INFLAMMATORY RESPONSE TO A HIGH-FORCE ECCENTRIC EXERCISE PROTOCOL IN ORAL CONTRACEPTIVE USERS AND NON-USERS

by

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GLOSSARY

17β-estradiol	The major estrogen hormone in humans
Acute Phase Response	The increase or decrease of proteins, usually from the liver, in response to inflammation
Alarm Cytokine	The first cytokines released at the initiation of inflammation; propagates an inflammatory response
Ethinyl Estradiol	A synthetic derivative of estradiol
Follicular Phase	The phase of the menstrual cycle controlled by estradiol in which ovarian follicles mature; ends with ovulation
Luteal Phase	The phase of the menstrual cycle controlled by progesterone in which the corpus luteum is formed; ends with pregnancy or menstruation

ACRONYMS

BMI	Body Mass Index
CAD	Coronary Artery Disease
CK	Creatine Kinase
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked ImmunoSorbent Assay
HDL-c	High Density Lipoprotein Cholesterol
HRT	Hormone Replacement Therapy
hs-CRP	High sensitivity-C-Reactive Protein
ICAM	Intracellular Adhesion Molecule
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LDL-c	Low Density Lipoprotein Cholesterol
Lp(a)	Lipoprotein(a)
MI	Myocardial Infarction
MCP	Monocyte Chemoattractant Protein
NF-κβ	Nuclear Factor-κβ
NO	Nitric Oxide
OC	Oral Contraceptive

ACRONYMS (CONTINUED)

PAI-1	Plasminogen-Activator Inhibitor
t-PA	Tissue Plasminogen Activator
TC	Total Cholesterol
TG	Triglyceride
TNF-α	Tumor Necrosis Factor-α
TNF-R1	Tumor Necrosis Factor-Receptor 1
VCA	Vascular Cell Adhesion Molecule

ABSTRACT

Researchers have demonstrated that oral contraceptive users have elevated serum C-reactive protein (CRP) levels. However, it is unclear whether or not CRP is elevated in response to inflammation. Results of preliminary studies indicate that individuals with elevated CRP exhibit diminished release of interleukin-6 (IL-6), an inflammatory mediator with anti-inflammatory properties. Low IL-6 may cause less control of inflammation and a resultant elevation of CRP. The inflammatory response was compared between female oral contraceptive (OC) users and non-OC users because of the known elevation of CRP in OC users. A high-force eccentric exercise protocol was used to induce muscle damage to the biceps brachii via 45 repetitions of the elbow flexors of the non-dominant limb. Blood was collected pre-exercise and at 4-, 8-, 12-, 24-, 48-, 96-, and 120-h post-exercise for analysis of muscle damage and inflammatory markers. At baseline, cortisol was greater in the OC group than the non-OC group. CRP was greater in OC users than non-OC users at all time points. Soluble tumor necrosis factor-receptor 1 (sTNF-R1) and cortisol decreased, and IL-6 increased, in the post-exercise phase, indicating an inflammatory response. Because IL-6 increased post-exercise while sTNF-R1 decreased, IL-6 was most likely released from skeletal muscle and acted as an anti-inflammatory agent. Although total cortisol was higher in the OC group, these findings may be controversial, as free cortisol may have greater implications on inflammation. CRP did not appear to be associated with the other inflammatory markers and IL-6 secreted from skeletal muscle has an anti-inflammatory effect.

CHAPTER 1

INTRODUCTION

Cardiovascular disease accounts for more deaths in the United States than any other cause (The European Society of Human Reproduction and Embyology [ESHRE] Capri Workshop Group, 1998). Although it is well recognized that men have a high risk for developing cardiovascular disease (CVD), women are likely to develop CVD as well, especially after menopause. Female hormonal influences delay increases in CVD risk until about ten years after the age-related increase in risk in men. Even though overall mortality from CVD has decreased about 25% overall since 1975, mortality though coronary heart disease is higher in women than in men (Dorner & Rieder, 2004). This statistic indicates a need to investigate female specific risks for developing cardiovascular disease.

Many risk factors for CVD are known and many are modifiable. The most well-known modifiable risks include smoking, hypertension, obesity, insulin resistance, sedentary lifestyle, poor diet, and dyslipidemia, which includes elevated triglycerides (TG), elevated levels of total cholesterol and low-density lipoprotein cholesterol (LDL-c), and low levels of high-density cholesterol (HDL-c) (Dorner & Rieder, 2004). As evidenced by an increased risk of cardiovascular disease in women after menopause, low estrogen levels may also pose an independent risk factor for CVD. Additionally, low-grade systemic inflammation has been cited as a relatively new risk factor for CVD (Pearson et al., 2003).

Of the inflammatory cascade markers, CRP is the most reliable inflammatory marker for predicting a cardiovascular event (Dvorakova & Poledne, 2004; Pearson et al., 2003). CRP is stable, and has a widely available assay (high sensitivity (hs)-CRP) that is standardized by the World Health Organization. Furthermore, CRP levels are related to a number of known CVD risk factors. High levels of CRP are associated with increasing body mass index (BMI) (Bermudez, Rifai, Buring, Manson, & Ridker, 2002; Dvorakova & Poledne, 2004; Raitakari, Mansikkaniemi, Marniemi, Viikari, & Raitakari, 2005; Visser, Bouter, McQuillan, Wener, & Harris, 1999; Williams, Williams, Milne, Hancox, & Poulton, 2004), systolic blood pressure (Bermudez et al., 2002; Williams et al., 2004), smoking (Bermudez et al., 2002; Raitakari et al., 2005), and fasting insulin (Pradham, Cook, Buring, Manson, & Ridker, 2003). CRP is inversely associated with cardiorespiratory fitness independent of weight status, blood pressure, and smoking status (Williams, Milne, Hancox, & Poulton, 2005). In fact, hs-CRP correlates with CVD risk independently of a host of other risk factors, and may contribute additional prognosis information beyond the traditional predictors of CVD development and progression (Dvorakova & Poledne, 2004; Pearson et al., 2003; Ridker, Rifai, Cook, Bradwin, & Buring, 2005).

Higher plasma CRP concentrations have been measured in women using oral contraceptives (OC's). In fact, OC use and BMI have the greatest influence over CRP level in healthy women between the ages of 24-39, and one-third of women taking OC's have high (>3.0 mg/L) CRP levels (Raitakari et al., 2005). Presently, the mechanisms by which hormones affect CVD are unclear; however, oral estrogens may possibly have an inflammatory effect. Although users of OC's have not shown increases in myocardial

infarctions in the absence of other risk factors, combining OC's with smoking increased CVD risk 30-fold, which is a greater increase in risk than in smokers who did not use OC's (The ESHRE Capri Workshop Group, 1998).

The general population taking oral contraceptives are young women, who have a low risk of heart disease and are not likely to demonstrate most known CVD risk factors. However, research has found that women taking OC's present with inflammatory markers; yet it is unknown whether these markers affect the endogenous control of the inflammatory response. Therefore, inflammation must be induced in order to observe the effects of oral contraceptive hormone therapy on regulating inflammation.

Skeletal muscle damage is known to create an inflammatory response that simulates inflammation, and eccentric contractions have been shown to induce more skeletal muscle damage than either concentric or isometric contractions (Miles, 2005). Therefore, an eccentric exercise protocol was implemented to induce inflammation. The response to muscle damage is initiated by mechanical disruption of the muscle fibers and the activation of local macrophages. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), proinflammatory cytokines, are then released. These cytokines then stimulate the release of interleukin-6 (IL-6) from the damaged muscle. IL-6 then propagates the acute phase response, inducing the release of CRP from the liver (Miles, 2005). CRP marks inflammation on a systemic level. The magnitude and duration of the presence of CRP can then be measured to determine the control of the inflammatory response. IL-6 serves as a negative feedback inhibitor of inflammation, and is partially responsible for this control.

Endogenous estrogen is known to protect muscle fibers (Stupka & Tiidus, 2001), as well as protect from CVD. Estrogen may also play a role in controlling inflammation, as suggested by studies comparing the response to eccentric exercise between men and women (MacIntyre, Reid, Lyster, & McKenzie, 2000; Stupka et al., 2000). However, it is unclear whether or not exogenous estrogens have similar effects. Therefore, the inflammatory response was compared between OC users and nonusers.

Statement of the Problem

Research has shown that OC users have high basal serum concentrations of CRP (Raitakari et al., 2005). CRP is an indicator of systemic low-grade inflammation. However, it is unclear whether or not CRP levels are elevated in OC users as a result of an acute phase inflammatory response or for other reasons. Results of preliminary studies show that individuals with elevated basal CRP have a diminished IL-6 response, and therefore, less control of inflammation (Miles, unpublished work). The objective of the present study is to compare the response to an acute stress elicited by eccentric exercise induced muscle damage between OC users and nonusers.

Purpose

The purpose of this study is to determine whether a difference exists in the inflammatory response to muscle damage induced by an acute bout of eccentric exercise between OC users and non-users.

Hypothesis

Preliminary studies on the inflammatory response in individuals with varying CRP levels indicate that those with high basal CRP levels have less inflammatory control, indicated by an attenuated IL-6 response, than those with normal CRP levels. Because women taking OC's have elevated basal CRP, it is expected that women taking OC's will have a lower IL-6 response and a higher CRP response to muscle damage compared to women not taking OC's.

$$HO: \mu OC = \mu non\text{-}OC$$

$$HA: \mu OC \neq \mu non\text{-}OC$$

where μOC is equal to the mean inflammatory responses in women not taking OC's and μnon-OC is equal to the mean inflammatory responses in women taking OC's.

Limitations

Certain conditions limit the generalizability of the study findings. Some women may not report heavy lifting outside of a specific exercise program if these activities are infrequent or occur only once. Performance of these activities may induce the repeated bout effect, thereby diminishing the inflammatory response to the exercise protocol. Secondly, diet may also influence the results. Antioxidants have the effect of reducing inflammation. Individual differences in consumption of foods containing antioxidants, especially vitamins C & E, may interfere with the results. Finally, the study measurements require participants to come to the lab for eighteen blood draws. Volunteers without much vested interest in the study may not be willing to partake in this

number of blood draws. The study findings are generalizable only to women between the ages of 18-40 who do not perform heavy eccentric exercise and who consume a diet average in antioxidants.

Delimitations

The inclusion criteria for this study specified women with similar characteristics and without indicators of inflammation. Only women between the ages of 18-40 were included in the investigation. Women any younger may not have regular menstrual cycles and estrogen status; women any older may have age-related inflammation and/or undiagnosed CVD. Women with known inflammatory conditions, diabetes, CVD, kidney disease were excluded; additionally, women who smoke, drink more than one alcoholic drink per day, or take certain medications were excluded as well. Women with anemia were also excluded, as low iron is associated with an immune response. Women with musculoskeletal limitations who were unable to perform the exercise were excluded. Finally, women who have weight trained in the last year were excluded because of the repeated bout effect. This effect states that muscle that has been exposed to high-force eccentric exercise will not respond in the same manner to a repeated bout of eccentric exercise for as long as several months. All women were free of known inflammation and are expected to have an inflammatory response to high-force eccentric exercise.

CHAPTER 2

REVIEW OF LITERATURE

Cardiovascular disease (CVD) is the leading cause of death in the United States for both men and women (CDC, 2005), yet most research to date on CVD has used male participants. In recent years, researchers have recognized a gender difference in the incidence and presentation of CVD (Eizagaechevarria, Perez, Ramos, & Garcia, 2006). Traditional risk factors for the development of CVD in women include smoking, a waist-to-hip ratio of greater than 0.8, triglyceride concentrations greater than 150 mg/dL, low-density lipoprotein cholesterol (LDL-c) concentrations greater than 115 mg/dL, high-density lipoprotein cholesterol (HDL-c) less than 45 mg/dL, ratio of LDL-c to HDL-c greater than 4.0, fasting glucose values greater than 100 mg/dL, hypertension, a high fat diet, and little physical activity (Castelli, 1999). The factors present additional risk in women at the onset of menopause, a time when natural estrogen production decreases.

Estrogen is known to positively affect a variety of risk factors for cardiovascular disease, particularly blood lipid levels, endothelial function, thrombosis, and inflammation. However, results from the Women's Health Initiative (WHI) showed no protective effect of hormone replacement therapy on risk of developing CVD, and in fact, researchers found that mortality from CVD is higher in women than in men (Eizagaechevarria et al., 2006). Additionally, oral contraceptive use has been associated with systemic inflammation; specifically, CRP levels are significantly higher in OC users versus nonusers. CRP has a direct role in the progression of atherosclerosis, suggesting that OC users with high CRP may be at increased risk for CVD. Therefore, the specific

role of estrogen in the progression of cardiovascular disease is not fully understood, warranting an investigation of the effects of estrogen on the cardiovascular disease process in women.

Estrogen and Cardiovascular Disease Risk

Researchers found that premenopausal women with menstrual irregularity have increased risk of developing CVD (Matthews et al., 2006; Solomon et al., 2002) providing evidence for an influence of estrogen on cardiovascular function. Solomon et al. reported greater body mass index, diabetes, hypertension, and hypercholesterolemia in women with irregular menstrual cycles compared to those women cycling normally. Irregular cycles indicate abnormal hormone levels; therefore, altered hormones may be linked to the higher prevalence of these risk factors for CVD in women with menstrual irregularities.

Menstrual irregularity and impaired hormonal response to menstrual cycle changes are not the only evidence suggesting that estrogen status invokes CVD risk. The National Heart, Lung, and Blood Institute (NHLBI) conducted the Women's Ischemia Syndrome Evaluation (WISE) study and angiographically observed atherosclerosis in the coronary arteries of premenopausal women (Bairey Merz et al., 2003). Women with further progression of atherosclerosis had hypoestrogenemia of hypothalamic origin (Bairey Merz et al.) indicating that estrogen plays a key role in prevention of coronary artery disease (CAD). In another group of women, those with CAD had lower estrogen concentrations than did healthy women; moreover, lower estrogen was also correlated with impaired flow-mediated and nitroglycerin-induced dilation (Li et al., 2004). Poor

vasodilation results in reduced blood flow. Accordingly, coronary flow velocity reserve was slower in the luteal phase of menstruation, when estrogen is low, than during the follicular phase, when estrogen is high (Hirata et al., 2001). Additionally, low estrogen status independently predicts all-cause and cardiac death and non-fatal myocardial infarction (MI) (Morise, 2006) and irregular menstrual cycles are associated with a 50% increase in risk of MI (Solomon et al., 2002).

Estrogen also has shown cardioprotective effects. Endogenous estrogen promotes vascular endothelial function. In a cynomolgus monkey model, premenopausal monkeys were found to have less coronary artery atherosclerosis than either males or surgically induced postmenopausal monkeys; furthermore, the hyperestrogenemic state of pregnancy further protected the coronary arteries from atherosclerotic progression (Clarkson, Anthony, & Klein, 1994). This protection may result from estrogen's effect on vasodilation, as estrogen has been shown to increase nitric oxide (NO) production (The ESHRE Capri Workshop Group, 1998), facilitating coronary relaxation, and flow-mediated dilatation and nitroglycerin-induced dilatation of brachial arteries (Li et al., 2004). Furthermore, estradiol decreases LDL-c uptake by arterial wall, reduces total cholesterol in the atherosclerotic plaque and in macrophages, and decreases fibrinogen, factor VII, and platelet aggregation, all part of the clotting mechanism (The ESHRE Capri Workshop Group, 1998).

