

The Effect of Estradiol on Insulin Concentration in Response to an
Acute Bout of Exercise

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ABSTRACT

CECILY LEHMAN: The effects of estradiol on insulin concentration in response to an acute bout of exercise.

(Under the direction of Anthony C. Hackney, PhD, DSc)

The purpose of this study was to determine the influence of changes in circulating estradiol levels on the insulin response to an acute bout of exercise, in college age women who were not using hormonal contraceptives. Ten eumenorrheic women completed exercise sessions (60 minutes, 65% of VO_2max) in the mid-follicular (days 3 – 7; low estradiol levels) and mid-luteal (days 20 – 25; high estradiol levels) phases of their menstrual cycle. Blood samples were taken pre-exercise, immediately post-exercise, and 30 minutes into recovery. Results indicate that there is not a significant change in resting or exercise insulin levels during the low and high estradiol phases of the menstrual cycle ($p = 0.415$). In addition, the added stress of exercise does not cause a significantly different percentage change (i.e., level of decrease) in insulin between the low and high estradiol phases of the menstrual cycle ($p = 0.494$).

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CHAPTER I

Introduction

Insulin is a polypeptide hormone secreted from the beta-cells of the pancreas. At rest, usually postprandial, insulin stimulates glucose uptake, resulting in a decrease in blood glucose levels (1). As blood glucose decreases, insulin release from the pancreas is inhibited through a feedback mechanism, allowing the level of glucose in the blood to be tightly regulated (2). Insulin promotes glycogen synthesis in muscle tissue, and fat synthesis in adipose tissue, creating energy stores within the body (3). Glucagon is the antagonistic hormonal counterpart to insulin, promoting glycogenolysis and gluconeogenesis. When comparing the two hormones, a high Insulin/Glucagon (I/G) ratio usually causes a decrease in blood glucose, while a low I/G ratio usually causes an increase in blood glucose (1).

During exercise the release of insulin is inhibited by the sympathetic nervous system in order to maintain adequate concentrations of blood glucose; i.e., to provide a means by which non-insulin dependent glucose uptake can be increased at skeletal muscle (4). The drop in insulin concentration decreases the I/G ratio and promotes the effects of glucagon, thereby increasing blood glucose. The decrease in insulin concentration with the onset of exercise has been well established (5, 6, 7). It has also been demonstrated that one hour of moderate exercise can immediately increase insulin sensitivity and responsiveness in untrained subjects, and the effects can last for 48 hours after the exercise bout (6). Therefore, exercise is a beneficial therapy for metabolic diseases, like type two diabetes mellitus, where the insulin response has been corrupted.

Estradiol (E_2) is an 18-carbon steroid hormone derived from cholesterol and is the main estrogen secreted by the ovaries in females (1). Estradiol helps to control ovulation, menstruation, and pregnancy, but can also have influence on blood vessels, bones, lungs, liver, intestines and metabolism (2). In eumenorrheic women, E_2 levels vary greatly over the course of the menstrual cycle, with concentrations being lower in the follicular phase (days 1 – 14), and increasing during the luteal phase (days 15 – 28) (1). E_2 is known to have many metabolic actions on a multitude of tissues (8). In general, the impact on energy metabolism is to reduce reliance on carbohydrate as an energy source while enhancing lipid reliance (8, 9). Estradiol brings about these actions by direct effects on tissues through interactions with E_2 receptors, and indirectly by its actions on other hormones in the endocrine system. For example, relative to this last point, elevated E_2 levels promote enhanced growth hormone release from the anterior pituitary, and growth hormone induces increased lipolysis activity (10). Figure 1 schematically depicts some of the direct and indirect physiologic means by which E_2 mediates these effects on energy metabolism (8).

One of the means by which E_2 indirectly affects energy metabolism is by altering the actions of the hormone insulin. While there is evidence indicating that E_2 has transient effects on insulin, the results from research studies are not universally in agreement. For example, some studies have indicated an increase in resting insulin, specifically in the luteal phase of the menstrual cycle (high E_2 levels), while other studies have shown no difference in insulin whether E_2 concentration is high or low (8, 11, 12). Similarly, studies on the effects of E_2 on the insulin response to an acute bout of exercise have also produced mixed results (11, 13, 14). Bonen et al. (11) observed similar concentrations of insulin between the follicular and luteal phases of the menstrual cycle. Lavoie et al. (13) observed a decreased

concentration of insulin in the luteal phase of the menstrual cycle. Finally, Hackney et al. (14) observed an increased concentration of insulin in the luteal phase of the menstrual cycle. The limited number of studies examining the issue of E_2 on insulin (especially relative to exercise), and the contradictory nature of findings, suggests more research is needed to create a better understanding of the hormonal interactions between E_2 and insulin during exercise. Therefore the present study was proposed.

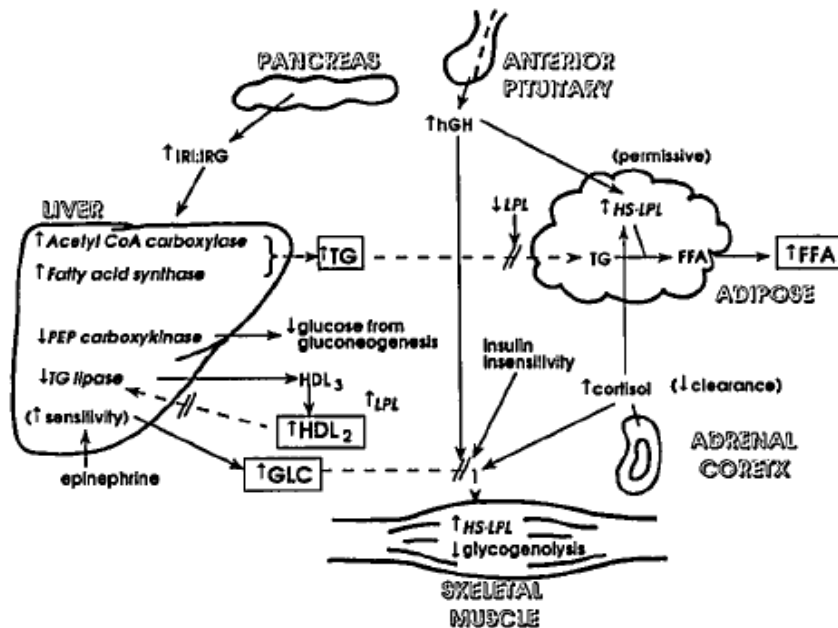


Figure 1—An integrated summary of the major direct and indirect metabolic/hormonal effects of estradiol on liver, skeletal muscle, and adipose tissues.

Taken from: Bunt J. Metabolic actions of estradiol: significance for acute and chronic exercise responses. *Med Sci Sports Exerc.* 1990; 22(3): 286-290.

Purpose of the Study:

The purpose of this study was to determine the influence of changes in circulating E_2 levels on the insulin response to an acute bout of exercise in college age women, who were not using hormonal contraceptives. Varying levels of E_2 were induced by exercising subjects

during the mid-follicular (low E₂) and mid-luteal (high E₂) phases of their menstrual cycles. This study required each participant to make three visits to the laboratory. The initial visit was an orientation and maximal oxygen uptake (VO₂max) test. The following two sessions (session I and II) were testing days and were scheduled during the mid-follicular (low E₂) and mid-luteal (high E₂) phases of the menstrual cycle. During sessions I and II, participants completed 60 minutes of treadmill exercise at 65% of their predetermined VO₂max (enough time and intensity to illicit a change in insulin responsiveness based on previous research)(6). Blood samples were taken at baseline, immediately post-exercise, and 30 minutes into recovery to determine E₂ and insulin concentrations.

Research Questions:

1. Was there a significant difference in blood insulin concentrations (μIU/mL) during the mid-follicular phase when compared to blood insulin levels during the mid-luteal phase?
 - a. Was there a significant difference in insulin concentration at baseline between the mid-follicular phase and mid-luteal phase?
 - b. Was there a significant difference in insulin concentration immediately post-exercise between the mid-follicular phase and mid-luteal phase?
 - c. Was there a significant difference in insulin concentration 30 minutes into recovery between the mid-follicular phase and mid-luteal phase?
2. Did the added stress of exercise cause a significant change in relative (percentage, %) blood insulin concentrations when comparing insulin concentrations pre- and post-exercise during the mid-follicular phase to insulin concentrations pre- and post-exercise during the mid-luteal phase?

- a. Was there a significant percent change in relative blood insulin concentration between baseline and immediately post-exercise when comparing the mid-follicular phase to the mid-luteal phase?
- b. Was there a significant percent change in relative blood insulin concentration between baseline and 30 minutes into recovery when comparing the mid-follicular phase to the mid-luteal phase?

Research Hypotheses:

- 1. There is a significant difference in blood insulin levels during the mid-follicular phase when compared to blood insulin levels during the mid-luteal phase.
 - a. Insulin concentration at baseline is significantly higher during the mid-luteal phase compared to the mid-follicular phase.
 - b. Insulin concentration immediately post-exercise is significantly higher during the mid-luteal phase compared to the mid-follicular phase.
 - c. Insulin concentration 30 minutes into recovery is significantly higher during the mid-luteal phase compared to the mid-follicular phase.
- 2. The added stress of exercise did not cause a significant change in blood insulin levels when comparing insulin concentrations pre- and post-exercise during the mid-follicular phase to insulin concentrations pre- and post-exercise during the mid-luteal phase.
 - a. There is no significant percent change in insulin concentration between baseline and immediately post-exercise when comparing the mid-follicular phase to the mid-luteal phase.

- b. There is no significant percent change in insulin concentration between baseline and 30 minutes into recovery when comparing the mid-follicular phase to the mid-luteal phase.

Definition of Terms:

Estrogen (E₂): an 18-carbon steroid secreted mainly from the ovaries, the major

estrogen is estradiol. Estrogen concentrations in the blood vary dramatically over the menstrual cycle with levels being lower in the mid-follicular phase and increasing in the mid-luteal phase (1). Estrogen status can be linked to changes in lipid and carbohydrate metabolism (8).

Insulin: a polypeptide hormone secreted from the cells of the pancreas. Insulin

stimulates glucose uptake throughout the body, resulting in a decrease in blood glucose levels. Exercise decreases insulin concentration in the blood (1)

Mid-follicular phase: Days 3 – 7 of the menstrual cycle, E₂ levels in the blood are low (1)

Mid-luteal phase: Days 20 – 25 of the menstrual cycle, E₂ levels in the blood are high (1)

Assumptions:

1. Subjects were honest about use of hormonal contraceptives and medications.
2. Subjects were not pregnant or trying to become pregnant. Pregnancy would alter the normal menstrual cycle E₂ response.
3. Subjects complied with exercise and dietary guidelines before exercise sessions.

Delimitations:

1. Healthy, recreationally active, premenopausal women between ages 18 – 30.
2. Subjects had been eumenorrheic for six months prior to the investigation.

3. Subjects were not using hormonal contraceptives or had not used any other hormonal therapy for six months prior to the study.
4. Subjects were not taking insulin or other medications that may affect the insulin response.

Limitations:

1. Subjects consisted of only eumenorrheic women abstaining from hormonal contraceptives; consequently the results are not applicable to men, amenorrheic women, postmenopausal women, and women taking hormonal contraceptives or hormone therapy.
2. The results are not applicable to persons who take insulin or have metabolic disorders such as diabetes.
3. Exercise sessions consisted of running so results may not be applicable to other forms of exercise.

Significance of Study:

The results of this study will add to the limited knowledge of the effects of E_2 on insulin concentrations in response to an acute bout of exercise. An increased knowledge of such effects in response to an acute bout of exercise can have significance in further understanding the metabolic role E_2 has on insulin, energy use, and the development of metabolic disease. Unfortunately, additional inquiries cannot be answered without increased familiarity of the interactions between E_2 and insulin in a young healthy premenopausal population. Increased understanding of the interactions between E_2 and insulin in a young healthy premenopausal population may lead to understanding the E_2 and insulin interaction in postmenopausal women. If E_2 and insulin interact in a menstruating population, then as E_2 concentrations

decrease with age and menopause begins, insulin concentrations should be affected. Due to the interaction of E_2 and insulin, and the variations of hormone concentrations with age, exercise prescriptions and nutrition recommendations may need to be altered to meet the changing demands of the aging female.

