significant difference in RPE between menstrual cycle phases was conducted in a population of sedentary women.²⁸ These less active subjects might experience a different level of exertion during exercise, as well as being less experienced in monitoring exercise exertion. The non-significant difference in lactate concentration immediately after exercise also agrees with other studies that have examined this relationship.²³,²⁵,²⁷ Collectively, the similar VO₂, HR, RPE, and lactate data confirm the subjects’ exercise sessions were highly alike and suggest any IL-6 response differences were not due to differing aspects of the exercise sessions.

In response to the 60 minute exercise, all values were elevated compared to the resting values. Oxygen uptake increased at each time point and then plateaued from minute 30 until the end of exercise. Thus, the second half of the exercise was at a steady state VO₂. Heart rate increased from rest and remained relatively level from 10 minutes to 30 minutes and then increased slightly at 60 minutes. Based on this rise in HR the end of the exercise session was more physiologically difficult compared to the beginning of the session. Based on the HR response, exercise was not at a steady state for the entire 60 minutes of running. An increase in HR towards the end of a long endurance exercise is, however, consistent with the cardiac drift phenomenon.²⁹ During prolonged exercise plasma fluid can be lost from the blood, thereby reducing venous return and cardiac output. As cardiac output declines, HR must increase in order to maintain a given VO₂.²⁹ From minute 30 to minute 60 of exercise as the VO₂ remained level, HR rose significantly.
Perceived exertion continued to rise at each measurement time point which demonstrates that the exercise became progressively more difficult during the 60 minutes, in agreement with the HR findings. Although the exercise was at a constant intensity, RPE increased throughout the prolonged running bout which could be attributed to the extended length of the exercise bout and potential subject fatigue.

Lactate concentration immediately post exercise was also not significantly different between the two sessions. This is consistent with other studies that have measured this variable. These studies did not have the same exact exercise component (i.e., intensity, duration) but they all had the same findings that lactate was the same between phases at all time points and intensities. Based on the extended duration of the exercise bout, and the subjects’ need to run below their lactate threshold (i.e., a moderate intensity), the observed lactate concentrations were similar to what was expected based on the literature.

Cytokine Response

Exercise Effects

The average resting concentration of IL-6 for all twenty exercise sessions was 7.35 ± 2.43 pg/mL. This resting concentration is higher than most other exercise study reports. In male subjects Ostrowski et al. reported resting IL-6 concentration as 1.5 ± 0.7 pg/mL, while Steensberg et al. had resting concentrations of 0.74 (range, 0.67-1.50) pg/mL, and Mendham et al. measured IL-6 at 1.31 ± 0.28 pg/mL. In non-oral contraceptive using females pre-exercise IL-6 concentration was 2.0 pg/mL as reported by Timmons et al. Chaffin et al. also had resting values less than 2.00 pg/mL, both of which are lower than the results of this study. The finding of elevations in resting IL-6 in the present subjects could be a result of excessive background interference in the assay outcomes due to a limited practical experience by the investigator performing the assays; nonetheless, the levels were relatively similar across and within all the subjects. This last point suggests there was good internal
validity in the data. An additional factor accounting for the elevated resting IL-6 may be related to the study design pre-exercise protocol. Holmes et al. report that the level of resting IL-6 is inversely related to the level of circulating free fatty acids (FFA). At rest, the circulating level of FFA is directly related to the duration of a post-prandial fast. The pre-exercise fast that the subjects were asked to complete was relatively short compared to most studies being only two hours. This means the subjects started the exercise trials with low FFA levels, and thus elevated IL-6. Holmes et al. reported resting IL-6 levels as $6.05 \pm 0.89$ pg/mL for their subjects having FFA levels at that expected for the present study subjects based upon their level of fasting. The IL-6 concentration of Holmes et al. match very similarly with that of the present study.