Additionally, Matthews et al. (2006) found that women with proper ovarian function, as evidenced by high estrogen levels in early follicular phase and high progesterone in the luteal phase of menstruation, have lower CVD risk (2006). Most of the decrease in risk involves metabolic and hemostatic factors. For instance, the

researchers found that the higher the estrogen level in the follicular phase, the lower the waist circumference, triglycerides, lipoprotein (a) (Lp(a)), and plasminogen-activator inhibitor-1 (PAI-1); and that the higher the progesterone in the luteal phase, the lower the waist circumference, blood pressure, triglycerides, Lp(a), insulin, PAI-1, and C-reactive protein (CRP) (Matthews et al., 2006). Lp(a) is a form of LDL-c, which is responsible for most of the fatty build up in atherosclerotic arteries. A low value for Lp(a) and for each of these factors indicates low risk for CVD. However, CVD risk increases when menstrual cycles become long or lack defined hormone surges in the proper phases.

Hormone Replacement Therapy and Cardiovascular Disease Risk

The beneficial effects of estrogen are lost in postmenopausal women. It has been determined that part of the increase in disease risk in older women is attributed to the loss of ovarian function and estrogen levels (The ESHRE Capri Workshop Group, 1998). To counteract these effects, women have turned to hormone replacement therapy (HT) to decrease their risk for disease. However, in the Women's Health Initiative studies, HT users demonstrated increased risk of cardiac events (Davison & Davis, 2003; Dorner & Rieder, 2004), and that a commonly used estrogen, conjugated equine estrogen, offers no protection from myocardial infarction or coronary death in healthy postmenopausal women (Hsia. et al., 2006). It is well documented that venous thombosis risk increases with use of conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA), a combination commonly used in HT (The ESHRE Capri Workshop Group, 1998; Smith et al., 2004). However, the type of estrogen and progesterone used, the active

concentrations of the estrogen, and the length of usage heavily influences the degree of risk.

The data collected on HT users may be indicative of the effects of oral contraceptive use in younger women, who have not been extensively studied in relation to CVD and the associated risk factors. Of the predictors of CVD risk that have been studied in premenopausal women, exogenous estrogen levels seem to act similarly between the two groups. It is expected that effects of postmenopausal HRT have indications for oral contraceptive prescription and the modulation of long-term CVD risk.

Oral Contraceptives and Cardiovascular Disease Risk

Although premenopausal women generally experience a protective effect of endogenous estrogen, use of oral contraceptives (OC's) interferes with this natural regulation. Evidence for altered cardiovascular function with OC use has grown from physical performance studies. While normal ovarian function does not seem to affect VO2max, oral contraceptives use decreases VO2peak (Vanheest, Mahoney, & Rodgers, 2005). This effect was seen in women using both monophasic and triphasic OC regimens. Monophasic OC's have a constant hormone level for 21 days of the cycle; whereas, triphasic OC's vary in the ratio of estrogen to progesterone throughout the cycle. Notably, the triphasic OC users decreased VO2peak faster, over a two to four month period, than did the monophasic OC users, over a six-month period (Vanheest et al.).

Lipids

One of the most common risk factors for CVD is dyslipidemia, or altered blood triglyceride and cholesterol levels. OC use is known to produce these effects. Although the research community has not met a consensus as to the specific effects of OC use on the lipid profile, many investigators reached similar findings. Total cholesterol is commonly elevated marker of CVD risk among OC users (Crook et al., 1993; Gaspard et al., 2004; Vaziri, Evans, Larson, & Wilson, 1993); LDL-c and Lp(a) have also shown to be elevated in OC users (Gaspard et al.; Oyelola, 1993). Elevated cholesterol is a major risk factor for CAD. Knopp (1986) reported that a 1% reduction in cholesterol levels reduced CAD by 2%; therefore, even small increases in cholesterol levels of OC users poses a risk for developing CVD. However, the effects of OC use on lipid levels are confounded when it comes to the two most commonly affected and measures of plasma lipids. Most researchers have shown that triglyceride levels increase with OC usage (Crook et al.; Gaspard et al.; Porkka et al., 1995; Vaziri et al.), increasing CVD risk; concurrently, HDL-c levels increase, decreasing CVD risk (Crook et al.; Doring, Frohlich, Lowel, & Koenig, 2004; Gaspard et al.; Porkka et al.; & Vaziri et al.). When the effects of OC hormones are differentiated, the synthetic estrogens, usually ethinyl estradiol, used in OC's may increase total cholesterol, triglycerides, and HDL-c, and decrease LDL-c, while the progestin component may decrease total cholesterol, triglycerides, and HDL-c and increase LDL-c (Vanheest et al., 2005). Each hormone produces both beneficial and detrimental effects on the plasma lipids; however, regardless of hormone ratio, lower dosages elicit lower CVD risk than higher dosages. Additionally, compared to nonusers, OC users have significantly higher plasma free fatty

acid (Vanheest et al., 2005) and apolipoproteins concentrations than nonusers (Gaspard et al., 2004; Porkka et al., 1995; Vaziri et al., 1993), further indicating altered lipid metabolism in OC users.

Cardiac Events

Elevated lipid levels contribute to the development of ischemia, myocardial infarction (MI), and stroke. Ischemia, or the decrease in blood supply and oxygen delivery, results from the build up of lipids in the subintimal layer of the arteries and poor endothelial function. Kovacs (2002) and Nightengale and Farmer (2004) and report that the use of combined OC's increases incidence of ischemic stroke. With high-dose estrogen and progesterone use, the relative risk of ischemic stroke is 5-15; and among users of newer, low-dose OC's, 15% of stroke incidences are attributable to OC use (The ESHRE Workshop Group, 1998). The effect of OC's on MI risk is less clear, and is highly influenced by smoking status. Women smokers taking more than 35 micrograms of ethinyl estradiol had significantly greater risk of MI compared to those taking greater dosages (The ESHRE Workshop Group, 1998; Kovacs, 2002). The progesterone component of the OC may also be to blame for the increase in MI risk, as Knopp (1986) found higher incidence of MI in women taking higher progestin potencies. More recently, researchers evaluated the third generation progestins, gestodene and desogestrel, with 30 micrograms ethinyl estradiol. They found that OC's with these progestins reduce the risk of MI to 1/3 of the risk among OC users with different progestin components (Crosignani & La Vecchia, 1999).

Thrombosis

Abnormal coagulation evidenced by thrombosis is responsible for much of the deleterious effects of OC use on the cardiovascular system. Researchers agree that the etiology of MI in OC users is due to thrombosis, and not atherosclerosis (Crosignani & La Vecchia, 1999; The ESHRE Capri Workshop Group, 1998). Thrombosis is the formation of a clot in a blood vessel that results in obstruction of blood flow, while a thromboembolism is when the clot dislodges from the vessel wall and travels in the vascular system until it lodges in a smaller vessel. Crosignani and La Vecchia found that venous thromboembolism relative risk is 3.89 for OC users compared to non-users. Castelli (1999) clarified that third-generation progestins desogestrel and gestodene pose a greater risk for thromboembolism than do other progestins. Changes in blood flow, changes in constituents of blood, and changes in the vessel wall can influence the risk of thrombosis. Porkka et al. (1995) found increases in systolic blood pressure, which reflects a change in blood flow, in women using both monophasic and triphasic OC's containing desogestrel or levonorgestrel.

Changes in the constituents of blood involve activation of two hemostatic processes: coagulation and fibrinolysis. Coagulation occurs with the onset of vascular wall damage, when blood platelets are activated, thrombin is formed causing fibrinogen to convert to fibrin, and a blood clot is formed (Widmaier, Raff, & Strang, 2004). Women who use OC's have altered platelet, coagulation factor, and fibrinogen levels, enhancing their risk of clot development and thrombosis. Norris and Bonnar (1994) demonstrated that women taking an OC with 30 mcg ethinyl estradiol had more platelet activation than those women taking 20 mcg ethinyl estradiol. In women taking OC's

versus women not taking OC's, other researchers have found higher levels of fibrinogen (Doring et al, 2004; Kovacs, 2002; Norris & Bonnar, 1997; Scarabin et al., 1995; Vanheest et al., 2005) and more activity of coagulation factor VIIa (Kovacs; Norris & Bonnar, 1997; Scarabin et al.; Vanheest et al.), which binds to tissue factor to initiate clotting; factor VIIIa (Kovacs), which serves as a cofactor in the IXa catalyzed conversion of factor X to Xa; factor Xa (Kovacs; Norris & Bonnar, 1996; Norris & Bonnar, 1997; Vanheest et al.), which converts prothrombin to thrombin, leading to blood clot formation; and factor Va (The ESHRE Capri Workshop Group, 1998; Vanheest et al.), which serves as a cofactor in the prothrombin to thrombin reaction. Increased levels of each of these coagulation components increase the risk of thrombosis in women using OC's.

The fibrinolytic system is responsible for dissolving the clot. Mechanisms to do so involve plasminogen, which, when properly activated by tissue plasminogen activator (t-PA), converts to plasmin, which digests the fibrin of the blood clot. NO, protein C, and antithrombin also contribute to anti-clotting activity. PAI-1 acts to inhibit fibrinolysis by preventing t-PA from binding to the clot, leading to fibrosis in the arterial wall. Women taking OC's are likely to have altered levels of these and other factors. Fortunately for OC users, plasminogen increases and PAI-1 decreases with OC use (Kovacs; Norris & Bonnar, 1996; Norris & Bonnar, 1997). With more plasminogen and less inhibition of t-PA, disintegration of blood clots occurs, reversing some of the enhanced coagulation activity. However, with altered functioning of the hemostatic processes, a clot may only partially disintegrate and become a thrombus. Other researchers have also noted decreases in anti-clotting mechanisms with OC use. Van

Vliet et al. (2004) found that women taking OC's, especially those containing cyproterone acetate and third-generation pills containing gestodene and desogestrel, were more resistant to activated protein C than both women taking OC's with levonorgestrel and non-users. Resistance to protein C inhibits inactivation of factors VIIIa and Va, causing superfluous production of factor Xa and therefore, thrombin. Finally, antithrombin, which inactivates thrombin, has been shown to decrease in OC users (Kovacs; Norris & Bonnar, 1997; Scarabin et al., 1995). Between shifts in the components of both the coagulation and fibrinolytic systems, women taking OC's have a higher risk of developing thromboembolism.

The platelet response can also be turned off in order to control hemostatic factors. Prostacyclins and NO are released from endothelial cells adjacent to damaged cells. These factors inhibit adhesion, activation, and aggregation of platelets, and therefore, reduce risk of thrombosis. NO is also a potent vasodilator and is released by endothelial cells. Estrogen has been shown to be protective of the endothelial tissue by increasing the production of NO from endothelial cells and monocytes (Pfeilschifter, Koditz, Pfohl, & Schatz, 2002; Widmaier et al.). Rickenlund, Eriksson, Schenck-Gustafsson and Hirschberg (2005) also found estrogen to be beneficial to endothelial function in amenorrheic athletes with low hormone levels. Taking a monophasic OC with 30 mcg ethinyl estradiol and 150 mcg of levonorgestrel produced the greatest increase in flow-mediated dilation in the amenorrheic athletes when compared to menstruating athletes and controls. The main effect of exogenous estrogen on the endothelium seems to be on nitric oxide synthase (NOS) protein; that is, 17β-estradiol up-regulates the synthesis of NO and produces a concentration dependent relaxation of previously constricted arteries

(The ESHRE Capri Workshop Group, 1998). However, when NO production is uncoupled from NOS activity, estrogen contracts the arteries, indicating a mechanism of estrogen-induced CVD risk (White et al., 2004).

Overview of Vascular Inflammation

In response to irritation of the vascular walls caused by cigarette smoking, hypertension, atherogenic lipoproteins, hyperglycemia (Pearson et al., 2003), local inflammation occurs that functions to eliminate foreign substances and initiate tissue repair (Widmaier et al., 2004). Signs of inflammation appear shortly after the adoption of a diet high in saturated fat and cholesterol (Blake & Ridker, 2002) or in rapid response to other arterial damage. First, damaged tissue cells become permeable to proteins and leukocytes by contracting, and then allow leukocytes and proteins to enter the interstitial fluid (Widmaier et al.). The damaged endothelium then expresses adhesion molecules that attract circulating leukocytes, initially neutrophils, to the damaged tissue via chemotaxis (Blake & Ridker; Widmaier et al.). The adhesion molecules include the selectins, intercellular adhesion molecule -1 (ICAM-1), which is expressed in monocytes and endothelial cells, and vascular adhesion molecule -1 (VCAM-1), which binds only to endothelial cells. As neutrophils tether to the arterial wall, more adhesion molecules appear, allowing collection of neutrophils at the site of damage. Monocyte chemoattractant protein -1 (MCP-1) then attracts leukocytes to the subendothelial space (Blake & Ridker), where unspecific blood monocytes are converted to macrophage foam cells (Widmaier et al.). Macrophages, endothelial cells, and vascular smooth muscle cells then produce the alarm cytokines, IL-1β and TNF-α (MacKinnon, 1999; Pfeilschifter et

al., 2002). These cytokines proceed to activate lymphocytes and stimulate the inflammatory response (MacKinnon).

The alarm cytokines further stimulate activation of macrophages and initiate IL-6 production by the smooth muscle cells. IL-1β also induces more production of TNF-α (MacKinnon, 1999). IL-6 is a messenger, or endocrine cytokine that synergizes with IL-1 and TNF-α. to perpetuate the acute-phase response. Specifically, IL-6 further activates fibrinogen (Rachon, Mysliwska, Suchecka-Rachon, Wieckiewicz, & Mysliwski, 2002), intracellular adhesion molecules (ICAM's) (MacKinnon) and also stimulates release of C-reactive protein (CRP), an acute phase glycoprotein, from the liver (MacKinnon; Rachon et al.). IL-1β, TNF-α, and IL-6 also increase the permeability of the vascular endothelial cell barrier (Pfeilschifter et al., 2002), allowing a greater collection of lipid molecules and macrophages in the subintimal tissue. IL-6 also provides a negative feedback loop to suppress further IL-1β and TNF-α production.

CRP is an acute phase reactant to systemic inflammation, and is mostly produced in the liver in response to IL-6 stimulation. One effect of CRP in the inflammatory process is to act as a nonspecific opsonin; in other words, CRP increases susceptibility of cells and molecules, particularly LDL-c, to phagocytosis by macrophages (Widmaier et al.). CRP plays another role in perpetuating the inflammatory response by increasing expression of ICAM and VCAM by endothelial cells and mediating MCP-1 action (Blake & Ridker, 2002). These activities attract leukocytes to the site of vascular injury and into the subintimal space, respectively. CRP also activates the complement cascade, which recruits more inflammatory cells to the site of vascular damage and directly causes

phagocyte adherence to arterial walls, ultimately causing deposition of foam cells and fats in the subintimal arterial wall (MacKinnon, 1999).