CHAPTER II

Review of Literature

This chapter is divided into seven sections. The first section is a brief summary of insulin and its effects on the body during rest. The second section gives an overview of studies documenting the interactive effects of insulin and exercise. Within the second section the effects of insulin during exercise are discussed in an acute bout of exercise in a healthy untrained population; then the effects of insulin and training are discussed. The third section is a brief summary of E_2 its effects on the body during rest. The fourth section gives an overview of studies documenting the interactive effects of insulin and exercise. Within the fourth section the effects of E_2 during exercise are discussed in an acute bout of exercise focusing on women and comparing women and men; then the effects of E_2 during exercise are discussed in women who are habitually active. The fifth section is a brief summary of the interaction of insulin and E_2 . The sixth section gives an overview of studies documenting the interactive effects of E_2 , insulin, and exercise. Within the sixth section the interactive effects of E_2 , insulin, and exercise are discussed in acute bouts of exercise varying in time from 60 minutes to 120 minutes, and varying in intensity from 50% to 80% of VO_{2max} . The sixth section moves from exercise without a glucose load, to exercise with a glucose load, and finally exercise comparing a fasted state to a glucose loaded state. The seventh and final section is a summary of the chapter.

Insulin

Insulin is a polypeptide hormone secreted from the beta-cells of the pancreas. At rest, the consumption of nutrients causes a release of insulin, stimulating glucose uptake into different body tissues (15). Insulin promotes glycogen synthesis in muscle tissue and fat synthesis in adipose tissue, creating energy stores within the body (3). Ultimately the actions of insulin result in a decrease in blood glucose levels (1). As blood glucose decreases, insulin release from the pancreas is inhibited through a feedback mechanism, allowing the level of glucose in the blood to be tightly regulated (2). Insulin concentrations in the blood never reach zero. Even during fasting conditions there is still a small concentration of insulin in the blood stream (15). The antagonistic hormonal counterpart to insulin is glucagon, which promotes glycogenolysis and gluconeogenesis. When comparing the two hormones, a high Insulin/Glucagon (I/G) ratio usually causes a decrease in blood glucose, while a low I/G ratio usually causes an increase in blood glucose (1).

Insulin and Exercise

To determine if insulin and exercise had an additive or synergistic effect, DeFronzo et al. (5) compared 10 healthy males, who had no history of diabetes and had completed a 12 – 24 hour fast, using four separate study protocols. Subjects all maintained a similar diet for three days leading up to each laboratory visit. Study one looked at the effects of insulin alone using an insulin clamp technique. Study two looked at the effects of exercise alone on glucose uptake after 30 minutes of cycling at 40% VO_2max . Study three combined protocols from study one and two. In study protocol three, subjects underwent the same insulin clamp technique as in study protocol one, afterwards subjects then cycled for 30 minutes at 40% VO_2max . Study four repeated the protocol in study three, but with additional catheters

placed in the femoral artery and femoral vein to monitor glucose activity within the legs. Results from all four protocols, indicated that insulin and exercise act synergistically to enhance glucose metabolism in skeletal muscle tissue.

Mikines et al. (6), used euglycemic hyperinsulinemic clamp tests to observe the effects of exercise and insulin after one hour of rest, one hour of cycling at 64% $\text{VO}_{2\text{max}}$, and 48 hours after one hour of cycling at 64% $\text{VO}_{2\text{max}}$, in seven untrained healthy males without a family history of metabolic disorder or diabetes. For three days prior to each laboratory visit, subjects ate a “weight-maintaining diet” and refrained from strenuous physical activity. Subjects refrained from tobacco and alcohol the day before, and fasted the night before each laboratory visit. Results showed that one hour of exercise increases insulin sensitivity and responsiveness in untrained subjects that lasts for 48 hours into recovery from the exercise (6).

King et al. (7) demonstrated that exercise training increases insulin sensitivity. Subjects, five men and four women, were all exercising as least 45 minutes per day, five to seven days per week. Diets were recorded for three days before the first euglycemic hyperinsulinemic clamp test, and the diet was repeated for three days before the second euglycemic hyperinsulinemic clamp test. All tests occurred after an overnight fast. The first euglycemic hyperinsulinemic clamp test took place while the subjects were still exercising, while the second test took place after the subjects had been physically inactive for 10 days. Results indicate after 10 days of physical inactivity, the increased insulin sensitivity that accompanies exercise training reverses when exercise stops (7).

Although the aforementioned studies have primarily male subjects, the same effects of exercise on insulin have been seen in women (4). During exercise, the release of insulin is

inhibited by the sympathetic nervous system in order to maintain adequate concentrations of blood glucose; i.e., to provide a means by which non-insulin dependent glucose uptake can be increased at skeletal muscle (4). The inhibition of insulin release decreases insulin concentration in the blood. However, insulin concentrations in the blood never reach zero (15). Therefore exercise can only force insulin concentration to decrease to the lower limit of the normal hormonal range. This phenomenon is referred to as the “basement effect” (16).

Estradiol

E₂ is an 18-carbon steroid hormone derived from cholesterol, and is the main estrogen secreted by the ovaries in females (1). E₂ helps to control ovulation, menstruation, and pregnancy, but can also have influence on blood vessels, bones, lungs, liver, intestines and metabolism (2). E₂ varies dramatically over the menstrual cycle, with concentrations being lower in the follicular phase (days 1 – 14) and higher in the luteal phase (days 15 – 28) (1). Both animal and human model research has shown during rest, E₂ can lead to increased lipolysis and decreased gluconeogenesis and glycogenolysis (8, 17).

Estradiol and Exercise

Hackney et al. (9) collected data on substrate utilization from nine eumenorrheic women performing 30 minutes of running which increased in intensity every 10 minutes from 35% VO₂max, to 60% VO₂max, and finally to 75% VO₂max. Exercise bouts were conducted during both the mid-follicular and mid-luteal phases of the menstrual cycle. Results showed that during exercise at 35% and 60% of VO₂max lipid utilization was significantly greater during the mid-luteal phase of the menstrual cycle when E₂ is elevated. There was no difference between substrate utilization and phase of the menstrual cycle during exercise at 75% VO₂max.

In a study performed by Bailey et al. (18), nine healthy eumenorrheic women, not using oral contraceptives, cycled at 70% of peak oxygen uptake ($\text{VO}_{2\text{peak}}$) until exhaustion. Exercise trials were performed twice during the follicular phase and twice during the luteal phase. Subjects were asked to refrain from caffeine and alcohol 12 hours prior to testing and strenuous exercise 24 hours prior to testing. On the day of exercise testing all subjects were fed a standard breakfast at the laboratory. The purpose of this investigation was to determine if E_2 influenced the effect of carbohydrate supplementation. Results indicated that carbohydrate supplementation is not influenced by menstrual cycle phase. It is interesting to note that increases in performance measured by duration to exhaustion, due to supplementation, were lower in women (11 – 14% increase) when compared to men exercising in the same conditions (24 – 32% increase).

It has been shown that during low to moderate intensity endurance exercise, 35% - 70% $\text{VO}_{2\text{max}}$, women utilize more lipids and fewer carbohydrates for metabolic energy when compared to men (19). The higher levels of E_2 in women are associated with a shift in energy substrate utilization towards lipids (8). When E_2 levels are experimentally increased in men; use of lipids as a substrate during exercise increases, while use of carbohydrates as a substrate during exercise decreases (19). Similar findings have been reported for male rats administered estrogens and then exercise trained; the use of lipids as an energy substrate increases (17). Furthermore, Hackney (20) performed muscle biopsies before and after 60 minutes of exercise (70% $\text{VO}_{2\text{max}}$) in women at the mid-follicular and mid-luteal points of their cycles, and found that muscle glycogen utilization was decreased in the luteal phase. This utilization difference was attributed to a greater reliance on lipids as a fuel source.

Finally, it has been documented that a negative correlation exists between habitual exercise, of moderate intensity, and salivary E_2 concentration (21). A relationship was also observed between activity level, E_2 concentration, and percentage body fat. Women who reported low activity levels, in both the moderate and high percentage body fat groups, had a statistically higher concentration of E_2 (21). Although the authors did not state this, it could be hypothesized that habitual exercise increases the sensitivity of E_2 which creates a greater usage of lipids during exercise.

Insulin and Estradiol

At rest, E_2 can inhibit gluconeogenesis and glycogenolysis, reducing the reliance on carbohydrate as an energy source while enhancing lipid reliance by increasing the I/G ratio (8, 9). If the I/G ratio is elevated for a prolonged amount of time, insulin sensitivity may decrease causing metabolic disorders like type two diabetes mellitus (8). Estradiol brings about these actions by direct effects on tissues through interactions with E_2 receptors and indirectly by its actions on other hormones in the endocrine system. For example, relative to this last point, elevated E_2 levels promote enhanced growth hormone release from the anterior pituitary and growth hormone induces increased lipolysis activity (10). There is some evidence that E_2 has direct effects on insulin receptors in adipocytes, however further research is merited (22). In mice, it has been shown that E_2 supplementation protects against beta cell apoptosis, while in women E_2 supplementation improves insulin sensitivity (22). Again, Figure 1 schematically depicts some of the direct and indirect physiologic means by which E_2 mediates these effects on energy metabolism (8).

Insulin, Estradiol, and Exercise

Horton et al. (12) studied glucose kinetics across three phases of the menstrual cycle; early follicular, mid-follicular, and mid-luteal. Thirteen eumenorrheic, active females not using oral contraceptives or other hormones, were recruited to cycle for 90 minutes at 50% of VO_2max . Diet and exercise were controlled for three days, with an overnight fast the night before exercise. Results showed significantly higher levels of insulin during the first 45 minutes of exercise in the mid-luteal phase when compared to either point in the follicular phase. Based on other measurements taken, glucose utilization appeared equivalent across the menstrual cycle but was coupled with higher levels of insulin within the mid-luteal phase. Although not statistically significant, resting levels of insulin were higher during the luteal phase than the early follicular or mid-follicular phases.

In an exercise study performed by Lavoie et al. (13), eumenorrheic, physically active females not taking oral contraceptives cycled for 90 minutes at 63% of maximal oxygen uptake (VO_2max) during the mid-follicular and mid-luteal phases of their menstrual cycles. Twenty-four hours before the exercise session, all subjects were restricted to a carbohydrate poor diet. Results indicated lower insulin values during the luteal phase of the menstrual cycle; however the concentration difference between phases was not statistically significant. In both phases insulin decreased significantly, and in a similar fashion, over the course of the exercise bout. It is interesting to note that these women were starting the exercise session glycogen depleted, following a carbohydrate poor diet and an overnight fast.

In a study conducted by Hackney et al. (14), eight recreationally active, eumenorrheic women performed 60 minutes of cycle ergometry at 70% of VO_2max after ingesting an oral glucose load. After consuming the glucose load subjects rested for 45 minutes before

beginning exercise. Testing occurred in both the follicular and luteal phases of the menstrual cycle and both diet and exercise were replicated before testing days. At the onset of exercise insulin decreased similarly in both the follicular and luteal phases. However, results from this study show a statistically significant higher peak insulin response before beginning exercise in the luteal phase than in the follicular phase. Indicating insulin response to a glucose challenges differs with the stages of the menstrual cycle.

Campbell et al. (23), observed eight endurance trained eumenorrheic women during the follicular and luteal phases of the menstrual cycle. None of the subjects were taking oral contraceptives. Diet was controlled before each exercise trial and no food was allowed the morning of a laboratory visit. Four exercise sessions were conducted consisting of two hours of cycling at 70% of VO_2max followed by a 4kJ/kg body weight time trial. Two sessions were supplemented with carbohydrate loaded beverages; the other two sessions were supplemented with a placebo. Results indicated that variations in E_2 levels throughout the menstrual cycle altered exercise metabolism, but that the alterations due to E_2 levels dissipate with glucose supplementation. Resting insulin levels were not significantly different between trials; however insulin tended to be higher during the luteal phase.