Interleukin-6 concentration increased from rest after the 60 minutes of running, but this change was not significant ($p=.072$; main effect time). There was a concentration of $26.13 \pm 9.03$ pg/mL immediately after exercise. This concentration continued to rise until 30 minutes of recovery from exercise where it was $26.92 \pm 5.54$ pg/mL, this concentration is significantly elevated compared to rest ($p<.05$). Most studies have found that IL-6 peaks immediately after exercise. However, there are various factors that can affect the cytokine response. These include the type of physical activity, the type of contraction, as well as the intensity and duration of exercise. The rise in IL-6 is most closely related to the duration of the exercise bout. The absolute IL-6 concentration after exercise in this study was higher than some previous studies but lower than others. The IL-6 response to aerobic endurance exercise in men has been reported by Ostrowski et al. as reaching a level of $94.4 \pm 12.6$ pg/mL after completion of a marathon. Timmons et al. found that IL-6 rose to approximately 7.0 pg/mL after cycling for 90 minutes at 65% of VO$_2$. There is far fewer data with respect to females who were not using oral contraceptives and underwent aerobic exercise. One study found that IL-6 rose to approximately 7.0 pg/mL after 90 minutes of cycling at 65% VO$_2$max. The difference in IL-6 concentration between these studies could be attributed to the type of exercise that the subjects were required to complete and the subjects training background. The non-significant
immediate post-exercise value was not expected but the fact that the recovery values were 
significantly greater than rest suggests that the exercise bout intensity and duration was sufficient to 
ilicit a rise in IL-6 (N.B., the slightly higher variable observed in the SD at the immediate versus 
recovery concentration most certainly affected the lack of detection of a significant increase).

The increase in IL-6 following exercise was about 3.5 fold for the current study. Most other 
studies do not report the percent change from rest but the fold increase is able to be determined. 
While the marathon studies have reported that IL-6 rose as much as a 128-fold, the current findings 
are close to studies with similar aerobic exercise components. In two studies Timmons et al. found 
that IL-6 had a 3-fold increase in concentration. Ives et al. found about a 3.5 fold increase as well in 
oral contraceptive using females, while Chaffin et al. had approximately a 4-5 fold increase in non-
oral contraceptive using females.

**Menstrual Phase Effects**

The primary research question of this study was to examine the relationship between estradiol 
and IL-6 in active females who do not use oral contraceptives. The results of this study suggest that 
there is no significant difference in IL-6 concentration in response to varying estradiol concentrations 
either at rest or in response to exercise. When examining the interaction effect between phase and 
time there were no significant findings. Although the IL-6 concentration was lower in the midluteal 
phase compared to the midfollicular phase at all time points, the difference was not large enough to 
be significant (p=.074; interaction effect). With respect to percent change, this finding was also not 
significant (p=.072). In both cases the effect nearly reached statistical significant and had there been 
less variability in the IL-6 response significance might have been reached based on the critical 
statistical criteria of 0.05.

In animal and *in vitro* studies, IL-6 concentration was consistently found to be lower in 
response to higher estrogen concentrations. Studies conducted in mice determined that estradiol
treatment significantly decreased the production of IL-6 and that estrogen treated mice had an attenuated IL-6 response. 13,16 In human cells, in vitro estradiol treatment lead to a smaller rise in IL-6 production as induced by other pro-inflammatory cytokines.15 An in vivo, human study has also found that IL-6 was attenuated by estrogen supplementation in healthy women who were exposed to endotoxin, which promotes pro-inflammatory cytokine responses.14

In response to exercise the results of some previous studies have not found that estrogen affects IL-6. Two separate studies required subjects to cycle at 90 minutes at 65% VO2 max. In one study, estrogen supplementation had no effect on the IL-6 response.8 The other study utilizing this exercise session reported that IL-6 levels were not different between menstrual cycle phase within female subject and that there was no difference in IL-6 responses between genders.17 Ives et al.5 tested oral contraceptive using females and did not find a significant interaction between phase and time or main effect for time; although, the reported changes did approach statistical significance as in the present study. Most recently, Chaffin et al.24 found that IL-6 concentration after exercise was not significantly different between phases or the interaction between menstrual phases and exercise time. This is the only other study found that looks at IL-6 changes in response to exercise solely in non contraceptive using females. The data from the current study agrees with the findings from previous exercise-related research in that although IL-6 concentrations are lower in the luteal phase compared to the midfollicular, these differences are not at a level to reach statistical significance.