Inflammation and Cardiovascular Disease

Many researchers have associated CRP levels with various traditional CVD risk factors. Raitakari et al. (2005) found associations between hyperlipidemia (TG, TC, LDL-c) and CRP. Other CVD risk factors associated with plasma CRP levels are obesity (Kathiresan et al., 2006; Raitakari et al.;Visser et al., 1999), hyperinsulinemia (Pradham et al., 2003; Tuzcu, Bahceci, Gokalp, Tuzun, & Gunes, 2005), diabetes (Karaduman et al., 2006), physical inactivity (Raitakari et al.), and smoking (Karaduman et al.; Raitakari et al.). In addition to being associated with most CVD risk factors (Khera et al., 2006), an elevated plasma CRP level has been established as a risk factor for CVD itself, independent of age, TC, HDL-c, smoking, BMI, hypertension, diabetes, and family history of CAD (Blake & Ridker, 2002; Pfeilscchifter et al., 2002; Raitakari et al.). Ridker et al. (2005) added that, in women, CRP adds information on CVD risk beyond that conveyed by lipid measures, and van Rooijen et al. (2006) found that CRP is the strongest predictor of MI in women. Individuals in the highest tertile of resting CRP concentrations have about a 50% greater risk of developing CVD than those in the lowest tertile (Raitakari et al.). This finding holds true even in those who have a CRP level well within the normal- or low-risk range. Currently, plasma CRP <1.0 mg/dL defines low CVD risk, 1.0-3.0 mg/dL for moderate risk, and >3.0 mg/dL for high risk (Raitakari et al.).

IL-6, the messenger cytokine, also contributes to the identification of those with CAD at high risk of death (Rachon et al., 2002). IL-6 is associated with obesity and the degree of adiposity (Pfeilscchifter et al., 2002). In fact, adipose releases IL-6, and IL-6 production is reduced with loss of fat mass (Raitakari et al., 2005). The messenger cytokine also has a proinflammatory effect on the endothelium (Raitakari et al.) and contributes to the formation of blood clots by invoking an increase in fibrinogen activity.

Indeed, CRP and other inflammatory markers are associated with not only risk factors for CVD, but also with incidence of MI, stroke, and CAD. Baseline ICAM-1 was elevated in healthy men who later suffered a MI; furthermore, ICAM-1 was identified as an independent risk factor for risk of future cardiac events (Blake & Ridker, 2002). Both ICAM-1 and VCAM-1 were elevated in those with CAD who later died; also, those in the highest quartile of plasma VCAM-1 concentrations had twice the risk of death compared to lowest quartile (Blake & Ridker). Increases in concentrations of IL-6 and TNF-α are also associated with recurrent cardiac events (Blake & Ridker).

In the cardiovascular system, blood vessel wall injury elicits the coagulation, fibrinolysis, and inflammation. Processes that irritate the vascular walls like cigarette smoking, hypertension, atherogenic lipoproteins, hyperglycemia (Bermudez et al., 2002; Pearson et al., 2003), and OC use (Doring et al., 2004; Dreon et al., 2003; Raitakari et al., 2005; Salkeld, MacAulay, Ball & Cannon, 2001) promote atherosclerosis and inflammation. Persistent low-grade inflammation exacerbates the response to vessel wall damage. Specifically, CRP induces a dose-dependent decrease in endothelial NOS (Raitakari et al.) and is associated with atherosclerosis, or the hardening of arteries that occurs due to the deposition of lipids and leukocytes in the inner layer of the arterial wall

(Widmaier et al., 2004). Inflammatory mechanisms mediate all phases of atherosclerosis, from recruitment of leukocytes to injury site to rupture of the fibrous plaque.

It is thought that the fatty streaks characteristic of atherosclerosis are initiated by the conversion of monocytes to macrophages and the deposition of lipoprotein into the arterial intima (van der Wal, Das, Bentz van de Ber, van der Loos, & Becker, 1989). Ongoing inflammatory processes then contribute to the growth of the atherosclerotic plaque. As atherosclerosis worsens, foam cells accumulate in the subintima of blood vessels forming a lipid pool and tissue factor, which binds with factor VIIa in the coagulation response to cellular injury and initiates an extrinsic coagulation response that results in a fibrous plaque (Widmaier et al., 2004). Up-regulation of CRP and complement in the atherosclerotic plaque contribute to the increased expression of tissue factor (Rachon et al., 2002) and enhanced opsonization of LDL-c and deposition of foam cells to injury site (Blake & Ridker, 2002). Smooth muscle cells then produce collagen to stabilize the plaque formed in the blood vessel wall. While platelet-derived growth factor increases collagen production, macrophages secrete metalloproteinases that degrade collagen and counterbalance its growth (Blake & Ridker). However, in an inflamed environment, cytokines induce nuclear-factor-κβ (NF- κβ) activity, increasing the expression of collagen matrix metalloproteinases (Pfeilschifter et al., 2002), which further destabilizes the plaque. With macrophage invasion of blood vessel walls, oxidization of LDL-c, and metalloproteinase domination over platelet activation, the fibrous plaque cap can weaken and rupture, resulting in thombosis.

CRP may be even more predictive of plaque rupture than extent of atherosclerosis (Blake & Ridker, 2002). Furthermore, the hazard ratio for developing CVD in the top

versus bottom quintiles of CRP was greater than the hazard ratio in the top versus bottom quintiles of HDL-c and apolipoproteins B100 (Ridker et al.,2005), suggesting that CRP may be a more important indicator of CVD risk than these two risk factors. Other researchers have confirmed CRP as an independent tool for the risk of developing a cardiac event (Blake & Ridker; Rachon et al., 2002).

Estrogen and Inflammation

Premenopausal women have a decreased risk of CVD compared to men and postmenopausal women. Many researchers have found that estrogen may be the connecting factor between young women and cardioprotection. Pfeilschifter et al. (2002) found that estrogen blocks NF- κβ from binding to regulatory regions on the IL-6 gene; and Rachon et al. (2002) noted that estrogen inhibits IL-1β and TNF-α-stimulated IL-6 secretion. Furthermore, the presence of luteal activity as well as high endogenous estrogen levels during the early follicular phase of menstruation is associated with lower fibrinogen and CRP levels (Matthews et al., 2006) Estrogen also inhibits lymphocyte maturation (Pfeilschifter et al., 2002).

The protective effects of endogenous estrogen in premenopausal women become confounded with an altered menstrual cycle and the onset of menopause. Altered cyclicity is associated with increased fibrinogen, t-PA-agonist, factor VII, and CRP in premenopausal women (Matthews et al., 2006). Pfeilschifter et al. (2002) added that with the onset of menopause and decrease in estrogen brings an increase in cytokine production via expansion of the bone marrow T-lymphocyte pool; additionally, macrophage-colony-stimulating-factor activity increases, resulting in greater conversion

of monocytes to macrophages. Inder and Wittert (2005) contributed that hypoestrogenemia correlates with decreases endothelial-dependent arterial dilation, and Rickenlund, et al. (2005) noted that amenorrheic athletes experienced endothelial dysfunction and had poor lipid profiles. Each of these effects poses a risk of atherosclerosis and thombosis. Menopausal women not taking HT also show irregular cytokine activity as well. With decreasing 17-β-estradiol, Rachon et al. (2005) found increasing plasma IL-6 concentrations in perimenopausal and early menopausal women. This research group also noticed greater bioactivity of IL-6 and more spontaneous production of IL-6 in this group of women compared to young controls. Correspondingly, Pfeilschifter et al. found increased levels of IL-1, TNF-α and IL-6 in menopausal women and increased responsiveness of cells to theses cytokines via increased expression of receptors and co-activators. Amenorrhea and menopause are not the only evidence of altered estrogen status and CVD risk.

It has been thought that sufficient and normal blood estrogen levels are responsible for lessening disease risk; therefore, many menopausal women use HT to mimic premenopausal hormonal status and maintain a low CVD risk level. However, results of many studies indicate that endogenous and exogenous estrogens have different effects on CVD risk in women. CRP significantly increased in women according to degree of HT use; levels were lowest in never-users, moderate in previous-users, and highest in current users (Bermudez et al., 2002). Interestingly, Pfeilschifter et al. (2002) discovered that IL-1β and TNF-α secretion from monocytes and macrophages decreases with exogenous estrogen treatment. Accordingly, IL-6 levels respond in an inverse relationship to level of HT use, and exogenous estrogen has been shown to inhibit IL-6

release from macrophages and endothelial cells (Pfeilschifter et al.). These finding suggests another mechanism of CRP activation besides the known inflammatory response. It should be noted that transdermal estrogen therapy with or without added progestins, in contrast to oral hormone replacement, does not increase CRP levels (Lakoski & Herrington, 2005). Oral administration of hormone replacement therapy requires estrogen to infiltrate the liver, the site of CRP production, via portal circulation before interacting with other tissues. This effect may alter the activity of exogenous oral estrogens in the way of inducing production of CRP independent of IL-6 (Bermudez et al.; Davison & Davis, 2003). Importantly, there was no difference in IL-6 gene expression between women taking oral versus transdermal HT (Rachon, et al., 2005). Regardless of the mechanism of CRP formation, the most reliable predictor of CVD in postmenopausal women is plasma CRP concentrations.

Oral Contraceptives and Inflammation

The use OC's is another way to alter the endogenous estrogen status. Currently used hormones in OC's are ethinyl estradiol and one of a few progestins. The most recent forms of progesterone are desogestrel and gestodene, termed the third-generation progestins. Second-generation progesterones, levonorgestrel and norethindrone are also still used. Regardless of hormonal composition and dosage, OC use is associated with immunologic and inflammatory responses. Timmons, Hamadeh, Devries, and Tarnopolsky (2005) found higher resting levels of neutrophils and cortisol in OC users versus non-OC users and men. Researchers have also found greater plasma CRP concentrations in OC users than non-users (Raitakari et al., 2005). Dreon, Slavin, and

Phinney (2003) reported CRP levels in OC users as 2x higher than non-users. OC use and elevated BMI together account for most of the elevated CRP in young women, and 35% of women taking OC's had CRP levels > 3.0 mg/dL, the cut-off for high CVD risk (Raitakari et al.) providing further evidence that OC increases risk for CVD, especially in women who are overweight.

Much of the effect of OC's on CRP has been attributed to progestin dosage. Researchers have found that the progestin dose increases plasma IL-6 levels and that CRP depends on bioavailable IL-6 and the presence of exogenous progestins (Salkeld, MacAulay, Ball, & Cannon, 2001). The third generation progestins, desogestrel and gestodene, versus other progestins and non-OC users, are correlated with elevated CRP, fibrinogen, plasma viscosity, and von Willebrand factor (Doring et al., 2004; Kluft, Leuven, Helmerhorst, Krans, 2002). Second-generation OC's containing levonorgestrel are known to elevate CRP and TNF-α and contribute to a poor lipid profile (Rickenlund et al., 2005). However, Lakoski and Herrington (2005) found no correlation between IL-6 and CRP levels in OC users, and a significant number of studies report no effect on IL-6 levels with OC use, with either second- (von Rooijen et al., 2006) or third-generation (Kluft et al.; von Rooijen et al.) progestins. Therefore, the mechanism of OC usage on the inflammatory response is unclear. It appears as though OC's may affect hepatic CRP production while bypassing the cytokine messenger system.

High-force Eccentric Exercise and Inflammation

High-force eccentric exercise damages muscle tissue, resulting in an inflammatory response. Eccentric exercise force production can create great tension per

individual cross bridge, causing mechanical disruption of the muscle structure (MacIntyre et al., 2000). Serum creatine kinase (CK) enzyme activity is an indicator of muscle damage (Miles & Clarkson, 1994). Researchers have found that amenorrheic female athletes have an enhanced CK response to muscle damage compared to ovulatory women (Thompson, Scordilis, & De Souza, 2006). This study will investigate whether exogenous estrogen induces a different response to muscle damage than does endogenous estrogen. Delayed onset muscle soreness (DOMS) also indirectly indicates muscle damage, as does loss of strength and muscle swelling (Miles, 2005; Stupka, Tarnoplosky, Yardley, & Phillips, 2001).

The inflammatory response to muscle damage elicits a response similar to that of other stressors, including vascular damage (Malm et al., 2000; Miles, 2005). Damaged muscular tissue first elicits local macrophage activation. The macrophages then induce the release of the alarm cytokines IL-1β and TNF-α, increasing their plasma concentrations. These alarm cytokines then elicit the release of plasma IL-6, which increases in large amounts. IL-6 then acts on the liver to induce the acute phase response and the release of CRP; additionally, IL-6 serves to control the inflammatory response via negative feedback. Exercise also increases the release of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (Miles, 2005). Therefore, a muscle damaging protocol provides a model to study an inflammatory response.

Researchers have demonstrated that eccentric exercise induced muscle damage does not respond the same when the exercise is repeated (Stupka, et al., 2001). This phenomenon is known as the repeated bout effect, and it can last for several months. Therefore, in this study, all participants may not have weight trained in the last year.

Summary

Premenopausal women with regular menstrual cycles and adequate estrogen levels minimal atherosclerosis, adequate vasodilatory function, and few CVD risk factors, while women with irregular menstrual cycles and low estrogen have progressed atherosclerosis, poor vasodilation, and elevated CVD risk. Because estrogen is protective to premenopausal women, postmenopausal women have taken HRT. However, HRT users experience increased cardiac events. The elevated risk of a cardiac event in HRT users versus postmenopausal non-HRT users suggests that OC users may have elevated CVD risk compared to non-OC users. This suggestion is evidenced by altered lipid profiles and coagulation mechanisms among OC users. These detrimental changes are expressed in poor physical performances and a greater likelihood of experiencing ischemia, MI, or stroke.

Damage to the arterial wall via lipid and coagulation changes in OC users instigates an inflammatory response. Macrophages, endothelial cells, and vascular smooth muscle cells produce alarm cytokines, which further permeate the vasculature to the influx of lipids and macrophages. The acute phase reactant CRP further enhances this response. Inflammatory markers, especially CRP, have proinflammatory effects on endothelium, increasing atherosclerosis and development of a thromboembolism. Estrogen in normally cycling women decreases IL-1β- and TNF-α-stimulated IL-6 secretion and CRP concentrations. An altered menstrual cycle and menopause initiate the inflammatory atherosclerotic process. Therefore, endogenous estrogen may protect women from CVD.

It is well supported that CRP is elevated in OC users; however, other inflammatory markers fluctuate in an unpredictable manner in various studies. Therefore, research on the effects of OC's on inflammation is warranted. In this study, high-force eccentric exercise was used to elicit an inflammatory response in young adult women in an attempt to reveal differences in the inflammatory responses between OC users and non-OC users.