Bonen et al. (11) divided 19 eumenorrheic women, not taking oral contraceptives, into three groups: a control group, a fasting group, and a glucose-loaded group. The glucose-loaded group consumed the glucose load 17 minutes before the onset of exercise. Subjects performed 30 minutes of walking on a treadmill at 40% of VO_2max followed by 30 minutes of walking up a steep incline at 80% of VO_2max . Exercise sessions were performed during both the follicular and luteal phases of the menstrual cycle. Diet was recorded before the first exercise session and repeated before the second exercise session. Results indicate that

when nutritional status is normal, insulin response to exercise is similar in both the follicular and luteal phase. In the glucose loaded group, insulin levels and response to exercise were similar between the two phases of the menstrual cycle. The group of women that fasted had significantly higher insulin levels in the luteal phase than in the follicular phase.

Summary

In summary, insulin stimulates glucose uptake and promotes glycogen synthesis in muscle tissue and fat synthesis in adipose tissue, creating energy stores within the body (1). Exercise and insulin work synergistically to enhance the metabolism of glucose, and these effects can last up to 48 hours after the bout of exercise (5, 6). Training increases insulin sensitivity but sensitivity will decline with the cessation of exercise (7).

Estradiol is the main estrogen secreted by the ovaries in females and can lead to increased lipolysis, decreased gluconeogenesis and glycogenolysis due, in part, to the influence E_2 has on increasing the I/G ratio (1, 8). Research has demonstrated that during low to moderate intensity exercise, lipids are oxidized more during the mid-luteal phase of the menstrual cycle. However as exercise intensity increases there is no difference between substrate utilization and menstrual cycle phase (9). At high exercise intensity there is no difference in substrate utilization when subjects supplement with carbohydrates (18). When compared to their male counterparts, women utilize lipids more than men during endurance exercise due to increased levels of E_2 (8, 19). Finally, physical activity has a negative relationship with E_2 , as activity increases; E_2 decreases (21).

There is varying information about the effects of E_2 , insulin, and exercise. Horton et al. (12) found significantly higher levels of insulin during the first 45 minutes of exercise in the mid-luteal phase when compared to the follicular phase whereas Lavoie et al. (13) found

no significant difference in insulin when comparing different phases of the menstrual cycle. Hackney et al. (14) showed a statistically significant higher peak insulin response after an oral glucose load but, before beginning exercise in the luteal phase than in the follicular phase; indicating insulin response to a glucose challenge differs with the stages of the menstrual cycle. However, it was found that at the onset of exercise insulin decreased similarly in both the follicular and luteal phases.

Variations in E_2 levels throughout the menstrual cycle alter exercise metabolism, but that the alterations due to E_2 levels dissipate with glucose supplementation (23). When subjects have not undergone an overnight fast or a pre-exercise glucose load, insulin response to exercise is similar in both the follicular and luteal phase. However, fasted subjects have significantly higher resting insulin levels in the luteal phase than in the follicular phase (11).

CHAPTER III

Methodology

This study required each participant to make three laboratory visits. The initial visit consisted of an orientation and maximal oxygen uptake (VO_2max) test. The following two sessions were submaximal exercise sessions and were scheduled during the mid-follicular (low E_2) and mid-luteal (high E_2) phases of the menstrual cycle. During the exercise sessions, participants completed 60 minutes of treadmill running at 65% of their predetermined VO_2max . Blood samples were taken at baseline, immediately post-exercise, and 30 minutes into recovery from exercise, to determine E_2 and insulin concentrations.

Subjects

Sample size was determined using PS: Power and Sample Size Calculation version 3.0.43 (Vanderbilt University, Nashville, TN). An α level of 0.05 required a total of ten subjects to achieve a power of 0.80 (β). Insulin concentrations used in the power calculation were taken from a previous investigation performed by Bailey et al. (Bailey et al., 2000). Healthy, recreationally active women between the ages of 18 – 30 were recruited. Subjects were premenopausal and eumenorrheic for six months prior to the investigation. Eumenorrhea was defined as having a consistent menstrual cycle lasting 24 – 35 days. Subjects could not be using hormonal contraceptives or have used any other hormonal therapy for six months prior to the study. Subjects could not be taking insulin or other medications that would affect the insulin response.

Subjects who were pregnant, taking insulin or any other medications that would affect the insulin response, and/or had sustained an injury in the last six months that would limit their ability to exercise were excluded from the study. If any contraindications to exercise given by the American College of Sports Medicine (24) were discovered during the physical examination or an irregular heart rhythm was discovered during the 12-lead electrocardiogram, the subject was excluded from the study and advised to follow-up with their physician (Appendix A)(24).

Instrumentation

Subject height was measured using a stadiometer (Perspective Enterprises, Portage, MI). Subject mass was measured using a mechanical scale (Detecto, Webb City, MD). Electrocardiograms were performed using a Schiller AT 10 Plus EKG unit (Schiller AG, Switzerland). A Lange skinfold caliper (Model 68902, Cambridge Scientific Industries, Inc., Cambridge, MA) was used to determine body composition. All treadmill exercise was performed on a Quinton MODEL Q65 treadmill (Cardiac Science Corporation Bothell, WA). Expired gas was collected using Parvo Medics TrueMax® 2400 Metabolic System (Parvo Medics, Salt Lake City, UT). Exercise heart rate was monitored using a Polar heart rate monitor (Polar Electro Inc., Lake Success, NY). Rating of perceived exertion (RPE) was determined using the Borg scale. Ovulation was determined using BabyHopes™ Ovulation One-Step test strips (BabyHopes.com). Hematocrit was determined using microcapillary tubes (Fisher Scientific International Inc., Hampton, NH), hematocrit tubes were sealed with critoseal capillary tube sealant (Krakeler Scientific Inc., Albany, NY), centrifuged with an Adams MCHT II microhematocrit centrifuge (Beckton Dickinson, Franklin Lakes, NJ), and read with an International Microcapillary Reader (International Equipment Company,

Needham Heights, MA). Hemoglobin was analyzed using the stat-site WT-9 hemoglobin meter (Stanbio Laboratory, Boerne, TX). Lactate was measured using a Lactate+ Analyzer (NOVA Biomedical, Waltham, MA) and glucose was measured using a ONE TOUCH glucometer (LifeScan, Milpitas, CA). To separate plasma and serum, an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA) was used. Estradiol levels were determined using an enzyme-linked immunoabsorbent assay (ELISA) (Abnova, Walnut, CA). Insulin levels were determined also using an ELISA (Abnova, Walnut, CA).

Protocol

Orientation/Maximal Oxygen Uptake Session

Subjects were asked to come to the Applied Physiology Laboratory at the University of North Carolina at Chapel Hill. The study protocol, schedule, inherent benefits, and potential risks were explained, and the subject was given an opportunity to ask questions before written informed consent was obtained (Appendix B). The subject then completed the Department of Exercise & Sports Science medical history questionnaire and completed a 12-lead electrocardiogram and physical examination (Appendix C). Height and body mass were obtained and the subject was fitted with a heart rate monitor. The subject then rested in a supine position for 10 minutes to obtain a resting heart rate. Next the subject was fitted for a mouthpiece used to collect expired gases. A Bruce treadmill protocol test was administered to determine VO_2max . Respiratory gases, for indirect calorimetry, and heart rate were monitored continuously throughout the test. Heart rate was recorded at the end of every minute, and rate of perceived exertion (RPE) was recorded at the end of every three minute stage. After completion of the VO_2max test subjects recovered actively until their heart rate

dropped below 120 beats per minute. Subjects were then asked to passively rest, if no adverse responses to exercise were observed the subject was allowed to leave once their heart rate decreased below 100 beats per minute.

The criteria to determine a maximal response to the VO_2max test were: a respiratory exchange ratio of 1.1 or greater, reaching age predicted maximal heart rate ($\pm 5\%$), a RPE of 18 or greater, and a decrease or plateau in VO_2 with increased workload. If all three criteria were not met then the test was classified as a $\text{VO}_{2\text{peak}}$. The highest VO_2 (max or peak) obtained during the test was used to calculate the running speed for the submaximal bouts of exercise. Running speeds to elicit 65% of VO_2max were determined using ACSM metabolic calculations (24).

Menstrual Phase Determination

Subjects informed the investigator on first day of menses, which was denoted as day one. The forward counting method was used to determine scheduling of the next two exercise sessions (25). The mid-follicular phase was 7 ± 2 days after menses. The mid-luteal phase was 20 ± 2 days after menses. The next two exercise sessions were scheduled using the onset of menses as day one and counting forward until reaching either the mid-follicular or mid-luteal phase. Urinary ovulation testing was used to support the forward counting method. At the end of the orientation session each subject was given an ovulation kit and instructions on how to determine the first day of ovulation (Appendix D). The mid-follicular and mid-luteal dates were based off of an average 28 day menstrual cycle. If a subject had a longer/shorter cycle length adjustments were made in the specific days of testing to correspond with the mid-follicular and mid-luteal points.

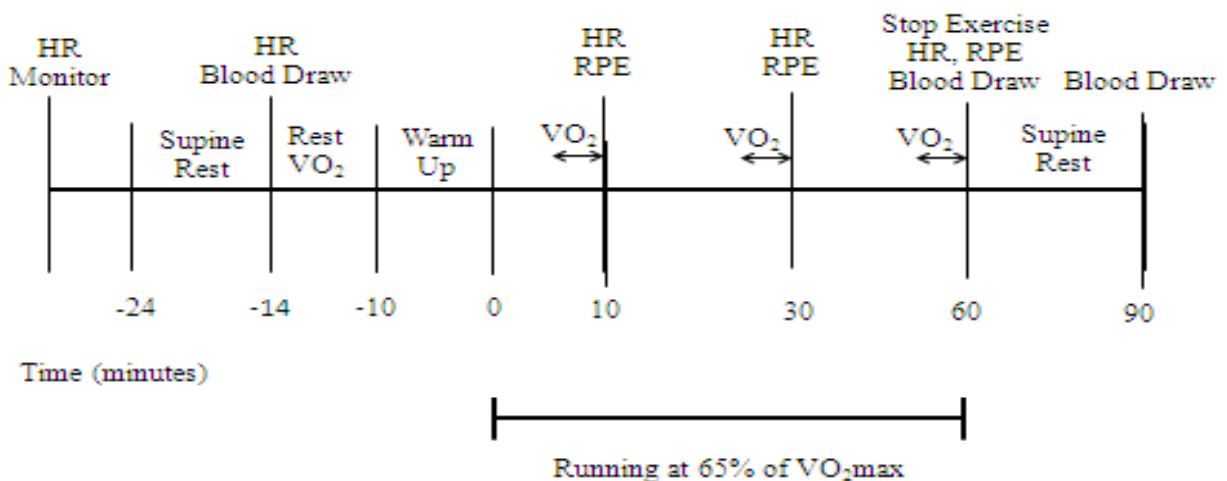
Exercise Sessions

The subject reported to the laboratory at a similar time for each exercise session. Twenty-four hours prior to each of the 60 minute 65% VO_2max exercise sessions, the subject was asked to drink plenty of water, refrain from caffeine and alcohol, record their food intake in a dietary log, and refrain from strenuous exercise. Two hours prior to the exercise session, subjects were asked to refrain from eating or drinking anything besides water. Upon arrival to the laboratory the subject was asked to complete a brief questionnaire to ensure that they had complied with physical and dietary guidelines (Appendix E). Dietary logs were reviewed and photo copied. The original dietary log was kept by the investigator and the copy was given back to the subject so they could repeat the same diet 24 hours prior to the second exercise session. Once compliance was established the subject was fitted with a heart rate monitor and asked to rest for 10 minutes in a supine position. After the 10 minutes, resting heart rate was recorded, and a three mL blood sample was collected by a certified phlebotomist using a venipuncture technique. The blood samples were placed into $\text{K}_2\text{-EDTA}$ blood collection tubes and immediately placed on ice. The blood samples were used to confirm menstrual cycle status and determine resting levels of E_2 and insulin, in analyses performed later. After the resting blood draw the subject moved to the treadmill and was fitted with a mouthpiece. Resting respiratory gases were collected for four minutes to determine resting VO_2 . Next, each subject had 10 minutes to warm-up; five minutes of walking on the treadmill and five minutes of stretching. Following the warm-up the subject ran on the treadmill at 0% incline and a speed that elicited 65% of their VO_2max . At six minutes, 26 minutes, and 56 minutes the subject was asked to insert the mouthpiece back into their mouth for collection of respiratory gases for four minutes. The four minute sampling

was to ensure running speed was appropriate and to allow adjustments in speed if needed. Any adjustments that occurred in the first 60 minute exercise trial were replicated for the second trial. Heart rate and RPE was recorded at 10 minutes, 30 minutes, and 60 minutes. The above procedures are depicted in Figure 2. Throughout the exercise session the subject was able to drink water at their convenience, listen to music, and have access to a fan to keep cool.