Conclusions

In conclusion, plasma IL-6 concentration does not appear to be significantly altered across the menstrual cycle in response to the fluctuating estradiol levels. Early studies that were conducted in vitro and in animal models found that estrogens attenuate the inflammatory response, as marked by lower IL-6 concentrations after treatment with estradiol.13,15,16 However, more recent studies exposed humans to exercise conditions and while some have approached significance, it has yet to be found
that IL-6 concentration varies across the menstrual cycle.\textsuperscript{5,8,17,24} Based on these as well as the current findings it cannot be suggested at this time that female athletes should alter their training program based on their hormonal status, such as decreasing training levels in the low estrogen, midfollicular phase of the menstrual cycle.

Limitations

A major limitation to this current study is that it appears underpowered. The typical accepted $\beta$ level for adequate power is .80, the percent change in IL-6 findings in this study were calculated to have a $\beta=.73$. Thus, if statistical significance was to be reached in the study, additional subjects would be required. Regrettably, the recruitment of any additional subjects was restricted by a limited pool of eumenorrheic women who met all inclusion criteria.

Another major limitation to the present study was the technical aspects of performing the IL-6 assay. As noted earlier, the investigator had limited practical experience in performing the assay and this may have resulted in great “background contamination” in the ELISA results of the assay. Additional practicing of the assay would have certainly been beneficial, but the cost of the cytokine assay reagents was extremely prohibitive for allowing such practice in the content of this study.
CHAPTER VI
Summary, Conclusions, and Recommendations

Summary

Ten active college-aged women who were eumenorrheic and had not used oral contraceptives for the previous six months were recruited for this study. Each subject reported to the laboratory on three separate occasions. On the first visit, subjects completed an incremental exercise test to volitional fatigue in order to determine VO₂ max and the appropriate experimental workloads. One of the remaining two sessions was conducted during the midfollicular phase of the menstrual cycle when estradiol levels are low, and the other session was conducted during the midluteal phase of the menstrual cycle, which was characterized by higher estradiol levels. This natural fluctuation of estradiol was used to determine the effect of estrogen on IL-6 concentration in response to exercise. These exercise sessions consisted of running at 61% VO₂ peak for 60 minutes followed by a 30 minute recovery period. Blood draws were taken before the start of exercise, immediately post-exercise, and 30 minutes into recovery from exercise. Physiological responses to the 60 minute exercise trials (VO₂, HR, RPE, and lactate) support that the exercise sessions were similar and did not differ from one another in responses.

Interleukin-6 concentration was significantly elevated at 30 minutes of recovery after exercise from rest levels (main effect time). However, no significant differences were found for the main effect of menstrual cycle phase or the interaction effect between phase and time; although, the main effect for menstrual cycle phase approached significance (p=.07; with lower IL-6 observed when estradiol was elevated).
Hypotheses (Accept or Reject)

Hypothesis 1: Plasma IL-6 concentration will be significantly increased from rest immediately post-exercise. (Reject)

Hypothesis 2: Plasma IL-6 concentration will be significantly increased from rest after 30 minutes of recovery from exercise. (Accept)

Hypothesis 3: Pre-exercise plasma estradiol will be significantly higher during the luteal trials compared to follicular trials. (Accept)

Hypothesis 4: The %Δ in plasma IL-6 concentration will be significantly lower during luteal trials compared to follicular trials from rest to immediately post-exercise. (Reject)

Hypothesis 5: The %Δ in plasma IL-6 concentration will be significantly lower during luteal trials compared to follicular trials from rest to 30 minutes of recovery. (Reject)

Future Research

Recommendations for future research would be, first to have a larger sample size. Power calculations suggest that additional subjects would be required to find significant differences in IL-6 across menstrual cycle phase (main effect). While all subjects are classified as active, exact aspects of training programs and regimes was not quantified. Future research should control for training status, as that could have contributed somewhat to the variability in IL-6 concentration. Another methodological factor would be to more closely control diet and the period post-prandial.