CHAPTER 3

METHODS

Subjects

All participants read and signed an Institutional Review Board approved informed consent form. The informed consent form briefly described the reasoning for the research and the implications of inflammation on cardiovascular disease. Participants included women ages 18 to 40 years old from the university population and surrounding community. Posted flyers and newspaper advertisements were used to recruit participants. This age group was chosen to eliminate the effect of aging on confounding variables in the assessment of estrogen's influence on CVD risk factors. Additionally, younger women normally have adequate circulating levels of estrogen that fluctuate regularly with the menstrual cycle and can be measured using laboratory analysis. Women less than 18 years old may be of a gynecological age (age since first menstruation) too young to have adequate and predicable hormonal cycles. Older women may have more advanced atherosclerosis and other cardiovascular risk factors, which makes this group dissimilar from the younger premenopausal women. Additionally, inflammatory markers are known to change with age, so choosing a younger cohort eliminated the need to correct for age-related inflammatory changes.

Unaccustomed muscle usage is known to cause muscle damage and an acute-phase immune response. Particularly after strenuous eccentric exercise, circulating inflammatory markers increase in concentration. However, with high-force eccentric

exercise training comes adaptations to neutrophil activation (Peake et al., 2005) and other inflammatory responses, such that exposure to this stress a second time within several months does not elicit the same acute-phase response. Therefore, women must not have weight trained and not have participated in high force activities on either arm for at least one year prior to the onset of the study.

Other exclusion criteria included known or latent anemia, musculoskeletal limitations, known inflammatory conditions, diabetes, heart disease, kidney problems (excluding kidney stones), smoking, alcohol use greater than one drink per day, binge alcohol drinking (greater than 4 drinks on a single occasion), chronic anti-inflammatory medication use (including over-the-counter non-steroidal anti-inflammatory drugs), lipid lowering medications, regular performance of physical activity in which muscle soreness or bruising occurs, and pregnancy.

Research Protocol

The investigation measured the changes in inflammatory markers resulting from muscle damage induced in the flexor muscles of one arm by high-force, eccentric resistance exercise. Inflammatory control response following muscle damage was assessed using multiple plasma inflammatory markers. The magnitude of the highest post-exercise response and the duration of inflammatory marker elevation were assessed. The dependent variables (inflammatory markers) were serum CK, CRP, and cortisol concentrations and plasma IL-6 and TNF-α concentrations. Other variables involved in the response to muscle damage were measured and include serum creatine kinase (CK)

enzyme activity, maximal force production, muscle soreness, and mid-brachial arm circumference.

Subjects completed both the experimental and control condition in randomized order, with three to eight weeks between each protocol. Measurement of the dependent variables occurred immediately pre-exercise, and at 4, 8, 12, 24, 48, 96, 120 hours post-exercise, and proceeded in the following order: perceived muscle soreness, mid-brachial arm circumference, blood collection, and maximal force production. The control group gave blood samples on an identical time course in order to correct for diurnal and day-to day variation.

Other measures were taken to minimize variability in the basal status of the participants. Morning blood collections occurred after an overnight fast and minimal physical activity before arriving at the research center. Blood collections for pre-exercise and at 24, 48, 96, and 120 hours post-exercise was scheduled at the same time each morning for each participant. Heavy physical exercise exceeding 60 minutes in duration or nearing maximal intensity were not allowed for the duration of the protocols. To account for the hormonal cyclicity of the young women, all participants began each of the two protocols within five days after the onset of menses. At this point the women's menstrual cycles were in the early follicular phase when blood estrogen and progesterone levels are both low and steady. Finally, participants suffering an infection or injury at any point of the study were excluded, as the inflammatory response to the infection would overshadow the exercise-induced inflammatory response.

Measurement Techniques

Anthropometrics

Height and weight were measured at the initial visit for each protocol. Weight was measured with a calibrated scale and height was measured with a calibrated stadiometer. These data was then used to calculate BMI, which is positively associated with CRP concentrations (Kathiresan et al., 2006; Raitakari et al., 2005; Visser et al., 1999).

Eccentric Exercise and Muscle Damage

High-force eccentric exercise stress elicits an inflammatory response (Smith & Miles, 2000). The inflammatory markers associated with an exercise-induced response are similar to the presentation of low-grade inflammation indicative of CVD.

Participants sat on an exercise machine, a Kin-Com, with the chest and posterior brachium against padded rests with the forearm secured in a padded lever. When cued, the participant exerted maximal resistance against the lowering of the lever on the Kin-Com. The lowering occurred at a rate of 45° per second in one movement over three seconds. This eccentric elbow movement was repeated for a total of forty-five repetitions using maximal resistance by the non-dominant arm (not used for writing). Three sets of fifteen repetitions were performed at a rate of one repetition per fifteen seconds with five minutes rest between sets.

Maximal Isometric Force

Maximal isometric force production for elbow flexion was measured before and after performance of the exercise protocol to ensure that a loss of strength occurred. Participants sat on the Kin-Com machine with their forearm attached to the padded lever so that the elbow was fixed at a 90° angle. When cued, participants exerted maximal isometric force against the lever, which remained in place. At each of the 0, 24, 48, 96, and 120-hour time points, three measurements of maximal isometric force were taken. The average force produced at each time point were used for analysis. This measurement added to evidence that the exercise produced muscle damage.

Muscle Soreness and Swelling

Muscle soreness was determined subjectively by each participant using an analog scale with a range from 'no soreness' to 'very, very sore.' Ratings were made by each subject while holding a one-pound weight during flexion and extension. Mid-arm circumference was measured at the mid-brachium with a tape measure to determine the extent of swelling. Each of these measurements were made during visits to the lab at 0, 4, 8, 12, 24, 48, 96, and 120 hours.

Blood Collection and Serum Separation

The pre-exercise blood collection was performed after an overnight twelve-hour fast. Blood collected at 4, 8, and 12 hours occurred as long after meals as possible. The 24, 48, 96, and 120-hour blood collections was performed at the same time each morning before meal consumption or exercise. Upon arriving at the research site for each visit, participants sat for 10 minutes to allow for metabolic blood markers to return to resting

levels. Blood samples were collected from an antecubital vein into evacuated tubes using a standard venipuncture technique. To measure concentrations of CK, CRP, and cortisol, blood were collected in one 7-ml vacuum tube without additive. For blood lipid, hs-CRP, and STNF-R1 measurements, blood were collected with one 7-ml vacuum tube containing K3-EDTA. Using a refrigerated 21000R Marathon centrifuge (Fisher Scientific, Pittsburgh, PA) at 2900 revolutions per minute and 12°C for 20 minutes, serum was separated from clotted blood and plasma separated from red blood cells; this procedure was performed 10 minutes after blood collection. Serum and plasma samples were kept at -80○C until analysis. Samples were analyzed when both protocols have been completed, and for each participant were analyzed in the same run of the assay. Therefore, intra-assay variability between conditions did not affect assay results.

Laboratory Analysis

Creatine Kinase

Creatine kinase is a commonly used marker of muscle damage (Miles & Clarkson, 1994). This enzyme reflects the neutrophil response and the extent of muscle damage (MacIntyre et al., 2000). Although CK activity does not correlate with inflammatory cytokines, it may be linked to the level of inflammation in response to high-force eccentric exercise. Measurement of this marker indicated that muscle damage has occurred and provided information on the extent of the inflammatory process.

ThermoTrace CK-NAC (N-acetyl-cysteine) Reagent (ThermoTrace, Victoria, Australia) was used to determine CK activity. The reagent was incubated to 37°C for two minutes and transferred to a NUNC plate. Immediately afterwards, the absorbance

was read in a spectrometer at 340 nm in four readings, each a minute apart. Corrections were made at 334 or 365 nm. Reported intra-assay sensitivity was 0.30 ΔMa/min per U/L. Intra-assay coefficient of variation was 0.89-1.75% within the range of expected values for women (< 2.3 μkat/L).

Interleukin-6

Interleukin-6 (IL-6) was measured to indicate the magnitude and duration of the inflammatory response to eccentric exercise-induced muscle damage. A serum IL-6 concentration was measured using the Quantikine HS Immunoassay, a 5.5-hour solid-phase ELISA kit (R&D Systems, Minneapolis, MN). This assay used the quantitative sandwich enzyme immunoassay technique in which IL-6 binds to antibody in microplate wells. Plates were read using a μQuant Universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) within 30 minutes at 490 nm. Readings were corrected for imperfections by subtracting readings at 650 or 690 nm. Reported sensitivity of the ELISA kit is 0.016-0.110 pg/mL with a mean of 0.039 pg/mL. Intra-assay coefficient of variation is 6.9-7.8% within the range of expected values (0.436 to 5.53 pg/mL).

C-Reactive Protein

Pre-exercise basal serum CRP was measured to distinguish between participants at low and high risk for CVD (Blake & Ridker, 2002; Pearson et al., 2003). Post-exercise, CRP levels were used to reflect the magnitude and duration of the inflammatory response to high-force eccentric exercise. Duplicate CRP concentration measurements were taken for each participant using a high sensitivity enzyme immunoassay (EIA) kit (ICN Pharmaceuticals, Costa Mesa, CA). Reported sensitivity of this kit was reported to

be 0.1 mg/L with an intra-assay coefficient of variation of 7.5-4.1% within the range of expected values (0.068 to 8.2 mg/L). Absorbance of each well was measured using a μQuant Universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 450 nm.

Soluble Tumor Necrosis Factor Receptor-1

STNF-R1 was measured to indicate the activity of the alarm cytokine TNF-α in response to the eccentric exercise protocol. The soluble receptor for TNF-α is more stable in the blood than the cytokine itself, so the receptor was chosen for measurement of the activation of the inflammatory response. Plasma sTNF-R1 was measured with the Quantikine Immunoassay, a 4.5 hour solid phase ELISA kit (R&D System, Minneapolis, MN). This assay used the qualitative sandwich enzyme immunoassay technique. Plates were read using a μQuant Universal microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT). Within 30 minutes of completion of the incubation period, the optical density of each microplate well was read at 450 nm, with corrections for optical imperfections made using readings at 540 nm. Reported sensitivity of the ELISA kit was 0.43-1.20 pg/mL with a mean of 0.77 pg/mL. Intra-assay coefficient of variation was 4.7-5.0% within the range of expected values (69.0-355 pg/mL).

Cortisol

Cortisol is a long-term stress hormone is a product of the hypothalamic-pituitary-adrenal axis (HPA axis). The HPA axis is a part of the neuroendocrine system that controls stress response and regulation of many body functions. In response to exercise stress, adrenocorticotrophic hormone (ACTH) and cortisol concentrations elevate,

increasing the activity of the HPA axis. Therefore, cortisol may influence the magnitude and/or duration of the inflammatory response to the eccentric exercise protocol. Duclos, Gouarne, and Bonnemaison (2003) found that exercise induces an increase in sensitivity of monocytes to cortisol and other glucocorticoids. As cortisol has an anti-inflammatory action, this mechanism may diminish muscle inflammatory reaction and cytokine synthesis and thereby reduce exercise-induced muscle damage and inflammatory response. For these reasons, cortisol levels may be helpful in interpreting the findings of this study.

Cortisol was measured using ACTIVE® Cortisol EIA Kits (Cortisol EIA, Diagnostic Systems Laboratories, Inc., Webster, TX). Reagents and samples were brought to 25°C prior to analysis. A microtitration plate reader was used for sample analysis. Absorbance was read within 30 minutes at a spectrometer setting of 450 nm with dual wavelength correction at 600 or 620 nm. Reported sensitivity was 0.1 μg/dL. Intra-assay coefficient of variation was 2.4-10.3% within the range of expected values (8.4-29.2 μg/dL).

Statistical Analysis

Data were analyzed using SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL). Descriptive statistics were used for all variables. Repeated measures ANOVA was used to determine the magnitude and duration of the inflammatory response and compared across groups over time. Post-hoc analysis was performed with an independent samples t-test. The Bonferroni alpha adjustment was used to correct for alpha levels.

CHAPTER 4

RESULTS

Participants in this study were drawn from a larger scale study that investigated the inflammatory response in both men and women. Seventy-five people enrolled in the large study, and 62 participants completed the exercise condition, which was a criterion for the present study. Six of these participants were eliminated from analysis for high pre-exercise hs-CRP. Of the remaining 56 participants, 26 were female. The OC user group was comprised of 9 participants and the non-OC user group was comprised of 16 participants. One female used a different form of steroid hormone contraception and was not included in the analysis. Other participants excluded from the study included those who presented with infection within a week of the start of the study and those with unstable baseline hs-CRP levels, defined as having baseline concentrations greater than 2.0 mg/L.

Baseline Participant Characteristics

Subjects in the non-OC user and the OC user groups were similar in age, weight, height, body mass index (BMI), lipid profile, maximal force production, arm circumference, and muscle soreness. IL-6 ($p<0.058$) and sTNF-R1($p<0.464$) were similar between groups at baseline. CK ($p<0.036$), hs-CRP ($p<0.028$), and cortisol ($p<0.011$) concentrations were significantly greater in the OC group than the non-OC group. (Table 1.1)

Table 1.1 Raw baseline characteristic data in the OC and non-OC groups.

Variable	OC User (n=9)	Non-OC User (n=16)	P value
Anthropometrics			
Stature (in)	66.0 ± 2.70	65.9 ± 2.26	0.937
Mass (#)	158.1 ± 41.6	141.5 ± 17.4	0.317
BMI (kg/m^2)	25.6 ± 6.87	22.9 ± 2.83	0.269
Lipids			
Triglycerides (mg/dl)	121.1 ± 52.9	95.40 ± 29.5	0.208
Total cholesterol (mg/dl)	169.8 ± 26.5	171.5 ± 23.5	0.870
HDL-c (mg/dl)	61.0 ± 22.7	53.6 ± 16.1	0.360
LDL-c (mg/dl)	84.6 ± 26.2	98.8 ± 20.2	0.148
Muscle Damage Markers			
Maximal Force Production (N)	51.17 ± 10.6	47.19 ± 13.3	0.457
Muscle Soreness (mm/100 mm)	3.00 ± 3.54	1.07 ± 2.63	0.140
Creatine Kinase (IU/l)	141.2 ± 53.0	102.1 ± 23.1	0.034
Inflammatory Markers			
IL-6 (pg/ml)	3.18 ± 5.32	0,984 ± 0.746	0.053
C-Reactive Protein (mg/l)	5.46 ± 5.37	1.02 ± 1.33	0.021
sTNF-R1 (pg/ml)	1090.4 ± 219.8	1181.2 ± 234.1	0.376
Cortisol (μg/dl)	51.34 ± 10.27	39.17 ± 9.864	0.011

Values = mean ± standard deviation

Analysis of Variance Results

Muscle Damage Markers

Maximal Force There were missing data for three of the 16 non-OC users and one OC user, as these participants missed a measurement. Because it was inappropriate to estimate this data, results from these participants were not included in the analysis of strength loss. Therefore, this analysis is based on n=13 for non-OC users and n=8 for OC users. Participants in both groups experienced a decrease in maximal muscle force production over time after engaging in the exercise protocol ($p<0.000$). Pair-wise comparisons indicate that at the 0-h, 24-h, and 48-h time points, maximal force production for the two groups combined was significantly lower than the initial maximal force production measurements ($p<0.000$ for all). The greatest strength loss occurred immediately following the exercise, which corresponded to the 0-h time point. By 96-h, maximal force production was no longer significantly lower than initial measurements, and strength recovered to baseline measurements by the 120-h time point. There were no significant differences between groups (Figure 4.1).