At the completion of exercise another three mL blood sample was collected, following the same procedure, placed in a K₂-EDTA blood collection tube and immediately placed on ice. The subject was then asked to rest in a supine position for 30 minutes. At the end of the 30 minutes of recovery, a final three mL blood sample was collected, following the same procedure, placed in a K₂-EDTA blood collection tube and immediately placed on ice. After the final blood draw, if no adverse reactions to exercise were observed, the subject was released from the laboratory if their heart rate was below 100 beats per minute. The subject repeated the protocol for both the mid-follicular and mid-luteal phases of the menstrual cycle.

Figure 2: Timeline of exercise sessions.



Blood Analysis

Hematocrit

Resting and post-exercise hematocrit were determined for each exercise session. Whole blood was drawn into a heparin treated microcapillary tube, sealed, and centrifuged for three minutes at 12,000 revolutions per minutes in a microhematocrit centrifuge. The microcapillary tube was then placed on a hematocrit wheel and the ratio of red blood cells within the blood was determined. Hematocrit measures were performed in triplicate and averaged.

Hemoglobin

Resting and post-exercise hemoglobin were determined for each exercise session. Twenty-five microliters of whole blood was pipetted onto a slide and analyzed using a handheld analyzer. Hemoglobin measures were performed in triplicate and averaged.

Plasma Volume

To ensure that changes in E_2 and insulin were not due to hemoconcentration effects alone, plasma volume shifts were calculated using the Dill and Costill method, which uses hematocrit and hemoglobin changes (26). The percent changes in the plasma volume shifts were compared to the percent changes in hormonal concentrations following exercise to determine the degree of hemoconcentration influence.

Lactate and Glucose

Post-exercise blood samples were analyzed for lactate concentration using a lactate analyzer. Pre-exercise, post-exercise, and recovery blood samples were analyzed for glucose concentration using a handheld glucometer.

Estradiol and Insulin

In order to separate the plasma, blood samples were centrifuged at 3000 X g for 15 minutes at 4° C. Plasma and serum were removed from the centrifuged samples and frozen at -80°C until analyzed later for E₂ and insulin concentrations. E₂ and insulin concentrations were determined using an ELISA and performed in duplicate. Specific directions of the assay procedures can be found in the appendix (Appendices F and G).

Statistical Analysis

All statistical analyses were done using SPSS statistical software (version 19.0, Chicago, IL). Significance was set a priori at an $\alpha < 0.05$. All values were displayed as means \pm standard deviations (SD).

To determine if there was significant difference in blood insulin concentrations (μ IU/mL) during the mid-follicular phase when compared to blood insulin concentrations during the mid-luteal phase, a 2x3 (menstrual phase [mid-follicular phase, mid-luteal phase] x exercise time [pre-exercise, immediately post-exercise, 30 minutes into recovery]) repeated measures model analysis of variance (ANOVA) was used with a Bonferroni post hoc test if a significant F-ratio was obtained.

Insulin concentration percent change from pre-exercise to post-exercise and pre-exercise to recovery was calculated. To determine if there was a significant difference in percent change a 2x2 (menstrual phase [mid-follicular, mid-luteal] x percent change in insulin [pre-exercise to immediately post-exercise, pre-exercise to recovery]) repeated measures model ANOVA was used with a Bonferroni post hoc test if a significant F-ratio was obtained. Figure 3 shows the calculations used to determine percent change in insulin concentration.

Figure 3: Calculations used to determine percent change in insulin concentration overtime.

Pre-exercise to immediately post-exercise:

$$(([\text{POST}] - [\text{PRE}]) / [\text{PRE}]) \times 100 = \text{percent change}$$

Pre-exercise to 30 minutes into recovery:

$$(([\text{REC}] - [\text{PRE}]) / [\text{PRE}]) \times 100 = \text{percent change}$$

[PRE] – concentration of insulin pre-exercise

[POST] – concentration of insulin immediately post-exercise

[REC] – concentration of insulin 30 minutes into recovery

CHAPTER IV

Results

Subject Characteristics

Twelve healthy women were recruited for this study. Two women were unable to complete the study due to personal reasons, leaving ten remaining women who completed all aspects of the study. All subjects met the inclusion criteria of healthy, recreationally active, ages 18 – 30, eumenorrheic (\geq six months) prior to the investigation, not using hormonal contraceptives or other hormonal therapy, and lastly not taking any insulin or other medication that may affect the insulin response. Subject physical characteristics can be found in Table 1.

Table 1. Subject physical characteristics (n = 10).

	Mean \pm SD	Range
Age (yr)	20.0 \pm 2.2	18 – 25
Height (cm)	164.1 \pm 5.9	156 – 177
Mass (kg)	58.7 \pm 8.3	43 – 69
Body Fat (%)	22.3 \pm 4.9	13 – 30
BMI (kg/m ²)	21.8 \pm 3.1	17.5 – 27.7

Maximal Oxygen Uptake Determination

The criteria for a maximal response to the VO₂max test were: a respiratory exchange ratio of 1.1 or greater, reaching age predicted maximal heart rate (\pm 5%), a RPE of 18 or greater, and a decrease or plateau in VO₂ with an increased workload. The VO₂max tests were stopped due to subject volitional fatigue. Therefore, only five subjects met all of the criteria for a maximal response to the VO₂max test. Since not all of the subjects met all of the criteria for a VO₂max test, the tests will be referred to from here on out as VO₂peak tests.

The mean (\pm SD) time of the VO_2peak tests were 846.5 ± 79.9 seconds. The mean respiratory exchange ratio was 1.19 ± 0.05 , the mean maximal heart rate was 190 ± 6 bpm, and the mean maximal RPE was 18 ± 2 . The mean VO_2peak was 50.7 ± 9.0 ml/kg/min with a range of 38 – 65 ml/kg/min.

Menstrual Phase Determination

Each subject was asked to use an ovulation kit to determine ovulation, which was used to help determine the dates for the first and second exercise sessions. The average length of the menstrual cycle was 30 ± 3 days. Testing during the mid-follicular phase, low E_2 concentrations, occurred on day 5 ± 3 of the menstrual cycle; while testing during the mid-luteal phase, high E_2 concentrations, occurred on day 24 ± 4 of the menstrual cycle. Mean E_2 concentration during the mid-follicular phase was 27.04 ± 6.12 pg/ml. Mean E_2 concentration during the mid-luteal phase was 67.64 ± 21.7 pg/ml. The difference in E_2 concentrations between the mid-follicular and mid-luteal phases was significant ($t = -5.695$; $p < 0.001$).

Exercise Sessions One and Two

Subjects ran for one hour at $69.7 \pm 7.3\%$ of their pre-determined VO_2peak during the mid-follicular phase and $67.6 \pm 7.9\%$ of their pre-determined VO_2peak during the mid-luteal phase. VO_2 during each exercise session was collected breath by breath but averaged over 15 second intervals. However, there was no significant difference in the running intensities between exercise sessions ($t = 0.617$; $p = 0.545$).

Heart rates at rest, and all three time points during exercise, were compared between both exercise sessions. Resting HR was 75 ± 13 bpm during the mid-follicular phase and 66 ± 12 bpm during the mid-luteal phase. There was no significant difference due to the

interaction effect of menstrual cycle phase and time point at rest ($p = 0.169$) and during exercise ($p = 0.169$) on HR between the mid-follicular and mid-luteal phases of the menstrual cycle ($F = 2.259$; $p = 0.169$). There was no significant difference found due to the main effect for differing phases of the menstrual cycle ($F = 0.098$, $p = 0.762$). There was a significant difference due to the main effect of time from rest to the exercise ($F = 59.564$; $p < 0.001$). A post hoc determined the exercise HR at all measurement points were greater than the resting HR ($p < 0.001$), but not different from one another ($p > 0.05$). Heart rates during exercise are reported in Table 2.

The RPE at all three exercise time points were compared between both exercise sessions (Table 2). There was no significant difference due to the interaction effect of menstrual phase and time during exercise on RPE between the mid-follicular and mid-luteal phases of the menstrual cycle ($F = 0.499$; $p = 0.625$). There was no significant difference found due to the main effect for differing phases of the menstrual cycle ($F = 3.115$; $p = 0.111$). There was a significant difference found due to the main effect of time during exercise ($F = 8.565$; $p = 0.010$). A post hoc determined all time points during exercise differed significantly from one another ($p < 0.05$).

Oxygen uptake averaged over each monitored time point was compared between both sessions (Table 2). No significant difference was found due to the interaction effect of menstrual cycle phase and time during exercise ($F = 1.859$; $p = 0.225$). No significant difference was found due to main effect between phases of the menstrual cycle ($F = 2.662$; $p = 0.137$). There was a significant difference due to the main effect between time points during exercise ($F = 82.615$; $p < 0.001$). A post hoc determined the specific significance

occurred when comparing 10 minutes to 60 minutes ($p = 0.028$) and 30 minutes to 60 minutes ($p = 0.017$).

The RER during the last minute of each monitored time period was compared between both exercise sessions (Table 2). There was not a significant difference found due to the interaction effect of menstrual cycle phase and time point during exercise ($F = 3.912$, $p = 0.065$). There was not a significant difference found due to the main effect between phases of the menstrual cycle ($F = 0.06$, $p = 0.813$). There was a significant difference in RER between the different time points during exercise ($F = 9.359$, $p = 0.008$). A post hoc analysis determined the specific significance was the RER at 10 minutes (0.91 ± 0.05 ; 0.90 ± 0.06) and 60 minutes (0.88 ± 0.06 ; 0.86 ± 0.07) as the RER decreased over time ($p = 0.004$).

Table 2. The HR, RPE, and VO_2 measurements during the one hour exercise sessions at 65% of VO_{2max} during the mid-follicular and mid-luteal phases of the menstrual cycle. The HR, RPE, and VO_2 were obtained at 10, 30, and 60 minutes during the exercise.

		10 minutes	30 minutes	60 minutes
Mid Follicular	Heart Rate (bpm)	152 ± 25	160 ± 19	179 ± 19
	RPE	10 ± 2	11 ± 2	13 ± 3
	VO_2 (ml/kg/min)	$30.90 \pm 8.1^{\&}$	$31.52 \pm 8.0^{\wedge}$	$33.13 \pm 8.4^{\&\wedge}$
	RER	$0.91 \pm 0.05^{\&}$	0.89 ± 0.06	$0.88 \pm 0.06^{\&}$
Mid Luteal	Heart Rate (bpm)	155 ± 18	164 ± 18	167 ± 19
	RPE	10 ± 2	12 ± 3	14 ± 3
	VO_2 (ml/kg/min)	$30.02 \pm 8.1^{\&}$	$30.95 \pm 8.2^{\wedge}$	$31.56 \pm 8.4^{\&\wedge}$
	RER	$0.90 \pm 0.06^{\&}$	0.89 ± 0.068	$0.86 \pm 0.07^{\&}$

[&]Indicates a significance between the 10 minute time point and the 60 minute time point, $p < 0.05$; [^]Indicates a significance between the 30 minute time point and the 60 minute time point, $p < 0.05$

Lactate and Glucose Response to Exercise

Blood lactate concentrations were measured immediately post-exercise. During the mid-follicular phase of the menstrual cycle lactate concentration was 3.0 ± 1.8 mM/L. During the mid-luteal phase of the menstrual cycle lactate concentration was 2.4 ± 1.3 mM/L. There was no significant difference between post-exercise lactate concentrations during the mid-follicular and mid-luteal phases of the menstrual cycle ($t = 0.787$; $p = 0.44$).

Blood glucose concentrations were measured pre-exercise, immediately post-exercise, and 30 minutes into recovery, during both the mid-follicular and mid-luteal phases of the menstrual cycle exercise sessions. There was no significant difference found due to the interaction effect of menstrual cycle phase and time point ($F = 0.179$; $p = 0.839$). There was a significant difference due to menstrual cycle phase ($F = 5.731$; $p = 0.040$), with levels being slightly greater in the mid-follicular phase. There was also a significant difference due to time point ($F = 8.767$; $p = 0.010$). A post hoc determined the specific significance due to time point was between post-exercise and recovery ($p = 0.007$; Table 3).