Another area of potential future research could be with respect to how progesterone affects inflammation. Estrogens are not the only fluctuating female hormones and it has been suggested that progesterone may play a role in immune function and be a regulator of IL-6 production as well as other cytokines.33,34
APPENDIX D

LEGEND MAX™ ELISA Kit with Pre-coated Plates
BioLegend, Inc.biolegend.com
ELISA Kit for Accurate Cytokine Quantitation from Cell Culture

Cat. No.
430507

Human IL-6

ELISA Kit for Accurate Quantitation of Human IL-6 from Cell Culture Supernatant, Serum, Plasma and Other Biological Fluids

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Introduction:

Human IL-6 (also known as B-cell stimulatory factor 2, Interferon beta-2, Hybridoma growth factor, and CTL differentiation factor) plays an essential role in the final differentiation of B-cells into Ig-secreting cells. It induces myeloma and plasmacytoma growth, nerve cells differentiation, and acute phase reactants in hepatocytes. IL-6 is expressed by T cells, B cells, monocytes, fibroblasts, hepatocytes, endothelial cells, and
keratinocytes. It has been shown that IL-6 plays a critical role in many physiological and pathological conditions, including autoimmune diseases and rheumatoid arthritis.

The BioLegend LEGEND MAX™ Human IL-6 ELISA Kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-6 from cell culture supernatant, serum, plasma, and other biological fluids. This kit is analytically validated with ready-to-use reagents.

**Materials Provided:**

<table>
<thead>
<tr>
<th>Description</th>
<th>Quantity</th>
<th>Quantity</th>
<th>Volume</th>
<th>Part #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Human IL-6 Pre-coated 96-well Strip Microplate</td>
<td>1 plate</td>
<td>5 plates</td>
<td></td>
<td>78180</td>
</tr>
<tr>
<td>Human IL-6 Detection Antibody</td>
<td>1 bottle</td>
<td>5 bottles</td>
<td>12 mL</td>
<td>78181</td>
</tr>
<tr>
<td>Human IL-6 Standard</td>
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<td>5 vials</td>
<td>lyophilized</td>
<td>78182</td>
</tr>
<tr>
<td>Avidin-HRP A Assay Buffer</td>
<td>1 bottle</td>
<td>5 bottles</td>
<td>12 mL</td>
<td>79131</td>
</tr>
<tr>
<td>Assay Buffer A Wash Buffer (20X)</td>
<td>1 bottle</td>
<td>5 bottles</td>
<td>25 mL</td>
<td>78232</td>
</tr>
<tr>
<td>Substrate Solution F Stop Solution</td>
<td>1 bottle</td>
<td>5 bottles</td>
<td>50 mL</td>
<td>79133</td>
</tr>
<tr>
<td>Plate Sealers</td>
<td>4 sheets</td>
<td>20 sheets</td>
<td></td>
<td>78101</td>
</tr>
</tbody>
</table>

**Materials to be Provided by the End-User:**

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 μL to 1,000 μL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

**Storage Information:**

Store unopened kit components at 4°C. Do not use this kit beyond its expiration date.

**Opened or Reconstituted Components**

Microplate wells

If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place
excess strips back in the foil pouch with the included desiccant pack and reseal. Store at 4°C for up to one month.

**Standard**
The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.

**Detection Antibody**
Store opened reagents at 4°C and use within one month.

- **Avidin-HRP A**
- **Assay Buffer A**
- **Wash Buffer (20X)**
- **Substrate Solution F**

**Stop Solution**

**Health Hazard Warnings:**

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend’s website for details (www.biolegend.com/support/#msds).

2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.

3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.