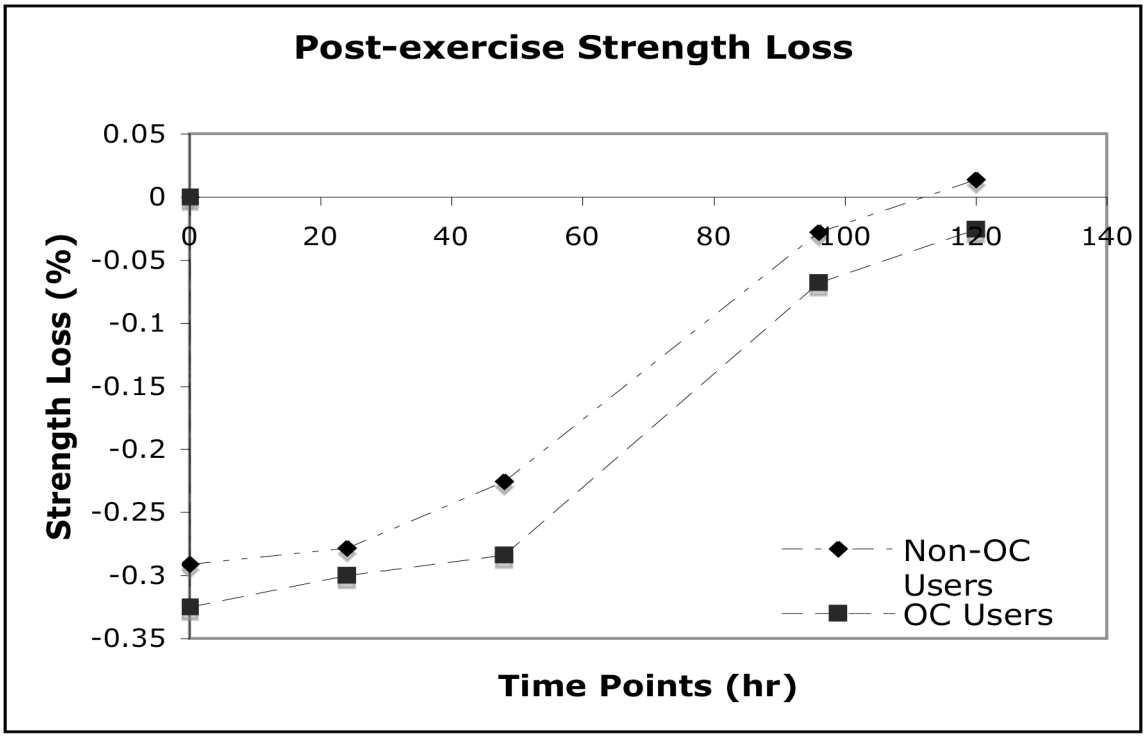

Figure 4.1. Change in strength in post-eccentric exercise phase. Values = mean ± SE. P<0.733 compared to the non-OC group. ^ = Significant difference relative to pre-exercise values (Bonferroni α adjustment = 0.0056).

Muscle Soreness There were missing data for two of the 16 non-OC users, as these participants missed a measurement. Because it was inappropriate to estimate this data, results from these participants were not included in the analysis of muscle soreness. Therefore, this analysis is based on n=14 for non-OC users and n=9 for OC users. A time main effect occurred in both groups (p<0.000) and all participants experienced soreness though the 96-h time point. Soreness gradually increased from pre-exercise values to peak soreness at the 48-h time point. Together, the groups experienced significantly greater muscle soreness at the 4-h (p<0.001), 8-h (p<0.001), 12-h (p<0.000), 24-h (p<0.000), 48-h (p<0.000), and 96-h (p<0.001) time points compared to pre-exercise measurements. Soreness was almost completely recovered by the 120-h time point.

Between groups, the OC users experienced greater soreness than did the non-OC users ($p<0.023$). In pair-wise comparisons, OC users showed a trend towards greater soreness than the non-OC users at the 24-h time point ($p<0.015$; α-adjusted significance level $p<0.0038$). (Figure 4.2).

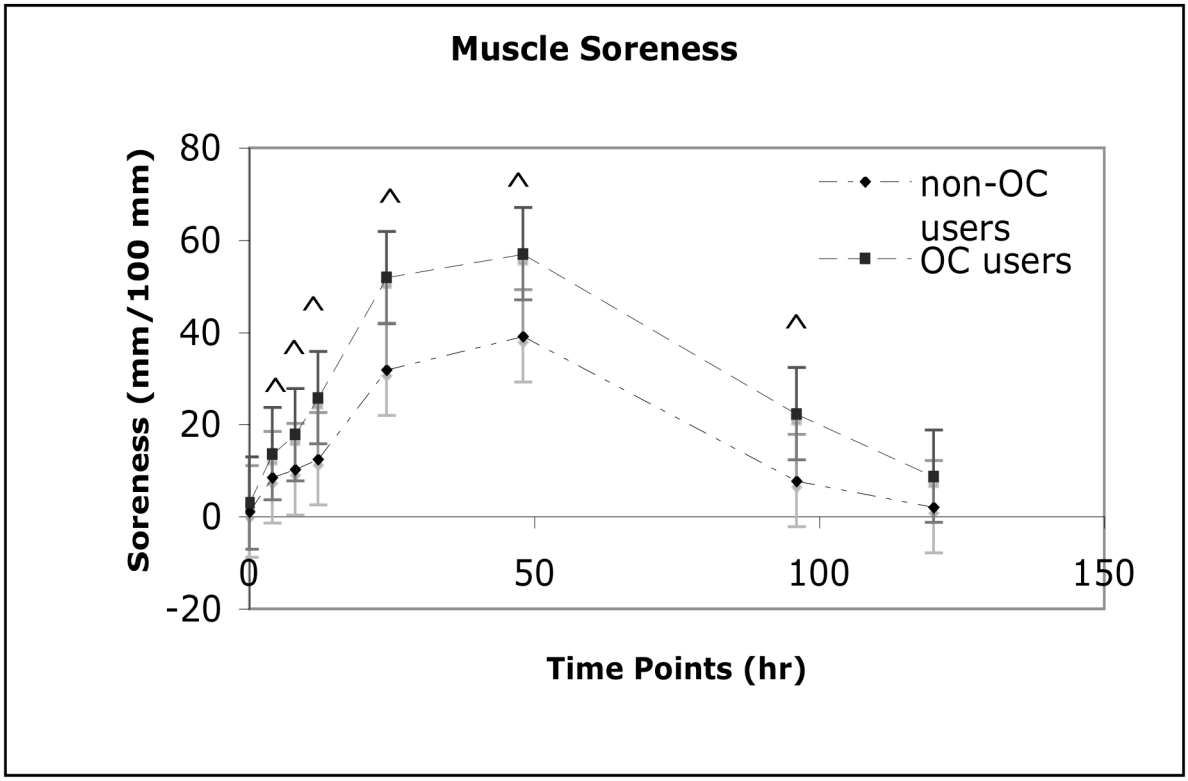

Figure 4.2. Muscle soreness (mm/100mm) progressing in post-eccentric exercise phase. Values = mean ± SE. $P<0.023$ compared to the non-OC group. ^ = Significant difference relative to pre-exercise values (Bonferroni α adjustment = 0.0038).

<u>Arm Circumference</u> There were missing data for three of the 16 non-OC users, as these participants missed a measurement. Because it was inappropriate to estimate this data, results from these participants were not included in the analysis of arm swelling. Therefore, this analysis is based on n=13 for non-OC users and n=9 for OC users. Throughout the experiment, participants in neither the non-OC group nor the OC user

group experienced a change in arm circumference (p<0.853). It was expected that the muscle damage invoked would cause muscular swelling, thereby increasing mid-brachial arm circumference. However, while the non-OC user group had a non-significant increase in arm swelling, the OC group had a non-significant decrease in arm circumference. (Figure 4.3).

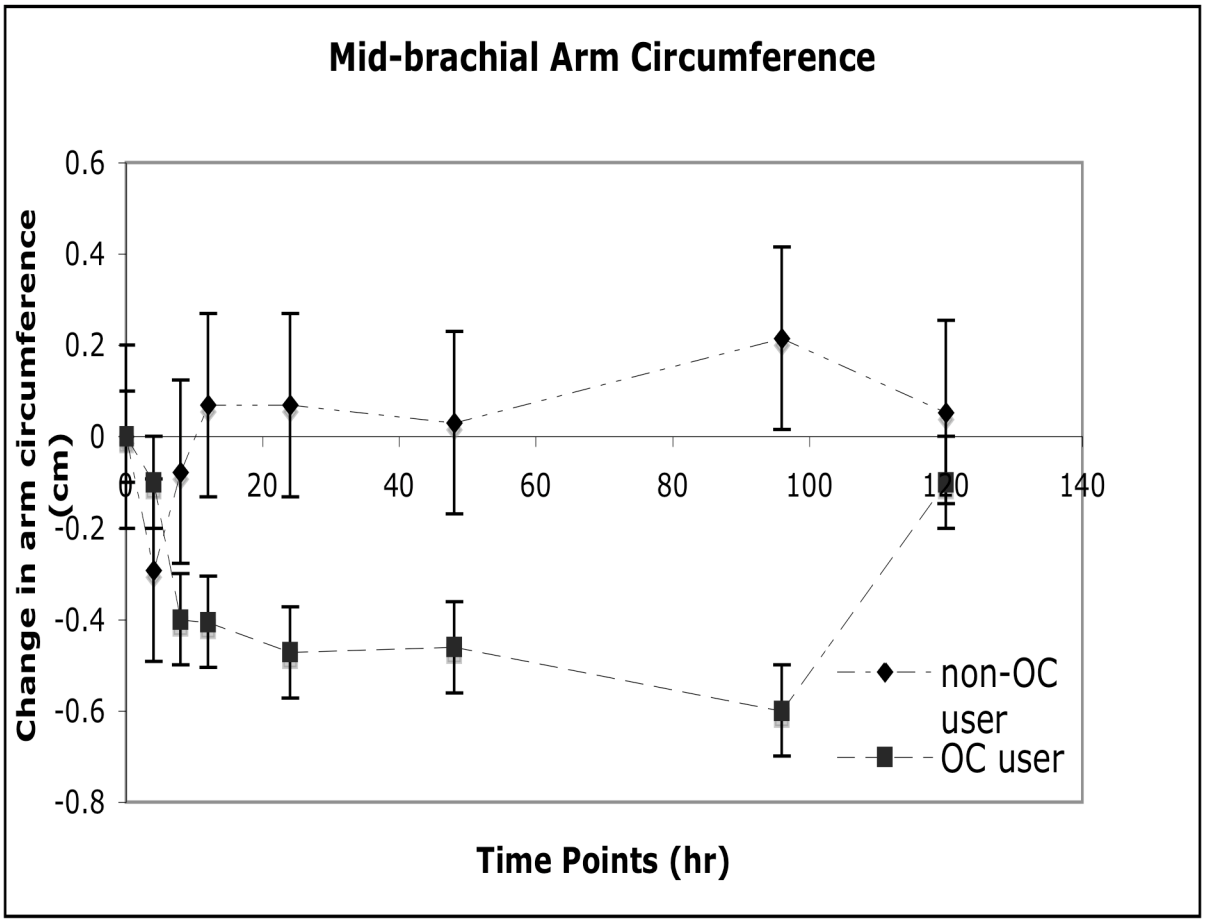

Figure 4.3. Change in mid-brachial arm circumference in the post-eccentric exercise phase. Values = mean ± SE. P<0.380 compared to the non-OC group.

<u>Creatine Kinase</u> Baseline measurements of CK were significantly greater among OC users than non-OC users (p<0.034). However, throughout the experiment, CK

responded similarly in the groups, increasing significantly after the eccentric exercise protocol ($p<0.000$). This finding indicated that muscle damage occurred. CK concentrations gradually increased each day, and at its highest concentrations, were significantly greater than pre-exercise values at the 96-h ($p<0.002$) and 120-h ($p<0.001$) time points (α-adjustment $p<0.0056$). Between groups, there was no significant difference in CK measurements in post-exercise measurements (Figure 4.4).

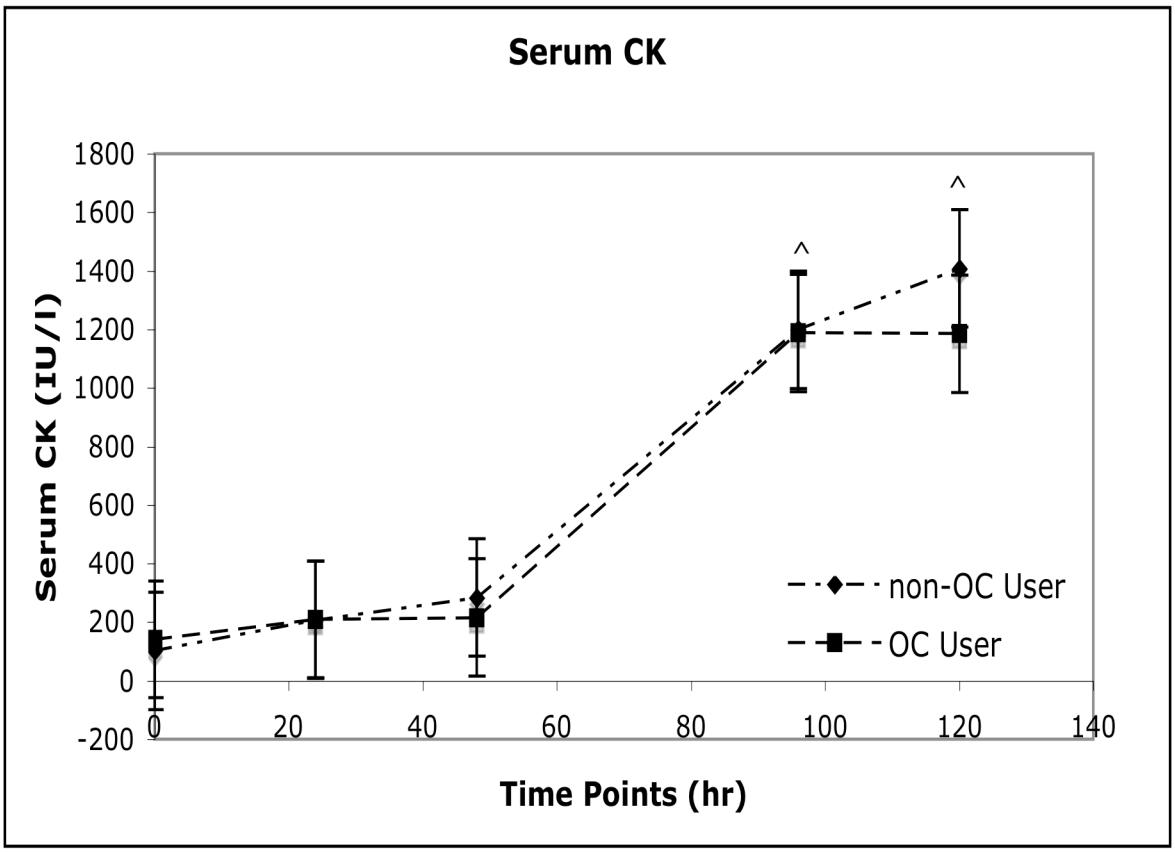

Figure 4.4. Serum creatine kinase concentration in the post-eccentric exercise phase. Values = mean ± SE. $P<0.930$ compared to the non-OC group. ^ = Significant difference relative to pre-exercise values (Bonferroni α adjustment = 0.0056).