Table 3. Blood glucose concentrations (mg/dL) pre-exercise, immediately post-exercise, and 30 minutes into recovery during the mid-follicular and mid-luteal phases of the menstrual cycle.

	Pre-Exercise (mg/dL)	Post-Exercise (mg/dL)	Recovery (mg/dL)
Mid-follicular	80 ± 13*	86 ± 15* [#]	78 ± 10* [#]
Mid-luteal	75 ± 11*	82 ± 10* [#]	76 ± 13* [#]

*Indicates a significant difference between the mid-follicular and mid-luteal phases of the menstrual cycle $p < 0.05$; [#]Indicates a significance between post-exercise and the recovery, $p < 0.05$

Insulin Response to Estradiol and Exercise

Blood insulin concentrations were measured pre-exercise, immediately post-exercise, and 30 minutes into recovery during both exercise sessions (see Table 4). No significant difference was found due to the interaction effect of menstrual cycle phase and time point ($F = 0.984$; $p = 0.415$). No significant difference was found in insulin concentration due to the main effect of menstrual cycle phase ($F = 0.247$; $p = 0.631$). In addition, no significant difference was found in insulin concentration due to the main effect of time point ($F = 0.685$; $p = 0.532$).

Insulin concentration percent change between pre-exercise to post-exercise and pre-exercise to recovery was calculated and compared (Table 5). No significant difference in

percent change was found due to the interaction effect of menstrual cycle phase and time point ($F = 0.507$; $p = 0.494$). No significant difference was found in percent change due to the main effect of menstrual cycle phase ($F = 0.861$; $p = 0.378$). Furthermore, no significant difference was found in percent change due to the main effect of time point ($F = 0.121$; $p = 0.736$).

Table 4. Blood insulin concentrations ($\mu\text{IU}/\text{ml}$) pre-exercise, post-exercise, and 30 minutes into recovery during the mid-follicular and mid-luteal phases of the menstrual cycle.

	Pre-Exercise ($\mu\text{IU}/\text{ml}$)	Post-Exercise ($\mu\text{IU}/\text{ml}$)	Recovery ($\mu\text{IU}/\text{ml}$)
Mid-follicular	3.85 ± 3.14	3.11 ± 1.64	2.69 ± 1.05
Mid-luteal	3.05 ± 1.18	2.85 ± 1.70	2.97 ± 1.09

Table 5. Insulin concentration percent change between pre-exercise to post-exercise and pre-exercise to recovery during the mid-follicular and mid-luteal phases of the menstrual cycle.

	Pre-Exercise to Post-Exercise (%)	Pre-Exercise to Recovery (%)
Mid-follicular	-6.31 ± 22.65	-8.13 ± 20.54
Mid-luteal	-1.00 ± 7.56	-0.46 ± 4.96

It should be noted that all hormonal values reported are not corrected for plasma volume shifts. This was done because no significant main effect differences ($p > 0.05$) were observed in the magnitude of the plasma volume movement because of menstrual cycle phase. Furthermore the data for hematocrit and hemoglobin, from which plasma volume shifts are calculated (26), are not reported due to this lack of significance existing.

Table 6. Individual measurements of VO_2peak , E_2 , and insulin concentration percent change during the mid-follicular and mid-luteal phases of the menstrual cycle for each subject. These data are previously reported as mean \pm standard deviation in earlier tables.

		Mid-follicular				Mid-Luteal			
Subject	VO ₂ peak (ml/kg/min)	E ₂ (pg/ml)	Insulin Pre-Exercise to Post-Exercise (%)	Insulin Pre-Exercise to Recovery (%)	E ₂ (pg/ml)	Insulin Pre-Exercise to Post-Exercise (%)	Insulin Pre-Exercise to Recovery (%)		
1	54.7	32.06	-4.08	-0.74	80.73	0.74	8.39		
2	38.4	27.00	0.14	0.82	44.66	-0.53	1.72		
3	53.8	20.74	19.45	1.20	37.41	-11.35	-7.86		
4	45.1	33.49	-0.32	-3.13	91.67	4.05	-0.45		
5	55.2	37.63	-16.32	-17.97	69.50	-6.76	-5.96		
6	63.2	26.81	-65.86	-63.90	102.81	0.75	-0.53		
7	42.4	18.93	3.78	-1.66	57.38	14.93	-4.67		
8	43.4	29.11	0.71	5.76	75.49	2.46	-1.68		
9	64.6	21.05	-1.62	-1.62	43.08	-8.17	1.48		
10	45.9	23.55	1.00	0.00	73.69	-6.07	5.01		

CHAPTER V

Discussion

The purpose of this study was to determine the influence of changes in circulating E₂ concentrations on the insulin response to an acute bout of exercise (60 minutes) in college age women, who were not using hormonal contraceptives. Circulating E₂ concentrations were manipulated by exercise testing subjects at the mid-follicular (low E₂) and mid-luteal (high E₂) phases of the menstrual cycle. There was not a significant difference in resting blood insulin concentration during the mid-follicular phase when compared to the mid-luteal phase of the menstrual cycle. Additionally, the added stress of exercise did not bring about a significant change in blood insulin concentrations during the mid-follicular or mid-luteal phase of the menstrual cycle. These findings were contrary to what had been hypothesized.

Ten subjects participated in this study which was the goal in order to achieve the pre-calculated statistical power of 0.80 (β). The subjects were healthy and met all the inclusion criteria. Furthermore, to the knowledge of the investigator all subjects complied with all of the study guidelines.

The physiological responses of the subjects for HR, VO₂, and RPE to the 60 minute exercise sessions showed the expected responses to such exercise, and were not significantly different between the phases of the menstrual cycle (4). These latter findings support that any observed changes in humoral or hormonal factors were more likely a function of menstrual phase and E₂ status than variability in exercise intensity.

Since the focus of this study was on metabolism, the discussion will be delimited to those measured outcomes that relate to energy metabolism as influenced by the hormone insulin. Thus, only blood lactate, glucose and insulin responses are discussed at length.

Lactate Response to Exercise

Blood lactate concentration was measured post-exercise, and no difference was found in concentrations between the mid-follicular and mid-luteal phases of the menstrual cycle. This is consistent with earlier work where blood lactate was measured either at rest, during exercise, or post-exercise (11, 12, 13, 23). In these studies blood lactate concentration significantly increased as exercise time increased but there was not a significant difference in blood lactate concentration due to changes in E₂ concentration (due to different phases of the menstrual cycle).

The observed lactate response, however, does not agree with all literature as some studies have reported reduced lactate responses to exercise in the luteal phase of the menstrual cycle due to an increased lipid mobilization induced by elevated E₂ (27, 28). The reason for the difference in findings between these and the present study is unclear, but may relate to the intensity of exercise utilized. The studies finding reduced lactate responses to exercise tended to use higher exercise intensities than the current study.

Glucose Response to Exercise

Blood glucose concentration was measured pre-exercise, immediately post-exercise, and 30 minutes into recovery. There was a main effect of menstrual cycle phase, resulting in blood glucose concentration being greater in the mid-follicular phase when compared to blood glucose concentration measured in the mid-luteal phase (mid-follicular > mid-luteal). A significant difference was also found across time of measurement too, as blood glucose

concentration between immediately post-exercise and 30 minutes into recovery differed (decrease from exercise to recovery). Previous published results are varied when looking at the effects of E₂ and exercise on blood glucose concentration.

In contrast with the current study, Horton et al. (12) found no difference in resting glucose values between different phases of the menstrual cycle in eumenorrheic women. However, there was a significant difference in the interaction effect of menstrual phase and exercise time, with blood glucose concentrations being higher in the first 45 minutes of exercise in the mid-luteal phase. Campbell et al. (23) discovered that in eumenorrheic women, menstrual phase had no effect on blood glucose concentration, but there was a significant decrease in blood glucose concentration at 120 minutes of exercise when subjects were not given a carbohydrate supplement during the exercise. Lavoie et al. (13) reported a significant interaction effect of menstrual phase and exercise time. In eumenorrheic subjects undergoing a 24-hour carbohydrate poor diet and an overnight fast, blood glucose concentration significantly decreased after 70 and 90 minutes of exercise in the luteal phase of the menstrual cycle but remained constant in the follicular phase of the menstrual cycle. Bonen et al. (11), however, reported a significant increase in blood glucose concentration during exercise but the change was similar in each phase of the menstrual cycle. Similar findings of Bonen et al. (11) were seen by Hackney et al. (14), who reported a similar blood glucose response to exercise and an oral glucose load in each phase of the menstrual cycle.

In the current study blood glucose decreased from immediately post-exercise into recovery, which contradicts some of the earlier results in studies where glucose increased. Differences between studies may be due to the time point at which blood sampling occurred. In many of the previous studies glucose was measured during exercise not into recovery.

At rest and during exercise, E_2 has been shown to increase lipolysis and decrease gluconeogenesis and glycogenolysis (8, 9, 17, 20). The increased concentration of E_2 and a greater reliance on lipids as an energy source during the mid-luteal phase may be the factor accounting for the significantly lowered blood glucose concentration in the current study (8). This interpretation is supported by the tendency towards a lower exercise lactate (non-significant) found in the mid-luteal phase as corroborated by other studies (27, 28). But, at this point, this view is speculative since lipid utilization was not measured in the current study.

Insulin Response to Estradiol and Exercise

Blood insulin concentration was measured pre-exercise, immediately post-exercise, and 30 minutes into recovery. No significant differences were found when comparing blood insulin concentrations between phases or across time points during exercise. Typically exercise is associated with a reduction in circulating insulin levels (4). This did occur in the present study, as immediately post exercise and 30 minutes into recovery insulin concentrations were reduced from pre-exercise, but not to a level to reach statistical significance. This lack of significance may be a function of a “basement effect” occurring within the data values (16). That is, the initial pre-exercise concentrations of insulin were low enough that a further reduction below a minimum background level (i.e., basement level) was not physiologically possible or analytically detectable.

These insulin findings are in agreement with some, but not all, studies as previous results are highly varied when looking at the effects of E_2 and exercise on blood insulin concentration. For example, Horton et al. (12) reported a significant interaction effect between menstrual phase and time point during exercise. Blood insulin concentration was

higher during the first 45 minutes of exercise in the mid-luteal phase when compared to the mid-follicular phase. Campbell et al. (23) reported a significant main effect of exercise time on insulin concentration (decreasing) and, although not significant, these investigators also reported a tendency for blood insulin concentration to be higher in the luteal phase of the menstrual cycle ($p = 0.07$). Lavoie et al. (13) also reported a significant decrease in insulin due to the main effect of exercise over time on insulin concentration ($p < 0.01$) in both the follicular and luteal phases of the menstrual cycle. Although not reaching statistical significance, Lavoie et al. did observe a trend towards lower insulin concentration during the luteal phase of the menstrual cycle. Bonen et al. (11) reported an opposite finding to Lavoie et al. (13), but somewhat similar to Campbell et al. (23), with slightly elevated levels of blood insulin concentration during the luteal phase of the menstrual cycle. Regrettably, the current findings do not add any clarity to the issue of the influence of E_2 and menstrual phase on insulin responses to exercise. These mixed findings within studies suggest further research is needed and warranted on this topic.

Insulin Percent Change

To determine if the added stress of exercise perhaps caused varying degrees of relative change in the insulin response during the different phases of the menstrual cycle, blood insulin concentration percent change was calculated between pre-exercise to immediately post-exercise and pre-exercise to 30 minutes into recovery. This means of expressing the insulin data also resulted in no significant differences between phases or across time points during exercise. Interestingly, all of the studies previously mentioned looked at the absolute insulin concentrations, and how concentrations were affected by varying levels of E_2 and the stress of exercise (12, 13, 23). It appears there have not been any

previous conducted menstrual cycle-exercise studies where insulin has been reported as calculated percent change. Obvious, the afore mentioned studies that did have significant absolute concentration changes would most likely have significant percent change responses too; and again, the current study data is at odds with some of these outcomes. This dissimilarity in results may be due to dissimilarities in the methods used between the studies. In the present study, subjects were asked to exercise for 60 minutes and in the other studies subjects were asked to exercise for at least 90 minutes or more. Methodology also differed on the acquisition of blood samples. In the present study, blood was drawn pre-exercise, immediately post-exercise, and 30 minutes into recovery. In the other studies discussed blood samples were taken pre-exercise and throughout the exercise bout, but not into recovery. Therefore although insulin was being monitored in each of the studies, the points at which the information was gathered could change the interpretation of the results obtained (29).