4. Stop Solution contains 2N sulfuric acid. **Wear eye, hand, and face protection.**

5. Before disposing of the plate, rinse it with an excess amount of tap water.

**Specimen Collection and Handling:**
Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

**Cell Culture Supernatant:** If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at < -70°C. Avoid repeated freeze-thaw cycles.

**Serum:** Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x g. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

**Plasma:** Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 10 minutes at 1,000 x g within 30 minutes of collection. Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles.

**Reagent and Sample Preparation:**

- **Note:** All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.

2. Reconstitute the lyophilized Human IL-6 Standard by adding the volume of Assay Buffer A indicated on the vial label to make the 20 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

3. In general, samples are analyzed without dilutions. However, if dilutions are required, use Assay Buffer A as the sample diluent.
**Assay Procedure:**

**Note:** Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 500 μL of the 500 pg/mL top standard by diluting 12.5 μL of the standard stock solution in 487.5 μL of Assay Buffer A. Perform six two-fold serial dilutions of the 500 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human IL-6 standard concentrations in the tubes are 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL). 4. Wash the plate 4 times with at least 300 μL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.

5. Add 50 μL of Assay Buffer A to each well that will contain either standard dilutions or samples.

6. Add 50 μL of standard dilutions or samples to the appropriate wells.

7. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

9. Add 100 μL of Human IL-6 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.

11. Add 100 μL of Avidin-HRP A solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

13. Add 100 μL of Substrate Solution F to each well and incubate for 15 minutes in the dark. Wells containing human IL-6 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.

14. Stop the reaction by adding 100 μL of Stop Solution to each well. The solution color should change from blue to yellow.

15. Read absorbance at 450 nm within 30 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

**Calculation of Results:**

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with cytokine concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.
If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample’s absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

**Typical Data:**
This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.

**Performance Characteristics:**

**Specificity:** No cross-reactivity was observed when this kit was used to analyze the mouse IL-6 and following recombinant cytokines/chemokines at up to 50 ng/mL.

**Sensitivity:** The minimum detectable concentration of IL-6 is 1.6 pg/mL.

**Recovery:** Recombinant IL-6 (250, 125 and 62.5 pg/mL) was spiked into 4 human serum samples, and then analyzed with the LEGEND MAX™ Human IL-6 ELISA kit. On average, 96.8 % of the cytokine was recovered from serum samples.

**Linearity:** Four human serum samples with high concentrations of IL-6 were diluted 1:2, 1:4, 1:8 with Assay Buffer A to produce samples with values within the dynamic range and then assayed. On average, 107 % of the expected cytokine was detected from serum samples.

**Intra-Assay Statistics:** Sixteen replicates of each of two samples containing different IL-6 concentrations were tested in one assay.

<table>
<thead>
<tr>
<th>Concentration Number of Replicates</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Concentration (pg/mL)</td>
<td>241.8</td>
<td>58.7</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>10.3</td>
<td>6.7</td>
</tr>
<tr>
<td>% CV</td>
<td>4.3</td>
<td>11.3</td>
</tr>
</tbody>
</table>
APPENDIX E

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:____
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.
APPENDIX F

Experimental Sessions

ID:_________ Date:____ Time:____ Session:_______ Phase:_______

Calculated Ex. Intensity:________mL/kg/min Speed:______mph Supine RHR_______bpm

Exercise Session (HR and RPE Recording)

<table>
<thead>
<tr>
<th>Minutes 6-10: VO₂ measurement</th>
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<tbody>
<tr>
<td>10 minutes</td>
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<tr>
<td>RPE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minutes 26-30: VO₂ measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
</tr>
<tr>
<td>RPE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minutes 56-60: VO₂ measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 minutes</td>
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<tr>
<td>RPE</td>
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Plasma Volume Shift Data

<table>
<thead>
<tr>
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<th>Immediate Post Hct and Hb</th>
<th>Recovery Hct and Hb</th>
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<tr>
<td>Hct</td>
<td>Hb</td>
<td>Hct</td>
</tr>
<tr>
<td>Pre 1</td>
<td></td>
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</tr>
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