Inflammatory Markers

Interleukin-6 IL -6 concentrations followed a significant change though time in all participants ($p<0.000$). After a non-significant decrease at the 4-h time point, IL-6 increased significantly from baseline in both groups at the 8-h time point ($p<0.001$). IL-6 concentrations returned to baseline by the 24-h measurement. The post-exercise IL-6 response was similar between both groups ($p<0.119$), despite that the OC user group displayed a trend towards greater IL-6 values at baseline ($p<0.053$). (Figure 4.5).

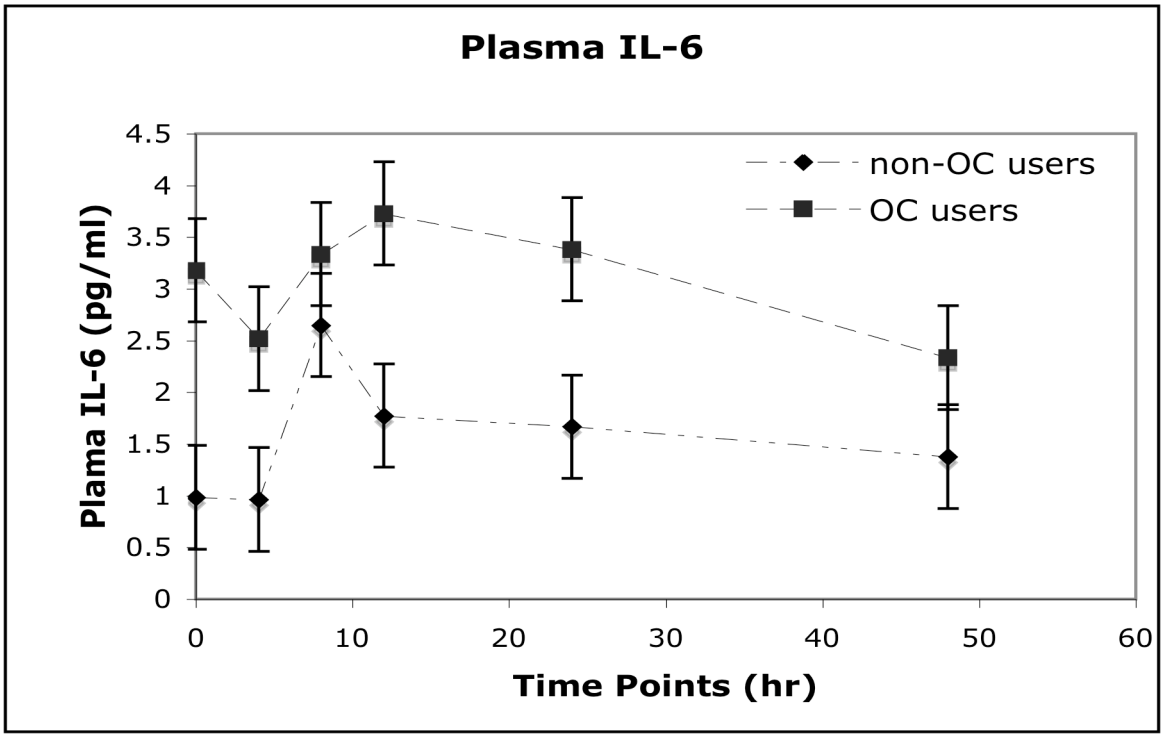

Figure 4.5. Serum interleukin-6 concentration in the post-eccentric exercise phase. Values = mean ± SE. $P<0.119$ compared to the non-OC group. ^ = Significant difference relative to pre-exercise values (Bonferroni α adjustment = 0.0045).

C-Reactive Protein CRP did not significantly differ with time in either group. In both groups, CRP concentrations peaked at the 12-h time point and followed a similar

pattern of change. However, the OC user group presented with baseline CRP values more than 5-fold greater than the non-OC user group (5.45 mg/l versus 1.02 mg/l, respectively) ($p<0.021$). In addition to greater baseline values, researchers found significantly greater post-exercise CRP concentrations in the OC user group compared to the non-OC user group ($p<0.036$). (Figure 4.6).

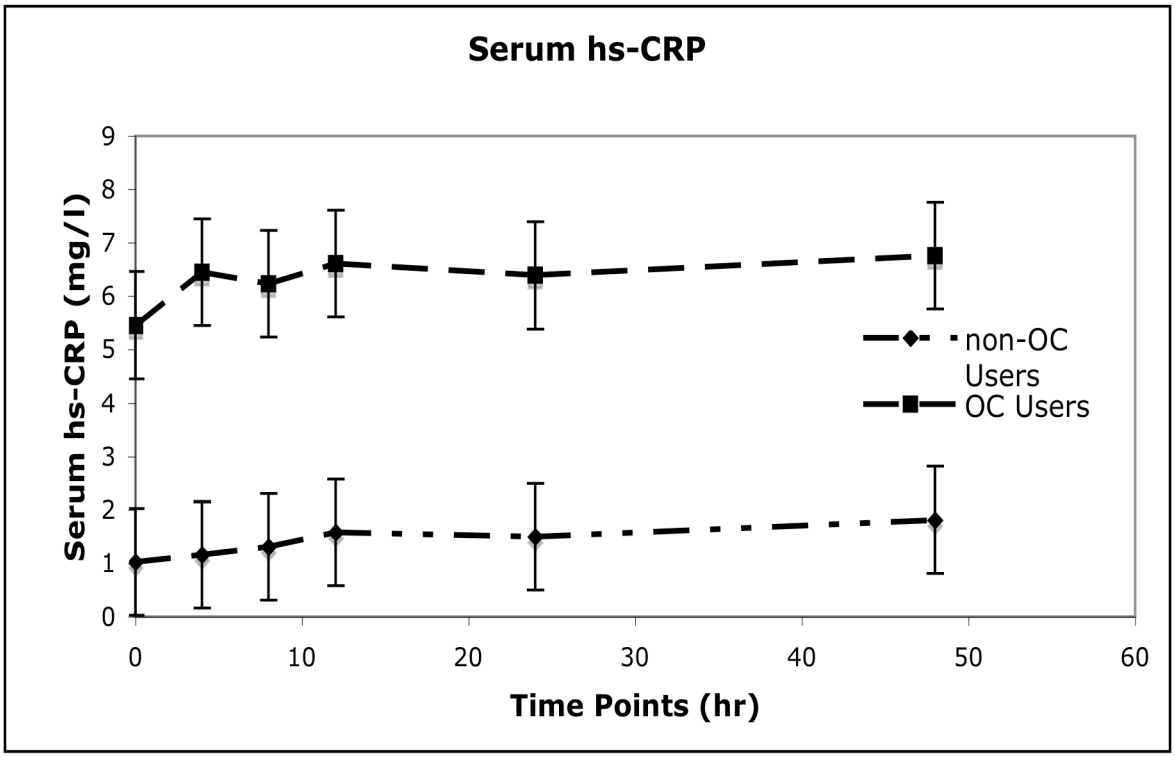

Figure 4.6. Serum hs-CRP concentration in the post-eccentric exercise phase. Values = mean ± SE. $P<0.036$ compared to the non-OC group.

Soluble Tumor Necrosis Factor-R1 There were missing data for one of the 16 non-OC users and one of the 9 OC users, as these participants missed a measurement. Because it was inappropriate to estimate this data, results from these participants were not included in the analysis of sTNF-R1 concentration. Therefore, this analysis is based on

n=15 for non-OC users and n=8 for OC users. sTNF-R1 followed a similar pattern of change over time in both groups (p<0.000). In pair-wise comparison, sTNF-R1 concentration was significantly less than pre-exercise values at the 4-h, 8-h, and 12-h (p<0.000 for all) in both groups. Both groups reached the lowest sTNF-R1 concentrations at the 12-h time point, and approached baseline measurements by the 96-h time point. There was no difference in sTNF-R1 post-exercise response between the groups. (Figure 4.7).

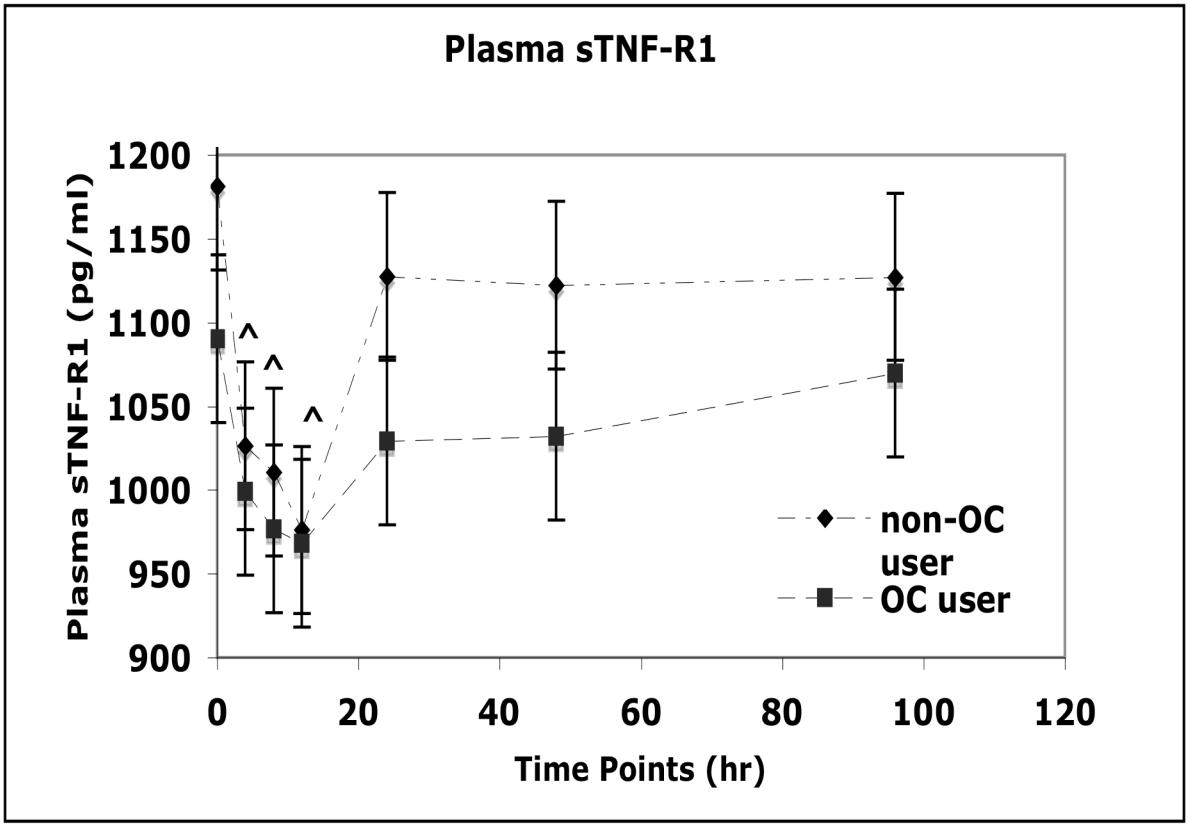

Figure 4.7. Plasma sTNF-R1 concentration in the post-eccentric exercise phase. Values = mean ± SE. P<0.541 compared to the non-OC group. ^ = Significant difference relative to pre-exercise values (Bonferroni α adjustment = 0.0038).

Cortisol There were missing data for one of the 16 non-OC users and one of the 9 OC users, as these participants missed a measurement. Because it was inappropriate to estimate this data, results from these participants were not included in the analysis of cortisol concentration. Therefore, this analysis is based on n=15 for non-OC users and n=8 for OC users. Researchers detected a significant time effect of cortisol concentrations throughout the testing period in both groups ($p<0.000$). Concentrations gradually decreased throughout the first day from the initial blood draw at time zero to the 12-h time point. In pair-wise comparison, researchers determined lower cortisol concentrations at the 4-h, 8-h, and 12-h time points ($p<0.000$ for all), compared to pre-exercise values. By the 24-h time point, cortisol returned to baseline levels and remained steady at each of the subsequent blood withdrawals, each taken at 7 a.m.

Researchers determined significant differences between groups at baseline ($p<0.011$) and in the post-exercise phase ($p<0.006$). In pair-wise comparison, cortisol concentrations were significantly greater in the OC group than the non-OC group at the 24-h time point (52.3 μg/dl versus 39.3 μg/dl, respectively; $p<0.001$) and the 96-h time point (51.4 μg/dl versus 37.2 μg/dl, respectively; $p<0.001$) (α-adjustment $p<0.0038$) (Figure 4.8).