Conclusion

It has been accepted that during rest E_2 has both direct and indirect effects on energy metabolism (8). There have only been a handful of studies investigating the relationship between E_2 and exercise metabolism, specifically examining the key metabolic hormone insulin. Horton et al. (12) showed that insulin was higher during exercise in the mid-luteal phase of the menstrual cycle when E_2 is elevated. Lavoie et al. (13) did not find significant difference in insulin levels during exercise after an overnight fast. Hackney et al. (14) demonstrated that after a glucose load (oral glucose tolerance test; OGTT) insulin was significantly higher during the luteal phase of the menstrual cycle at rest and throughout exercise. It is evident from even these few examples in some situations that a relationship

exists between E₂, insulin, and exercise. However, the current study suggests that varying levels of E₂ brought about by normal hormonal fluctuations during the menstrual cycle do not have an effect on insulin during an acute bout of exercise. The methodologies for the studies that exist vary greatly between exercise modalities, intensities, length, diet restrictions, and when blood sampling occurred. The results obtained in this study may not coincide with previous works due to these methodological differences. The inconclusive nature of the present study and the contradictory findings in the research literature suggest that further research is warranted and should be conducted to allow for a greater understanding of exactly how E₂ influences insulin during exercise.

Hypothesis Outcomes

Based on the findings of this study, the following indicates the acceptance or rejection of the research hypotheses:

1. There is a significant difference in blood insulin levels during the mid-follicular phase when compared to blood insulin levels during the mid-luteal phase – Reject.
 - a. Insulin concentration at baseline is significantly higher during the mid-luteal phase compared to the mid-follicular phase – Reject.
 - b. Insulin concentration immediately post exercise is significantly higher during the mid-luteal phase compared to the mid-follicular phase – Reject.
 - c. Insulin concentration 30 minutes into recovery is significantly higher during the mid-luteal phase compared to the mid-follicular phase – Reject.
2. The added stress of exercise did not cause a significant change in blood insulin levels when comparing insulin concentrations pre and post exercise during the

mid-follicular phase to insulin concentrations pre and post exercise during the mid-luteal phase – Accept.

- a. There is no significant percent change in insulin concentration between baseline and immediately post-exercise when comparing the mid-follicular phase to the mid-luteal phase – Accept.
- b. There is no significant percent change in insulin concentration between baseline and 30 minutes into recovery when comparing the mid-follicular phase to the mid-luteal phase – Accept.

Study Limitations

There are several factors that directly influence the data within this study and are thus limitations to the research. Diet and hydration status greatly effects insulin and unfortunately these factors varied greatly between the subjects, although each subject was instructed to repeat their exact diet-hydration practices for the 24 hours before each 60 minute exercise session. To the knowledge of the investigator, all subjects complied with this request and indicated so when asked about compliance.

The insulin ELISA may not have been sensitive enough to capture the small, changes in insulin concentration since pre-exercise resting levels were very low. This issue is always a possibility in biochemical assays, however, the ELISA used in this study was classified as an ultra-sensitive type by the manufacturer which in the realm of modern technology categorizes it as being able to measure the insulin hormone at the smallest detectable concentrations in humans. Physiologically, it is also possible with the additional stress of exercise the insulin concentration (which normally decreases) could not decrease

substantially further since the levels started at the low end of concentrations found in the normal physiological range (i.e., basement effect).

Finally, it is certainly possible the sample size for this study was too small. A power calculation on the observed absolute insulin concentrations and the changes observed suggested a β of approximately 0.36 was achieved and that an additional 14 subjects would have been necessary to reach the more typically accepted β value of 0.80. Thus, the current study has to be viewed as underpowered statistically. Although a power calculation was done prior to beginning the investigation, differences in study methodology may account for the differences in calculated β . That is, the article used to calculate power compared insulin concentrations during exercise at differing phases of the menstrual cycle using a carbohydrate supplement or a placebo (18). The current study did not use any supplementation. In addition, the study used for the power calculation measured insulin concentration using a radioimmunoassay which may not have measured insulin concentrations as sensitively as the ELISA used in the current study. These discrepancies in methodology between the two studies may account for why the current study was underpowered.

Future Directions

Future research should look at not only insulin during exercise but also glucagon in order to observe the influence E_2 has on the Insulin/Glucagon ratio during exercise. Insulin promotes glucose uptake and glycogen synthesis while glucagon promotes glycogenolysis and gluconeogenesis (4). It has been demonstrated in animal models that E_2 can cause a decrease in glucagon secretion (30). Due to the antagonistic relationship of insulin and glucagon, the metabolic changes seen during exercise may be due to decreased glucagon

concentration which would mimic increased insulin concentration. Research should also be performed with different populations including hormonal contraceptive users, persons with metabolic diseases, older pre-menopausal women, and post-menopausal women. The information gathered incorporating such items may help to better explain the overall effects E_2 has on insulin and exercise.

APPENDIX A: Indications for Terminating Exercise Testing
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BOX 5-2 Indications for Terminating Exercise Testing*

ABSOLUTE INDICATIONS

- Drop in systolic blood pressure of >10 mm Hg from baseline[†] blood pressure despite an increase in workload, when accompanied by other evidence of ischemia
- Moderately severe angina (defined as 3 on standard scale)
- Increasing nervous system symptoms (e.g., ataxia, dizziness, or near syncope)
- Signs of poor perfusion (cyanosis or pallor)
- Technical difficulties monitoring the ECG or systolic blood pressure
- Subject's desire to stop
- Sustained ventricular tachycardia
- ST elevation ($+1.0$ mm) in leads without diagnostic Q-waves (other than V₁ or aVR)

RELATIVE INDICATIONS

- Drop in systolic blood pressure of >10 mm Hg from baseline[†] blood pressure despite an increase in workload, in the absence of other evidence of ischemia
- ST or QRS changes such as excessive ST depression (>2 mm horizontal or downsloping ST-segment depression) or marked axis shift
- Arrhythmias other than sustained ventricular tachycardia, including multifocal PVCs, triplets of PVCs, supraventricular tachycardia, heart block, or bradyarrhythmias
- Fatigue, shortness of breath, wheezing, leg cramps, or claudication
- Development of bundle-branch block or intraventricular conduction delay that cannot be distinguished from ventricular tachycardia
- Increasing chest pain
- Hypertensive response (systolic blood pressure of >250 mm Hg and/or a diastolic blood pressure of >115 mm Hg).

*See reference 1: Modified from Gibbons RJ, Balady GJ, Bricker JT, et al. ACC/AHA 2002 Guideline Update for Exercise Testing; a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines Committee on Exercise Testing, 2002. American College of Cardiology web site: www.acc.org/clinical/guidelines/exercise/dirIndex.htm

[†]Baseline refers to a measurement obtained immediately before the test and in the same posture as the test is being performed.

Taken from: Whaley MH, Brubaker PH, Otto RM, editors. American College of Sports Medicine's guidelines for exercise testing and prescription. 7thed. Baltimore: Lippincott Williams & Wilkins; 2006.

APPENDIX B: Informed Consent

Study #: 10-2109

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects
Biomedical Form

THIS CONSENT DOCUMENT SHOULD BE USED ONLY
BETWEEN 1/6/12 AND 12/17/12
APPROVED BY
INSTITUTIONAL REVIEW BOARD, UNC-CHAPEL HILL

IRB Study # 10-2109

Consent Form Version Date: 9/29/11

Title of Study: Influence of Estrogen on Cytokine Response to prolonged Treadmill Running

Principal Investigator: Dr. Anthony C. Hackney
UNC-Chapel Hill Department: Exercise and Sport Science
UNC-Chapel Hill Phone number: 919-962-0334
Email Address: ach@email.unc.edu
Faculty Advisor: None

Study Contact telephone number: 919-962-0334
Study Contact email: 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.

Research studies are designed to obtain new knowledge that may help other 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 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 ($\text{VO}_{2\text{max}}$), two 60-minute running bouts at approximately 65-70% $\text{VO}_{2\text{max}}$, 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 $\text{VO}_{2\text{max}}$ 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?

If you decide to be in this study, you will be one of approximately 20 people in this research study.

How long will your part in this study last?

You will be enrolled in the study for approximately 8 weeks. Within the 8 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 with a before and immediately, and 30 minutes after exercise blood draws performed by a certified phlebotomist (NCPT 56147), 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 Dr. Anthony C. Hackney.
- 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 K₃-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 VO₂.
- 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% VO₂max. 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 at your convenience, and listen to music.
- At the completion of exercise, another 1-teaspoon blood sample will be drawn following the same procedure, placed in K₃-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 72 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 and immune markers. At the completion of the study the specimens will be stored for 3 years. This is further discussed in another consent form you will be asked to sign.

What are the possible benefits from being in this study?

Research is designed to benefit society by gaining new knowledge. You will not receive any direct benefit from participating in the study.

What are the possible risks or discomforts involved with 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.

In addition, there may be uncommon or previously unknown risks that might occur. You should

report any problems to the researchers.

What are the risks to a pregnancy or to a nursing child?

If you are a woman and you are planning to get pregnant, you should not be in the study.

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 your privacy be protected?

Following phone 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.

No subjects will 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. However, by signing this form, you do not give up any of your legal rights.

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?

If you enroll in this study, the only cost to you will be transportation to the research site.

What if you are a UNC student?

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.

What if you are a UNC employee?

Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

What if you have questions about this study?

You have the right to ask, and have answered, any questions you may have about this research. If you have questions, complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?

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.

Title of Study: Influence of Estrogen on Cytokine Response to Prolonged Treadmill Running
Principal Investigator: Dr. Anthony C. Hackney, ach@email.unc.edu, (919) 962-0334

Subject'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.

Study #: 10-2109

Signature of Research Subject

Date

Printed Name of Research Subject

Signature of Research Team Member Obtaining Consent

Date

Printed Name of Research Team Member Obtaining Consent

University of North Carolina at Chapel Hill
Consent for Storing Biological Specimens With Identifying Information

IRB Study #: 10-2109

Consent Form Version Date: 10/06/11

Title of Study: Influence of Estrogen on Cytokine Response to Prolonged Treadmill Running

Principal Investigator: Anthony C. Hackney

UNC-Chapel Hill Department: Exercise and Sport Science

UNC-Chapel Hill Phone number: (919) 962-0334

Email Address: ach@email.unc.edu

Study Contact telephone number: (919) 962-0334

Study Contact email: ach@email.unc.edu

What are some general things you should know about research?

Research is designed to gain scientific information that may help other people in the future. You may not receive any direct benefit from participating. There also may be risks.

You may refuse to take part in research. If you are a patient with an illness, you do not have to be in research in order to receive treatment.

Details are discussed below. It is important that you understand this information so that you can make an informed choice. 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 specimen repository or "biobank?"

Research with blood, tissue or body fluids (specimens) can help researchers understand how the human body works. Research can also answer other questions by using specimens. Researchers may develop new tests to find diseases, or new ways to treat diseases. In the future, research may help to develop new products, such as drugs. Specimens are commonly used for genetic research. Sometimes researchers collect and store many specimens together and use them for different kinds of research, or share them with other scientists; this is called a specimen repository or "biobank."

The purpose of this particular repository or biobank is to determine the influence of estrogen on the immune response to prolonged running. Within this study, analyses are limited to two pro-inflammatory cytokines as a result of the study being self-funded and resources limited. If more resources become available in the future, further analyses on other immune markers will be conducted to develop a better understanding of the influence of estrogen on immune response. Blood will be collected and separated by centrifuging and frozen until later analysis. The plasma will be analyzed for estrogen concentration and immune markers.

How will the specimens be collected?

Blood samples will be collected by a certified phlebotomist (NCPT 561471) via venipuncture method, placed into a 5ml K₃-EDTA blood collection tube and immediately put on ice. The blood samples will be separated by centrifuging, plasma will be frozen at -80°C until later analysis.

What will happen to the specimens?

Blood samples will be stored in a secured ultra-freezer behind an access code protected door within a laboratory involving only electric ID card access. These specimens will be encoded and labeled in a fashion that no personal identifying information will be revealed. Only the principal investigator and faculty advisor will have access to the samples. The specimens will be destroyed within 3 years of the completion of the study.

What are the possible benefits to you?

Benefits to you are unlikely. Studies that use specimens from this repository may provide additional information that will be helpful in understanding the effect of estrogen on the immune response to prolonged running.

What are the possible risks or discomforts involved with the use of your specimens?

In the process of collecting the sample, risks associated with blood draws include infection, hematoma or bruising of the area around the needle insertion, and dizziness/fainting. 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. 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. Research technicians are first aid, CPR, and AED certified.

There is a risk of breach of confidentiality. If this research involves genetics, there is also a potential risk for some of your relatives and other members of your ethnic group, since they share some of your genetic makeup.

Will there be any cost to you for storage of the specimens?

There will be no cost to you for the storage and use of the specimens for research purposes.

Will you receive anything for the use of your specimens?

You will not receive anything for taking part in this research.

Who owns the specimens?

Any blood, body fluids, or tissue specimens obtained for this purpose become the exclusive property of the University of North Carolina at Chapel Hill. This organization may retain, preserve or dispose of these specimens and may use these specimens for research that may result in commercial applications. There are no plans to compensate you for any future commercial use of these specimens.

How will your privacy be protected?

Following phone 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. The identification number will consist of a unique number for each individual subject, 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 and faculty advisor will have access to the records. 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 within a laboratory involving only electric ID card access. These specimens will be encoded and labeled in a fashion that no personal identifying information will be revealed.

You will not be identified in any report or publication about research using your specimens. 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 could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

Will researchers seek approval from you to do future studies involving the specimens?

By signing this consent form, you are giving your permission for researchers to use your specimens as described above. Current and future research is overseen by a committee called the Institutional Review Board (IRB). The role of the IRB is to protect the rights and welfare of research participants. In some cases, the IRB may require that you be re-contacted and asked for your consent to use your specimens in a specific research study. You have the right, at that future time, not to participate in any research study for which your consent is sought. Refusal to participate will not affect your medical care or result in loss of benefits to which you are entitled.

Will you receive results from research involving your specimens?

Most research with your specimens is not expected to yield new information that would be meaningful to share with you personally. There are no plans to re-contact you or other subjects with information about research results.

Can you withdraw the specimens from the research repository?

If you decide that you no longer wish for the specimens to be stored, you should contact the researchers on the front page of this form. It is best to make your request in writing.

Any analysis in progress at the time of your request or already performed prior to your request being received by the researcher will continue to be used as part of the research study. Once the researchers have been notified, your remaining specimens would be destroyed. If you do not make such a request, the specimens may be stored forever. The researchers may choose to destroy the specimens at any time.

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 having your specimen collected. 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. However, by signing this form, you do not give up any of your legal rights.

What if you have questions about this research?

You have the right to ask, and have answered, any questions you may have about this research. If you have questions, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?

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 you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by e-mail to IRB_subjects@unc.edu.

Subject'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. I agree to my specimen(s) being stored with the identifying code(s).

Signature of Research Subject

Date

Printed Name of Research Subject

Signature of Research Team Member Obtaining Consent

Date

Printed Name of Research Team Member Obtaining Consent

APPENDIX C: Medical History Questionnaire

Department of Exercise and Sport Science Medical History

Subject: _____ ID: _____ Telephone: _____

Address: _____

Occupation: _____ Age: _____

	YES	NO
<u>Patient History</u>		
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: _____

APPENDIX D: Ovulation Kit Testing Protocol



WHEN TO BEGIN TESTING

To find out when to begin testing, determine the length of your normal cycle. The length of your cycle is from the beginning of one period (the day of first bleeding) to the day before the beginning of the next. Count the first day of bleeding or spotting as day one (1). If your cycle length is irregular, that is, if it varies by more than a few days each month take the average number of days for the last 3 months. Use the chart shown to work out the day you should begin testing. The day you begin testing is listed opposite the number of days in your normal cycle.

Length of Normal Cycle (Total days)	Start testing this many days after your last period began	Length of Normal Cycle (Total days)	Start testing this many days after your last period began
21	5	31	14
22	5	32	15
23	6	33	16
24	7	34	17
25	8	35	18
26	9	36	19
27	10	37	20
28	11	38	21
29	13	39	22
30	13	40	23

For example, if your period normally begins every 28 days, you should begin testing eleven (11) days after the beginning of your last period. A sample calendar is on the back.

INTERPRETATION OF THE TEST

As the LH One Step Ovulation Test Strip begins to work, a colored band will appear in the upper section of the result area to show that the test is working properly. This band is the Control Band (C). The lower section of the result area indicates the test results. If another colored band, the Test Band (T), appears at the lower section of the result area and is of equal or darker color than the Control Band, the results should be interpreted as a positive test.

Positive Result

If two (2) color bands are visible and the test band is equal to or darker than the control band, ovulation will probably occur in the next 24–48 hours.

To maximize your chances of getting pregnant, intercourse should take place between 24 and 48 hours after a positive test.

Negative Result

Only one color band appears on the control region or the test band is present but lighter in color intensity than the control band. There is no LH surge even if two lines present as long as test line is fainter than the control line, the result is negative.

Invalid Result

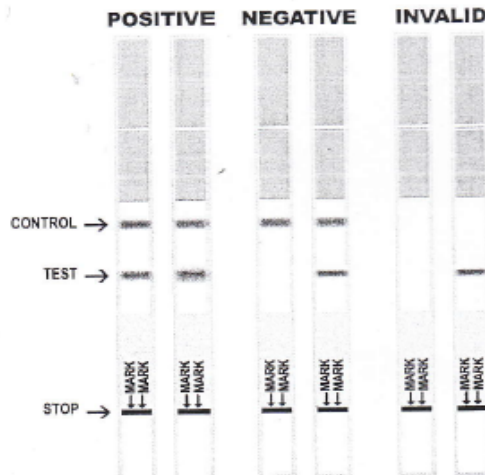
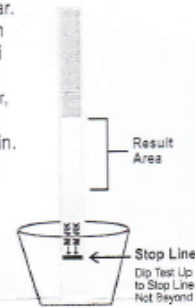
No visible bands in the control and test regions. Make sure to follow the specified instructions below for optimum results. You have to repeat the test by using a new LH One-Step Ovulation Test Strip.

When to Stop Testing

Unless otherwise specified by your doctor, stop testing once the LH surge is detected.

Instructions

1. Bring the test pouch to room temperature (18–30°C). Collect urine into a clean container (such as plastic cup). The best way to collect sample is by placing a cup in the middle of urination process. To begin testing, open the sealed pouch by tearing along the notch. Remove the test from the pouch when you are ready to use it.
2. Immerse the strip into the urine with the arrow end pointing toward the urine. Do not immerse past the MAX (maximum) line. Take the strip out after 5 seconds and lay the strip flat on a clean, dry, non-absorbent surface (e.g., mouth of the urine container). Do not immerse for longer than 7 seconds.
3. Wait for colored bands to appear. Depending on the concentration of LH in the test specimen, positive results may be observed in as little as 40 seconds. However, to confirm negative results, the complete reaction time of 10 min. is required. Do not read results after more than 30 minutes.
4. After interpreting the test results, discard the test strip.



APPENDIX E: Exercise Session Questionnaire

Exercise Session Questionnaire

Have you had anything to eat or drink, besides water, within the last **2** hours?

Yes No

Have you has 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)

Estradiol (Human) ELISA Kit

Catalog Number

KA0234 96 assays

Version: 03

Intended for research use only

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Introduction

Intended Use

For the quantitative determination of Estradiol (E2) concentration in human serum.

Background

Estradiol (E2) is a C18 steroid hormone with a phenolic A ring. This steroid hormone has a molecular weight of 272.4. It is the most potent natural Estrogen, produced mainly by the ovary, placenta, and in smaller amounts by the adrenal cortex, and the male testes (1,2,3).

Estradiol (E2) is secreted into the blood stream where 98% of it circulates bound to sex hormone binding globulin (SHBG). To a lesser extent it is bound to other serum proteins such as albumin. Only a tiny fraction circulates as free hormone or in the conjugated form (4,5). Estrogenic activity is effected via estradiol-receptor complexes which trigger the appropriate response at the nuclear level in the target sites. These sites include the follicles, uterus, breast, vagina, urethra, hypothalamus, pituitary and to a lesser extent the liver and skin.

In non-pregnant women with normal menstrual cycles, estradiol secretion follows a cyclic, biphasic pattern with the highest concentration found immediately prior to ovulation (6,7). The rising estradiol concentration is understood to exert a positive feedback influence at the level of the pituitary where it influences the secretion of the gonadotropins, follicle stimulating hormone (FSH), and luteinizing hormone (LH), which are essential for follicular maturation and ovulation, respectively (8,9). Following ovulation, estradiol levels fall rapidly until the luteal cells become active resulting in a secondary gentle rise and plateau of estradiol in the luteal phase. During pregnancy, maternal serum Estradiol levels increase considerably, to well above the pre-ovulatory peak levels and high levels are sustained throughout pregnancy (10).

Serum Estradiol measurements are a valuable index in evaluating a variety of menstrual dysfunctions such as precocious or delayed puberty in girls (11) and primary and secondary amenorrhea and menopause (12). Estradiol levels have been reported to be increased in patients with feminizing syndromes (14), gynaecomastia (15) and testicular tumors (16).

In cases of infertility, serum Estradiol measurements are useful for monitoring induction of ovulation following treatment with, for example, clomiphene citrate, LH-releasing hormone (LH-RH), or exogenous gonadotropins (17,18). During ovarian hyperstimulation for in vitro fertilization (IVF), serum estradiol concentrations are usually monitored daily for optimal timing of human chorionic gonadotropin (hCG) administration and oocyte collection (19).

Principle of the Assay

The E2 EIA is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol. In the incubation, goat anti-rabbit IgG-coated wells are incubated with 25 µl E2 standards, controls, patient samples, 100 µl Estradiol-HRP Conjugate Reagent and 50 µl rabbit anti-Estradiol reagent at room temperature (18-25°C) for 90 minutes. During the incubation, a fixed amount of HRP-labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled E2 in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The E2 concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve.

General Information

Materials Supplied

List of component

Component	Amount
Goat Anti-Rabbit IgG-coated microtiter wells	96 wells
Estradiol Reference Standards: 0, 10, 30, 100, 300, and 1000 pg/ml. Liquid, ready to use.	0.5 ml each
Rabbit Anti-Estradiol Reagent (pink color)	7 ml
Estradiol-HRP Conjugate Reagent (blue color)	12 ml
Estradiol Control 1, Liquid, Ready to use	0.5 ml
Estradiol Control 2, Liquid, Ready to use	0.5 ml
TMB Reagent (One-Step)	11 ml
Stop Solution (1N HCl)	11 ml

Storage Instruction

Unopened test kits should be stored at 2-8°C upon receipt and the microtiter plate should be kept in a sealed bag with desiccants to minimize exposure to damp air. Opened test kits will remain stable until the expiration date shown, provided it is stored as described above.

Materials Required but Not Supplied

Precision pipettes: 25 µl , 50 µl, 100 µl, 200 µl, and 1.0 ml. Disposable pipette tips.

Distilled and deionized water.

Vortex mixer or equivalent.

Absorbent paper or paper towel. Linear-linear graph paper.

A microtiter plate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 O.D. at 450 nm wavelength is acceptable for use in absorbance measurement.

Precautions for Use

1. Test methods are not available which can offer complete assurance that Hepatitis B virus, Human Immunodeficiency Virus (HIV/HTLV-III/LAV), or other infectious agents are absent from the reagents in this kit. Therefore, all human blood products, including patient samples, should be considered potentially infectious. Handling and disposal should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation, where it exists (e.g., USA Center for Disease Control/National Institute of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories," 1984)(22).
2. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. Serum samples demonstrating gross lipemia, gross hemolysis, or turbidity should not be used with this test.