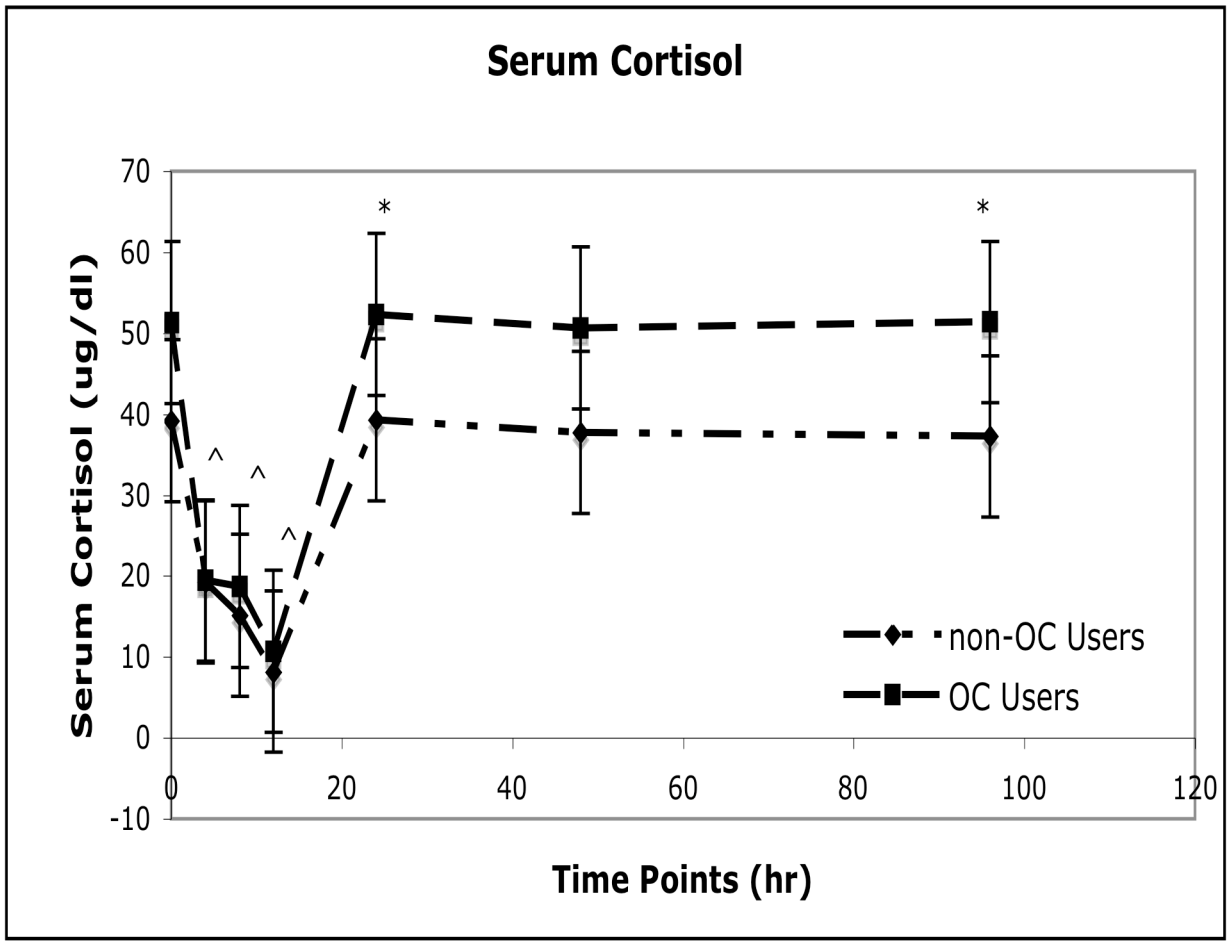

Figure 4.8. Serum cortisol concentration (μg/dL) in post-eccentric exercise phase. Values = mean ± SE. P<0.006 compared to the non-OC group. ^ = Significant difference relative to pre-exercise values; * = Significant difference relative to non-OC group (Bonferroni α adjustment = 0.0038).

CHAPTER 5

DISCUSSION

The present study compared an eccentric exercise-induced inflammatory response between women taking OC's and women not taking OC's or receiving hormonal therapy. Previous researchers concluded that women taking OC's have increased risk for cardiovascular disease (Crook et al., 1993; The ESHRE Workshop Group, 1998; Gaspard et al., 2004; Kovacs, 2002; Nightengale & Farmer, 2004; Oyelola, 1993; Porkka et al., 1995; Raitakari et al., 2005; & Vaziri et al., 1993), and persistent, low-grade inflammation is now a recognized risk factor for CVD (Blake & Ridker, 2002; Pfeilscchifter et al., 2002; Raitakari et al., van Rooijen et al., 2006). Therefore, this study aimed to elucidate differences in an experimentally induced inflammatory process between OC users and non-OC users.

Anthropometrics and Blood Lipids

All baseline anthropometric and blood lipid characteristics were similar between the OC users and non-OC users. These findings are contradictory to conclusions from previous studies in which researchers concluded that blood lipids are altered in response to oral contraceptive use. Casazza et al. (2004) found that OC use increased TG mobilization during cycle ergometry at 45 and 65% peak VO_2. Triglycerides were also elevated after 6 months of drospirenone-containing OC use (Taneepanichskul & Phupong, 2007). However, in the same cohort, TC remained neutral while HDL-c increased 25.7% and LDL-c decreased 9.9% (Taneepanichskul & Phupong).

Conclusions on the effects of OC's on lipids are further complicated by findings by Yahaya & Afonja (2005), in which the use of an OC containing norethindrone with ethinyl estradiol increased TC and TG significantly more so than did an OC containing norgesterol and ethinyl estradiol. However, the norgesterol-containing OC users increased HDL-c levels more so than non-OC users, while the norethindrone-containing group showed no change in HDL-c levels. It appears that the type of progesterone in the OC determines the effect of OC use on lipid concentrations.

Because the hormonal profile of each type of OC was not investigated in the present study, the possibly inconsistent hormonal types may have exerted effects that were cancelled out, leaving no significant difference between OC users and non-OC users. The lack of differences in lipid profiles between groups suggests that lipid differences were not likely to affect the inflammatory response to eccentric exercise between OC users and non-OC users.

Muscle Damage Markers

Maximal Force Production

Maximal force production was tested before the eccentric exercise protocol (initial), immediately after the protocol (0-h), and at each proceeding 24-hour interval (24-h, 48-h, 96-h, and 120-h). Previous research has shown that eccentric exercise-induced strength loss correlates with muscular damage. After performing 70 maximal eccentric contractions of the elbow flexors, 26 young women suffered strength loss for 11 days post-exercise; the strength loss correlated with other known markers of muscle damage (Cleak & Eston, 1992). Other researchers tested whether strength loss

associated with eccentric contractions was attributed to central neural drive or peripheral function (Hubal, Rubinstein, & Clarkson, 2007). These authors concluded that strength loss was attributable to peripheral functions, indicating that strength loss results from local muscular damage and not systemic neural function. The significant strength loss that occurred in both groups at 0-h, 24-h, and 48-h in the present study indicates that muscle damaged occurred as a results of the eccentric exercise protocol. Participants in both groups experienced the greatest strength loss at 0-h and gradually increased strength to normal by the 96-h time point. In the Cleak and Eston study, strength loss peaked at 24 and 96 hour post-exercise; however, the participants from that study incurred greater muscular damage by performing 1.5 times the eccentric repetitions. It may be suggested that the extent of muscle damage may correlate with the length of time until peak strength loss.

In another study using only female participants, Savage and Clarkson (2002) compared the responses between OC users and non-OC users to 50 eccentric muscle contractions of the elbow flexors. These researchers found that strength recovery took twice as long in the OC users versus the non-OC users. Conversely, Lebrun, Petit, McKenzie, Taunton, and Prior (2003) concluded that in highly trained athletes with prior regular ovulatory cycles, oral contraceptive use had no effect on isokinetic strength. Regardless of incongruent results in prior studies on the effects of OC use on strength change, in the present study, both groups experienced significant strength loss, indicating that muscle damage occurred. No differences between groups were measured.

Muscle Soreness

Muscle soreness was analyzed in 23 participants at each time point throughout the study. Participants in both groups rated the magnitude of soreness as increasing steadily and significantly with a time main effect at each time point from 4-h to 96-h, peaking at the 24-h and 48-h time points. Soreness incurred during eccentric exercise has previously been shown to correspond with muscle damage (Brown, Child, Day, & Donnelly, 1997; Stauber, Clarkson, Fritz, & Evans, 1990). In the present study, significant muscle soreness in both groups indicated that muscle damage occurred post-exercise.

Although the two groups followed a similar pattern of change in soreness over time, the OC group experienced a significantly greater magnitude of soreness than the non-OC group. This finding is in contrast to findings from other studies. After 50 minutes of continuous step aerobic exercise, OC users had less soreness than non-OC users when evaluated on days 2, 3, and 5 post-exercise (Thompson, Hyatt, De Souza, Clarkson, 1997). Another study found no differences in soreness between OC users and non-OC users after participants performed 50 eccentric contractions of the elbow flexors (Savage & Clarkson, 2002). It is inconclusive whether muscular soreness induced by eccentric contractions is affected by the use of OC's. In the present study, soreness was identified as being greater among OC users versus non-OC users. If OC use is the cause of increased soreness, the hormonal therapy may cause soreness via enhanced muscle damage from the eccentric exercise protocol, delayed muscular repair, or a diminished pain tolerance. Some researchers suggest that soreness is related to degree of inflammation (Miles & Clarkson, 1994; Stauber et al., 1990). Eccentric exercise-induced inflammation may vary between OC users and non-OC users; therefore, soreness

differences with OC use may reflect inflammatory responses rather than simply the magnitude of muscle damage.

Arm Circumference

Twenty-two subjects contributed measurements usable for analysis of mid-brachial arm circumference. Measurement of the mid-brachium reflects the degree of muscular swelling. Significant swelling indicates muscular damage (Stauber et al., 1990). In the present study, swelling did not occur over time with either group, nor did OC use affect changes in arm circumference when compared to non-OC users. Although a lack of swelling suggests that muscular damage may not have occurred, results from maximal force, soreness, and CK evaluations indicated that the eccentric exercise protocol elicited muscle damage. Furthermore, the arm circumference measurements may not have been accurate. Inter-experimenter variability may have been large enough to eliminate any significant differences in arm circumference. Other than mid-brachial location of measurement, there was no training on measurement technique.

Creatine Kinase

At the baseline blood draw at 0-h, OC users presented with significantly greater CK values. There was a time main effect, as CK increased throughout the study, with a significant surge in magnitude between 48-h and 96-h. Other researchers identified an increase in CK post-eccentric exercise in blood samples (Miles & Clarkson, 1994) and in muscle biopsy at 48-hours post-exercise (Malm et al., 2004) that indicates muscle damage. The CK response in both groups of the present study coincided with previous findings, suggesting that muscle damage occurred post-exercise.

Both groups in the present study experienced similar muscle damage, as there was no difference in serum CK enzyme activity between groups. However, a previous study concluded that OC users, measured at mid-luteal phase when estrogen levels are high, express a lower CK response at 72 hour post-exercise than do non-OC users, measured at mid-follicular phase when estrogen levels are low (Carter, Dobridge, & Hackney, 2001). Lower CK concentrations imply less muscle damage in OC users, suggesting that hormonal therapy in young women protects muscle cells from injury during elevated estrogen phases.

Endogenous hormones may also affect the CK activity in young exercising women. Amenorrheic exercising women expressed greater CK concentrations than did eumenorrheic exercising women or eumenorrheic sedentary women (Thompson et al., 2006). This finding showed that a lack of hormones lead to elevated CK. Furthermore, when the exercising ovulatory women were divided into a normal steroid function group and a suppressed progesterone luteal phase group, the normally ovulating women, as well as the amenorrheic women, expressed greater CK concentrations than did the suppressed progesterone women (Thompson et al.). This finding suggests that low progesterone levels may be more protective of muscle damage than having a balanced ratio of estrogen to progesterone, whether in an environment of low hormone levels (amenorrheic women) or normal hormone levels (normally ovulating women). Taken together with the conclusions from the Carter et al. (2001) study, it may be that high estrogen and low progesterone levels provide the greatest protection from muscle damage than other hormone ratios. This protection may result from the known anti-oxidant activities of

estrogen (Demirbag, Yilmaz, & Erel, 2005; Joo et al., 2004; Reyes, Sifuentes-Alvarez, & Lazadle, 2006).

Regardless of apparent protective effects of hormones on muscle damage, in the present study, the OC group and non-OC group displayed no differences in CK concentrations, which indicate the degree of muscle damage. Because cellular damage induces an inflammatory response, and the OC group and the non-OC group experienced similar muscle damage effects, it may be assumed that the two groups would have similar inflammatory effects.

Inflammatory Markers

Interleukin-6

At baseline, there was no difference in IL-6 concentrations between groups. This finding coincides with other studies in which researchers have concluded that although CRP is elevated in OC users, resting IL-6 concentrations are not affected by OC use, regardless of type or generation (Chatterton, Geiger, Mateo, Helenowski, & Gann, 2005; Kluft et al., 2002; Lakoski and Herrington, 2005; Timmons et al., 2005; von Rooijen et al., 2006).

IL-6 increased significantly in both groups in the post-exercise phase. At the 8-h time point, IL-6 concentrations reached peak levels, which were significantly greater than baseline measurements. The appearance of IL-6 in the blood follows the known response of inflammatory markers to exercise. Unlike an inflammatory response to an infectious agent, in which IL-6 is secreted mostly from monocytes and macrophages, the majority of IL-6 is released from skeletal muscle during exercise (Febbraio & Pederson, 2005;

Mackinnon, 1999; Mooren, Volker, Pedersen, Schulz, & Teschemacher, 2005). Post-exercise, the first cytokine to appear in plasma is IL-6, which increases 100-fold (Mooren et al., 2005).

Immediate elevation of IL-6 is found in both endurance and eccentric exercise modes. Most researchers have concluded that after 60 minutes of endurance exercise at 65-75% VO2max in trained athletes, IL-6 peaks immediately post-exercise and return to baseline levels within 5 hours. Of note, moderately fit individuals also express peak IL-6 immediately post-exercise, yet return to baseline within 2 hours; whereas, untrained individuals do not increase IL-6 in the post-exercise phase (Mackinnon, 1999). These findings suggest that endurance training may affect the IL-6 response to exercise. Because endurance training and aerobic fitness was not controlled for in the present study, it is possible that a training effect could have affected the IL-6 response to the eccentric exercise elicited in this study.

Eccentric exercise research studies revealed a similar time course of IL-6 elevation as found in the present study. In well-trained men performing a 10% downhill run for 45 minutes at VO2max, plasma IL-6 was elevated 460% from baseline immediately post-exercise, and remained at 410% elevation from baseline 1 hour post-exercise (Peake et al., 2005). Subsequent blood samples were not taken again until 24 hours post-exercise, so IL-6 concentrations were not available at 4, 8, or 12-hours post-exercise. Another study revealed that after 300 maximal eccentric quadriceps contractions in healthy men, IL-6 concentrations peaked at 6 hours post-exercise (Paulsen et al., 2005). This finding is similar to the finding of the present study, in which IL-6 peaked at the 8-h time point. Overall, participants in this study responded as expected to

the eccentric exercise, which elicited peak IL-6 levels later in the post-exercise phase compared to post-endurance exercise responses.

The initial peak in IL-6 after endurance and eccentric exercise induces production in the anti-inflammatory markers IL-1ra, sTNF-R1, IL-10 (Mooren et al., 2005; Steensberg, 2003), and cortisol (Mackinnon, 1999). IL-1ra, sTNF-R1, IL-6, as well as cortisol, inhibit the pro-inflammatory cytokine IL-1; while sTNF-R1, IL-10, and IL-6 inhibit the pro-inflammatory TNF- α; and sTNF-R1 prevents IL-6 from performing its pro-inflammatory role. Together, these anti-inflammatory molecules reduce elevated exercise-induced IL-6 to baseline levels by 24 hours post-exercise (Mackinnon, 1999; Malm et al., 2004; Peake et al., 2005). As expected, plasma IL-6 returned to baseline levels by 24-hours in the present study.

There was no difference in changes in post-exercise IL-6 concentrations between OC users and non-OC users. Conversely, other researchers identified differences between OC users and non-OC users in post-endurance exercise IL-6. Timmons et al. (2005) found that after cycling for 90 minutes at 65% maximal power, OC users experienced an 80% lesser IL-6 increase than non-OC users in the follicular phase of the menstrual cycle, when estrogen is high. During the luteal phase of menstruation, when progesterone is high, there was no difference in plasma IL-6 between the OC and non-OC groups.

In both follicular and luteal phases, OC users had lower estrogen and progesterone levels than did non-OC users. Because estrogen has an anti-inflammatory effect (Pfeilschifter et al., 2002; Rachon et al., 2002; Tiidus, 2005), possibly via inhibition of inflammatory cells and a reduction of TNF-α production (Salem, Hossain, &

Nomoto, 2000), and OC users had lower estrogen than non-OC users, it may be assumed that the non-OC users would have greater protection from inflammation than would OC users. However, non-OC users had greater IL-6 than did OC users post-exercise (Timmons et al., 2005), suggesting that estrogen had a minor impact on the IL-6 response to endurance exercise. A study of men supplemented with estrogen provides further evidence that estrogen has little effect on IL-6 responses to exercise. When men were treated with 2 mg/d 17β-estradiol for 8 days, and then performed 90 minutes of endurance exercise at 65% VO2max, researchers failed to find an influence of estrogen on systemic inflammation (Timmons, Hamadeh, & Tarnopolsky, 2006).

Estrogen's anti-inflammatory effects may hold true only under resting conditions. In an in vitro study of inoculation of female monocyte and macrophage cells with 17β-estradiol, estrogen modulated the production of TNF-α, IL-1, and IL-6 (Kramer, Kramer, & Guan, 2004). In an in vivo study, postmenopausal women were given 50 μg 17β-estradiol for 12 months. Spontaneous production of IL-6 from activated monocytes and macrophages decreased significantly (Rachon et al., 2002). Other researchers have also concluded that exogenous estrogen in post-menopausal women inhibits the secretion of IL-6 from macrophages and endothelial cells (Pfeilschifter et al., 2002). These findings indicate that the anti-inflammatory effects of estrogen may occur only during resting conditions when IL-6 production by the skeletal muscle is not a factor. In the present study, no group effect may be attributed to a lack of exercise-induced IL-6 response to estrogen, whether endogenously or exogenously produced.

Progesterone has not received much attention from researchers in relation to inflammation; however, this hormone may have more influence than estrogen on the IL-6

response during endurance exercise. In the Timmons et al. (2005) study, the non-OC users endogenously produced greater than 5.5-fold more progesterone in the luteal phase than in the follicular phase, as expected with a normal menstrual cycle, and produced a greater IL-6 response. The OC group had no flux in progesterone between follicular and luteal phases, suggesting that either natural hormonal flux or elevated progesterone was responsible for the enhanced IL-6 response in non-OC users. The present study may have found greater differences between groups if measurements were taken during the luteal phase rather than the early follicular phase of the menstrual cycle.

Research performed during resting conditions has revealed conflicting results. Some researchers have identified a positive correlation between IL-6 and OC progesterone dose (Salkeld et al., 2001). Furthermore, in a study of men given 200 mg norethisterone for 52 weeks, progesterone was positively correlated with IL-6 (Zitzman, Erren, Kamischke, Simoni, & Nieschlag, 2005). On the other hand, in another study when non-OC users were studied in the luteal phase during resting conditions, there was no change in monocyte-derived IL-6 concentration in spite of an increase in the alarm cytokines IL-1 and TNF-α (Willis, Morris, Danis, & Gallery, 2003). Therefore, it remains unclear whether progesterone or estrogen has an effect on IL-6 production. It appears as though estrogen exerts anti-inflammatory effects only during resting conditions while findings on progesterone effects are inconclusive. It is likely that knowledge of hormonal dosages and endogenous hormone levels are necessary to interpret the effects on inflammation. The present study required participants to initiate the study within the first five days of the menstrual cycle, when hormone levels of both estrogen and progesterone were low. However, blood hormone levels were not measured, nor were OC dosages

monitored; therefore, the influence of estrogen and progesterone on IL-6 concentrations was not clearly elucidated. It cannot be determined whether hormonal levels had an influence on IL-6 production, possibly explaining why no group effect was detected.

C-Reactive Protein

At baseline, the OC group had significantly elevated hs-CRP concentrations when compared to the non-OC group. This finding was expected, as most researchers agree that hs-CRP concentration is higher in OC users than non-OC users (Dreon, Slavin, & Phinney, 2003; Raitakari et al., 2005). In addition, current hormone replacement therapy users have greater hs-CRP than previous users, who have greater hs-CRP than non-users (Bermudez et al., 2002). It is unlikely whether the elevated hs-CRP in exogenous hormone users is an acute phase response because IL-6, the primary inducer of the acute phase response, was not elevated in resting OC users (Chatterton, et al., 2005; Kluft et al., 2002; Lakoski & Herrington, 2005; Timmons et al., 2005; von Rooijen et al., 2006).

There was no time main effect for hs-CRP response to the eccentric exercise. Although exhaustive endurance exercise has shown to induce elevated hs-CRP immediately and up to 3-5 days post-exercise (Mackinnon, 1999), conclusions from eccentric exercise studies differ. Much evidence supports a lack of CRP response to maximal eccentric exercise. After maximal eccentric elbow flexion that induced muscle damage, untrained males displayed no change in CRP immediately post-exercise (Milias, Nomikos, Fragopoulou, Athanasopoulos, & Antonopoulou, 2005) or up to 5 days post-exercise (Mackinnon). Additionally, in untrained males and females, after a 1-h, 45-min downhill run at 70% age-predicted heart rate max, no CRP response was elicited

immediately or up to 5 days post-exercise (Mackinnon). Simpson et al. (2005) found similar results after a muscle damaging 7-km run with equal uphill and downhill portions in which participants had no change in CRP post-exercise.

The elevation of IL-6 in the post-exercise phase may have greater anti-inflammatory effects than acute phase response implications. In a review of exercise studies, Steensberg (2003) concluded that IL-6 does not elevated CRP, while the cytokine does increase IL-1ra, IL-10, and cortisol, which are all anti-inflammatory agents. The anti-inflammatory signal may be strong enough to halt the acute phase response before acute phase proteins, such as CRP, can be released from the liver.

In response to the eccentric exercise in the present study, OC users expressed a significantly greater hs-CRP concentration than the non-OC group. This effect may be attributed to higher baseline hs-CRP in the OC users, regardless of an inflammatory response to the exercise. The primary acute phase response inducer, IL-6, increased with time in the post-exercise phase. However, this cytokine exerted greater anti-inflammatory than pro-inflammatory roles post-exercise, as evidenced by a significant decrease in the sTNF-R1. sTNF-R1 inhibits the inflammatory cytokines IL-1, TNF-α, and IL-6 (Mooren et al., 2005). A decrease in sTNF-R1 signifies that anti-inflammatory agents are no longer necessary, as the inflammatory response ceases. The concurrent lack of time effect of hs-CRP suggests that the inflammatory process ceased before acute phase proteins were released from the liver. The greater hs-CRP response post-exercise in OC users versus non-OC users likely reflects homeostatic factors associated with OC use, rather than factors elicited via exercise.

Soluble Tumor Necrosis Factor Receptor-1

At baseline, the two groups had similar plasma sTNF-R1 concentrations. When sTNF-R1values were measured from 0-h to 96-h, there was a time main effect, in that sTNF-R1 decreased from the time of exercise until the 12-h time point. Values were significantly lower than baseline at the 4-h, 8-h, and 12-h time point, and nearly returned to baseline levels by 96-h. The decrease in sTNF-R1 throughout the day reflects the diurnal fluctuation of sTNF-R1 and may not be associated with the eccentric exercise.

sTNF-R1 inhibits the actions of inflammatory cytokines; however, sTNF-R1 did not increase in response to the exercise protocol and appeared to not be associated with markers of muscle damage or inflammation. IL-6 stimulates the release of sTNF-R1; yet, IL-6 increased while sTNF-R1 decreased in the post-exercise phase of the present study. Researchers from previous studies have identified a small increase in sTNF-R1 in the post-eccentric exercise phase (Miles et al., press). These findings are contradictory; however, it may be true that a larger IL-6 response than found in this study is necessary to elicit sTNF-R1 release. If the exercise stimulus and muscle damage were greater, sTNF-R1 may have increased in order to hinder the pro-inflammatory IL-6 response. Further, because IL-1 and TNF-α are not strongly produced in the inflammatory response to eccentric exercise (Hirose et al., 2004; Mackinnon, 1999), and sTNF-R1 acts to inhibit these cytokines, the requirement for sTNF-R1 action was not high. Additionally, the elevated IL-6 acted to reduce TNF-α, further decreasing the need for sTNF-R1.

There was no significant difference in sTNF-R1 between the OC group and the non-OC group response to the eccentric exercise protocol. Prior researchers have reported conflicting findings in relation to exogenous hormones and sTNF-R1. With

exogenous estrogen therapy, monocytes and macrophages have increased secretion of the alarm cytokines IL-1 and TNF-α (Pfeilschifter et al., 2002). Conversely, other researchers did not find any significant relationship between either endogenous or exogenous 17β-estradiol or progesterone and IL-1β or TNF-α production (Bouman, Schipper, Heineman, & Faas, 2004). The present study was designed to detect the TNF receptor, which has a greater half-life in the plasma the TNF-α cytokine itself. Regardless of method of detection, it does not appear that TNF-α or sTNF-R1 is affected by OC usage.

Cortisol

At baseline, cortisol was higher in OC users than non-OC users. This finding is similar to those of other researchers, who have found elevated cortisol in OC users when compared to non-OC users. In resting participants, OC users had cortisol levels 2.5-fold greater than that of non-OC users and men (Meulenberg & Hofman, 1990; Timmons et al., 2005); in another study, OC users had greater cortisol concentrations than non-OC users both at rest and after performing leg ergometry for 60 minutes at 45-65% VO2peak (Casazza et al., 2004).

Post-eccentric exercise, cortisol varied similarly in both groups with time; it was highest in the morning and decreased significantly throughout day 1, as expected with diurnal variation, and reached to a low at the 12-h time point. By 24-h cortisol returned to baseline levels. Although the OC users and non-OC users changed cortisol concentrations on a similar time course, a group effect was detected as the OC users had significantly greater cortisol levels in the post-exercise phase. In a recent study, OC users

were found to have twice as high total cortisol than non-OC users after interval exercise (Klose et al., 2007). However, in the same group, free cortisol was significantly lower in the OC versus the non-OC group. Another research group also discovered that free cortisol was lower in OC users than non-users, while total cortisol was equal between groups (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). Because free cortisol is available for binding to receptors, it may have more implications for physiologic differences found between groups.

Cortisol is part of the anti-inflammatory response. It initially is stimulated by the alarm cytokines IL-1, TNF-α, as well as IL-6. These cytokines act on the hypothalamus to cause corticotropin-releasing hormone (CRH) secretion, and the anterior pituitary gland to stimulate adrenocorticotropic hormone (ACTH) secretion. CRH then acts on the anterior pituitary to stimulate ACTH secretion; ACTH then travels to the adrenal glands to cause release of cortisol to the blood stream. Free cortisol then exerts its anti-inflammatory effects by decreasing the production of IL-6 (Yeager et al., 2005) and the pro-inflammatory cytokines TNF-α and IL-8 (Straub et al., 2002). Elevated cortisol levels in OC users suggest that the inflammatory response would be lower in this group. However, sTNF-R1 and IL-6 were similar between groups in the present study. It is likely that free cortisol was lower in OC users than non-OC users, as found in other studies, which would explain elevated inflammation in OC users. If the elevated hs-CRP in OC users is in fact a result of an inflammatory process, low free cortisol may explain the difference. Limited anti-inflammatory effects of cortisol may also explain the greater soreness experienced by OC users.

Researchers have demonstrated a delayed peak cortisol concentration time during diurnal variation in OC users versus non-OC users (Meulenberg & Hofman, 1990). A delay in cortisol responsiveness may also occur during the post-exercise phase when cortisol rises. In the present study OC users had greater serum cortisol than non-OC users at the 24-h and 96-h time points. If the cortisol peak is delayed after exercise as it is during normal diurnal variation, then the delay in action could explain why OC users had higher cortisol at days 1 and 4 post-exercise, when the non-OC group cortisol levels were returning to baseline levels.

Limitations

A major limitation in the present study is the magnitude of the inflammatory response. The exercise protocol consisted of 45 repetitions of elbow flexion of one arm by high-force, eccentric resistance. The muscle damage elicited may not have been of a magnitude to inflict an inflammatory reaction significant enough to show differences between OC user and non-OC users. To create a greater inflammatory reaction, a series of contractions of the triceps muscles, in addition to the biceps brachii contractions, could be added to the exercise protocol. Performing high-force eccentric muscle contractions to both muscle groups would increase the total muscle mass damaged. Additionally, in daily activity the triceps are used less than the biceps; therefore, damaging the triceps will elicit a greater relative inflammatory response compared to the biceps, which are used regularly. Additionally, eccentric exercise contractions performed at a fast velocity have created more muscle damage, as evidenced by CK values, than have slow eccentric contractions (Chapman, Newton, Sacco, & Nosaka, 2006). The present study may have

elicited a greater inflammatory response if more muscle mass was damaged and if the eccentric contractions were performed at a faster rate.

Another limitation involves hormone regulation between the two groups. The hormonal composition of the oral contraceptives taken by the women in the study was not regulated. Women were taking both mono-phasic and tri-phasic hormonal regimes. The variation in estrogen and progesterone ratios between each OC user may have influenced the results of the study. Additionally, the women in the non-OC group were not tested for hormone levels. The assumption that all the non-OC users had regular hormonal cycles may be false.

Finally, experimenter variability may have influenced the results. The exercise protocol was facilitated by five different researchers to the 24 participants. Each participant may have responded differently to each of the researchers, as this was not a constant variable.

Conclusions

Height, weight, BMI, and lipids appeared to have no influence over the inflammatory response to exercise in OC users and non-OC users. Muscle damage occurred in both groups as a result of the eccentric exercise protocol, as evidenced by decreased maximal force production, soreness, and elevated serum CK concentrations. Enhanced soreness in OC users may reflect the systemic presence of CRP among these women, rather than a response to the exercise, since OC users did not have a greater magnitude of response in other muscle damage or inflammatory markers. A small inflammatory response occurred in both groups; however, greater muscle damage may

have enhanced the inflammatory response and elucidated more significant findings over time and between groups. Eccentric exercise caused elevation of IL-6 in the plasma without an increase in sTNF-R1, indicating the skeletal muscle was the predominant secretor of IL-6. hs-CRP did not change in response to the exercise; rather, it remained 5-fold higher in the OC group than the non-OC group throughout the experiment. Therefore, it may be assumed the hs-CRP was not a result of an acute phase response. Measurement of total cortisol rather than free cortisol obscures conclusions about the effects of cortisol on inflammatory response differences between OC users and non-OC users. In both OC users and non-OC users, the IL-6 secreted from skeletal muscle during exercise has an anti-inflammatory effect that may explain the health benefits of regularly exercise performance. hs-CRP does not respond to eccentric exercise; however, causes and affects of elevated levels of this acute phase protein in OC users require further investigation.

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