Assay Protocol

Sample Preparation

1. Only human serum should be used.
2. No special pretreatment of sample is necessary.
3. Serum samples may be stored at 2-8°C for up to 24 hours, and should be frozen at -10°C or lower for longer periods. Do not use grossly hemolyzed or grossly lipemic specimens.
4. Please note: Samples containing sodium azide should not be used in the assay.
5. Samples with expected Estradiol concentrations over 1000 ng/ml may be quantitated by dilution with diluent available from Abnova, Inc.

Assay Procedure

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

□ Quality Control

Good laboratory practice requires that controls are run with each calibration curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. We recommend using Bio-Rad Lyphochek Immunoassay Control Sera as controls. Abnova Estradiol EIA kit also provides with internal controls, Level 1 and 2.

Data Analysis

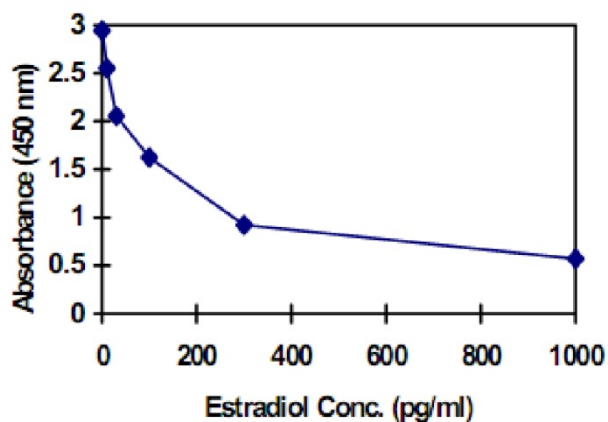
Calculation of Results

1. Calculate the mean absorbance value (A_{450}) for each set of reference standards, controls and samples.
2. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in pg/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis.
3. Use the mean absorbance values for each specimen to determine the corresponding concentration of Estradiol in pg/ml from the standard curve.
4. Any values obtained for diluted samples must be further converted by applying the appropriate dilution factor in the calculations.

□ Example of Standard Curve

Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against Estradiol concentrations shown in the X axis. Note: This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each experiment.

Estradiol (pg/ml)	Absorbance (450 nm)
0	2.943
10	2.551
30	2.055
100	1.624
300	0.925
1000	0.571



Expected Values and Sensitivity

Each laboratory should establish its own normal range based on the patient population. Abnova Estradiol EIA was performed on randomly selected outpatient clinical laboratory samples. The results of these determinations are as follows:

Males:		< 60 pg/ml
Females:	postmenopausal phase	< 18 pg/ml
	ovulating, early follicular	30-100 pg/ml
	late follicular	100-400 pg/ml
	luteal phase	60-150 pg/ml
	pregnant, normal	up to 35,000 pg/ml
	prepubertal children, normal	< 10 pg/ml

Performance Characteristics

□ Sensitivity

The minimum detectable concentration of the Abnova Estradiol ELISA assay as measured by 2 SD from the mean of a zero standard is estimated to be 10 pg/ml..

□ Specificity

The following materials have been checked for cross reactivity. The percentage indicates cross reactivity at 50% displacement compared to Estradiol.

Data on the cross-reactivity for several endogenous and pharmaceutical steroids are summarized in the following table:

Cross-reactivity (%) = Observed Estradiol Concentration / Steroid Concentration × 100.

Steroid	Cross-Reactivity
Estradiol	100%
Estrone	2.10%
Estriol	1.50%
17a Estradiol	0.30%
Cortisol	<0.01%
Cortisone	<0.01%
Progesterone	<0.01%
Testosterone	<0.01%
DHEA-Sulphate	<0.01%
5a-Dihydrotestosterone	<0.01%

Precision

Within-run precision was determined by replicate determinations of four different serum samples in one assay. Within-assay variability is shown below:

Samples	1	2	3	4
# Replicates	24	24	24	24
Mean Estradiol (pg/ml)	13	73	247	633
S.D.	3	7	10	31
C.V. (%)	24.1	10.3	4.1	4.9

Between-run precision was determined by replicate measurements of six different serum samples over a series of individually calibrated assays. Between-assay variability is shown below:

Samples	1	2	3	4
# Replicates	20	20	20	20
Mean Estradiol (pg/ml)	14	82	264	691
S.D.	4	8	17	45
C.V. (%)	26.7	10.3	6.4	6.6

□ Spiking Recovery

Various patient serum samples of known Estradiol levels were combined and assayed in duplicate. The mean recovery was 101.3%.

PAIR NO.	EXPECTED [Estradiol] (pg/ml)	OBSERVED [Estradiol] (pg/ml)	% RECOVERY
1	580	599	103.3
2	810	733	90.4
3	280	249	89.2
4	265	285	107.5
5	81	91	112.6
6	62	67	107.3
7	18	18	98.7

Resources

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Insulin (Human) ELISA Kit

Catalog Number

KA0921 96 assays

Version: 02

Intended for research use only

Introduction and Background

A. Overview

Insulin is the principal hormone responsible for the control of glucose metabolism. It is synthesized in the β -cells of the islets of Langerhans as the precursor, proinsulin, which is processed to form C-peptide and insulin. Both are secreted in equimolar amounts into the portal circulation. The mature insulin molecule comprises two polypeptide chains, the A chain and B chain (21 and 30 amino acids respectively). The two chains are linked together by two inter-chain disulphide bridges. There is also an intra-chain disulphide bridge in the A chain. Insulin concentrations are severely reduced in insulin-dependent diabetes mellitus (IDDM) and some other conditions such as hypopituitarism. Insulin levels are raised in non-insulin-dependent diabetes mellitus (NIDDM), obesity, insulinoma and some endocrine dysfunctions such as Cushing's syndrome and acromegaly.

B. Test Principle

The Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with enzyme (HRP)-conjugated anti-insulin antibody and anti-insulin antibody bound to micro-titration well. A simple washing step removes unbound enzyme labeled antibody. The bound HRP complex is detected by reaction with TMB substrate. The reaction is stopped by adding acid to give a colorimetric endpoint that is read using ELISA reader.

C. Notice for Application of Kit

This kit has been configured for research use only and is not for diagnostic and clinical use.

D. Application

The Insulin ELISA Kit is intended for the quantitative measurement Insulin in human serum or plasma.

Material and Method

A. List of component

	96 Tests
Microwells coated with Insulin MAb	12x8x1
Insulin Standard 1:1 vial (Ready to use)	2 ml
Insulin Standards 2-6: 5 vials (Ready to use)	1 ml
Insulin Enzyme Conjugate: 1 vial	1 ml
Assay Diluent: 1 bottle (Ready to use)	14 ml
TMB Substrate: 1 bottle (Ready to use)	12 ml
Stop Solution: 1 bottle (Ready to use)	12 ml
20X Wash concentrate: 1 bottle	25 ml

B. Additional Required Materials But Not Provided

- 1. Distilled or deionized water**
- 2. Precision pipettes**
- 3. Disposable pipette tips**
- 4. ELISA reader capable of reading absorbance at 450nm**
- 5. Absorbance paper or paper towel**
- 6. Graph paper**

C. Stability and Storage

- 1. Store the kit at 2-8°C.**
- 2. Keep microwells sealed in a dry bag with desiccants.**
- 3. The reagents are stable until expiration of the kit.**
- 4. Do not expose reagent to heat, sun, or strong light.**

D. Warnings and Precautions for Users

- 1. Potential biohazardous materials:**

The calibrator and controls contain human source components, which have been tested and found non-reactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents. However there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent. These reagents should be handled at the Biosafety Level 2, as recommended in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories" 1984.

2. This test kit is USA FDA exempt product.
3. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.
4. The components in this kit are intended for use as an integral unit. The components of different lots should not be mixed.
5. It is recommended that standards, control and serum samples be run in duplicate.
6. Optimal results will be obtained by strict adherence to the test protocol. Accurate and precise pipetting as following the exact time and temperature requirements prescribed are essential. Any deviation from this may yield invalid data.

E. Specimen Collection and Handling

1. Collect blood specimens and separate the serum immediately.
2. Specimens may be stored refrigerated at (2-8°C) for 5 days. If storage time exceeds 5 days, store frozen at (-20°C) for up to one month.
3. Avoid multiple freeze-thaw cycles.
4. Prior to assay, frozen sera should be completely thawed and mixed well.
5. Do not use grossly lipemic specimens.

F. Reagent Preparation and Storage

1. **20X Enzyme Conjugate:** Prepare 1X working dilution at 1:20 with assay diluent as needed, e.g. 0.1 ml of the stock conjugate in 1.9 ml of assay diluent is sufficient for 20 wells. The diluted conjugate has to be used the same day.
2. **20X Wash Buffer Concentrate:** Prepare 1X wash buffer by adding the contents of the bottle to 475 ml of distilled water. Store 1X wash buffer at room temperature.

G. Protocol

Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before use.

1. Place the desired number of coated strips into the holder.
2. Pipette 25 µl of Insulin standards, control and patient's sera into appropriate wells.
3. Add 100 µl of working Insulin Enzyme Conjugate to all wells.
4. Thoroughly mix for 10 sec., it is important to have a complete mixing in this step.
5. Incubate for 60 minutes at room temperature (18-26°C).
6. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbent paper towels.
7. Add 100 µl of TMB substrate to all wells.

8. Incubate for 15 minutes at room temperature.
9. Add 50 μ l of stop solution to all wells. Shake the plate gently to mix the solution.
10. Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of Results

The standard curve is constructed as follows:

1. Check Insulin standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit.
2. To construct the standard curve, plot the absorbance for the insulin standards (vertical axis) versus the insulin standard concentrations in μ U/ml (horizontal axis) on a linear graph paper. Draw the best curve through the points.
3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.
4. Value above the highest point of the standard are retested after diluting with "0" standard.

Example of Standard Curve

	OD 450 nm	Conc. μ U/mL
Standard 1	0.05	0
Standard 2	0.11	6.25
Standard 3	0.22	12.5
Standard 4	0.49	25
Standard 5	1.00	50
Standard 6	2.11	100

Expected Values

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

In a study conducted with apparently normal healthy adults, using the Insulin ELISA the following values are observed: < 25 μ U/ml.

Limitation of the Test

1. The test results obtained using this kit serve only as an aid to diagnosis and should be interpreted in relation to the patient's history, physical findings and other diagnostic procedures.

2. Do not use sodium azide as preservative. Sodium azide inhibits HRP enzyme activities.

Performance Characteristics

1. Correlation with a Reference ELISA kit:

A total of 62 sera were tested by this ELISA and a reference ELISA kit. Results were as follows:

Correlation	Slope	Intercept
0.91	0.80	0.24

2. Precision

a. Intra-Assay

Sample	No. of Replicates	Mean $\mu\text{IU/ml}$	Standard Deviation	Coefficient of Variation (%)
1	10	9.26	0.58	6.3
2	10	7.01	0.57	8.1

b. Inter-Assay

Sample	No. of Replicates	Mean $\mu\text{IU/ml}$	Standard Deviation	Coefficient of Variation (%)
1	10	6.79	0.58	8.5
2	10	9.27	0.69	7.4

3. Linearity

Two different patient samples were diluted with the "0" calibrator to 1:2, 1:4, 1:8. Insulin values were assayed and results were corrected with the dilution factor. The results of these dilution tests are as follows:

Original Value		Percentage of Recovery		
Serum	($\mu\text{IU/ml}$)	1/2	1/4	1/8
1	30	95.1	91.5	88.6
2	71	106	95.5	105

4. Sensitivity

The sensitivity was determined by calculating the mean plus 2SD of the standard zero point tested 20 times in the same run.

Serum	No. of Replicates	Mean ($\mu\text{IU/ml}$)	Standard Deviation	Mean + 2SD (Sensitivity)
Zero Standard	20	0.477	0.495	1.467

5. Specificity

The antibodies employed in this kit cross react with bovine insulin (20-25%) and porcine insulin but not with proinsulin of any species or any other insulin complexes.

6. Recovery

Samples have been spiked by adding Insulin solutions with known concentrations in a 1:1 ratio.

Expected value (μIU/ml)	Recovered (μIU/ml)	Percentage of Recovery
9.85	8.80	89.3
41.1	40.4	98.3
53.7	54.2	100.9

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