2008

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Your file Votre référence
ISBN: 978-0-494-38715-3
Our file Notre référence
ISBN: 978-0-494-38715-3

EFFECTS AND MECHANISMS OF FEMALE SEX HORMONE INFLUENCE ON SKELETAL MUSCLE INFLAMMATION

by

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Bachelor of Science, Biochemistry, University of Waterloo, 2005

THESIS

Submitted to Kinesiology and Physical Education, Faculty of Science

in partial fulfillment of the requirements for

Master of Science

Wilfrid Laurier University

Abstract

Previous work from our laboratory has demonstrated that estrogen will attenuate leukocyte infiltration into skeletal muscle following eccentric exercise. However the mechanisms by which estrogen exerts its effects are still uncertain. In experiment one, we investigated the role of estrogen receptor (ER) influence on muscle leukocyte infiltration following eccentric exercise through administration of the ER antagonist ICI 182,780 following downhill running in ovariectomized female rats with (E+) or without (E-) estrogen supplementation. At 24 hours post-exercise, soleus and white vastus muscles were removed and immunostained for HIS48 (neutrophil) and ED1 (macrophage) positive cells. The increase in number of fibres positive for HIS48 in soleus and positive for ED1 in the soleus and white vastus was significantly attenuated ($p<0.05$) in E+ relative to E- rats at 24 hours post-exercise. E+ rats administered the ER agonist also had significantly ($p<0.05$) attenuated 24 h post-exercise increases in HIS48 and ED1 positive soleus and white vastus muscle fibres similar ($p>0.05$) to those without ER antagonist. This suggests that the ability of estrogen to attenuate post-exercise leukocyte infiltration into skeletal muscle is not ER-mediated.

In experiment two, we examined the interaction effects of estrogen and progesterone on skeletal muscle in response to injury in ovariectomized female rats. At 24 hours post-exercise, soleus and white vastus muscles were removed and immunostained for HIS48 (neutrophil) and ED1 (macrophage) positive cells. Leukocyte infiltration 24 hours post-exercise in the soleus (red) and white vastus muscles were significantly decreased in ovariectomized rats treated with progesterone alone relative to unsupplemented ovariectomized rats. Following exercise the percentage of leukocyte

infiltration also decreased significantly with estrogen supplementation alone in both muscle types, 67-69% and 63-74% in neutrophils and macrophages, respectfully. Furthermore, the combination of estrogen and progesterone supplementation did not significantly alter the attenuation observed by estrogen alone. These results demonstrate that skeletal muscle in female rats benefited from both progesterone or estrogen treatment alone; effects of progesterone with estrogen following exercise were neither greater nor less than with estrogen alone.

Acknowledgements

I would like to thank the following people for helping me during my Masters:

I would first like to thank my advisor Dr. Peter Tiidus for taking me on as his first graduate student and providing me with support during my Masters. I am grateful that you gave me the freedom to explore and discover things for myself, while still being available to answer all my questions. I would also like to thank Dr. Debbie Enns for training me with the skills required to perform successfully in the laboratory.

My fellow graduate students also deserve thanks for keeping me sane and entertained. In particular I would like to thank my office mate, Anthony, for his sarcastic encouragement and keeping me humble. Also to Anne, you made the writing process immensely easier by always checking up on me and making sure that we celebrating the completion of every chapter we wrote.

Just as important as the people in school are the people outside. To my best friend Linda, I truly appreciate your constant encouragement. To my oldest friends Janice and Rachelle, that have always been there for me to hear about my problems in and out of the lab, thank you. Also, I am grateful to Rebecca for introducing me to research and for supporting me throughout this process.

Finally I would like to thank my mother, who taught me the definition of hard work and dedication.

"Spend your life doing something you love"

Sobia

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List of Abbreviations

Ca^{2+}- calcium

CK- creatine kinase

E2 - estradiol 17β

EC - coupling-excitation-contraction coupling

ERα - estrogen receptors

ERβ - estrogen receptors

FGF - fibroblast growth factor

HGF - hepatocyte growth factor

HSP - heat shock protein

IGF-1 - insulin like growth factor

ILs - interleukins

I/R - ischemia reperfusion

LIF - leukemia inhibitory factor

NO - nitric oxide

SR- sarcoplasmic reticulum

TGF - transforming growth factor

TNF - tumour necrosis factor

List of Definitions

Ad libitum	Free access. For example, animals that have free access to feed and water.
β-glucuronidase	An indirect indicator of muscle damage. The release of this lysosomal enzyme into blood is a biochemical marker for muscle damage.
Chemoattractant	A chemical agent that induces an organism/cell/leukocyte to migrate towards it.
Creatine kinase	A metabolic enzyme located intracellular in muscles. As a result of muscle damage, muscle cells release their contents, which include CK, into the bloodstream.
Cytokines	Cell signaling molecules used for cellular communication.
Feedback loop	An output product from the system signals back to the system. This is often used to control the behaviour of the system.
Fibroblasts	Provide the structural framework in muscles and play a critical role in the repair process
Fibroblast growth factor	Family of growth factors. They are important in the process of proliferation and differentiation in cells.
Homeostasis	Self-regulating process that tends to maintain stability while adjusting to conditions.
Hypertrophied	Enlargement of tissue, as a results of an increase in size rather than the number of constituent cells.
Immunohistochemical	The use of antibodies to detect certain proteins on sections of tissue. The primary unlabeled antibody binds to antigens on the protein of interest. Then a labeled secondary antibody is used to detect the positive antibody-antigen interaction
Insulin-like growth factor	Family of growth hormones. They are important in the regulation of normal physiology.
Interleukins	A group of cytokines. Predominately used in the immune system as a means of communication.

In Vivo	Experiments performed in living tissue of a whole, living organism.
In Vitro	Experiments performed outside a living organism, in a controlled environment.
Ischemia/Reperfusion	Eliminating blood flow to specific part of the body. The absence of oxygen and nutrients from the blood can result in damage to that area of the body.
Leukocytes	Also known as white blood cells. Cells in the immune system that defend against infection and foreign material.
Monocytes/macrophages	Phagocytes that engulf and digest cellular debris and pathogens.
Myogenic satellite cell	Undifferentiated mononuclear cells. In response to injury, disease and as a part of normal growth they differentiate and are capable of fusing with existing muscle fibres or fuse together to form new muscle fibres.
Neutrophils	The most abundant type of leukocyte. They are usually the first respondents to an infection or injury. These short lived cells are active phagocytes.
Phenolic Ring	6 carbon benzene ring with an attached hydroxyl (OH) group.
Prostaglandins	Group of lipid compounds. They are important in inflammation and contraction of smooth muscles.
Transforming growth factor	Family of growth hormones consisting of TGF-α and TGF-β. TGF-α stimulates growth of endothelial cells. TGF-β are important in tissue regeneration, cell differentiation, and regulation of the immune system.
Tumour necrosis factor	Family of cytokines, produced by monocytes and macrophages.

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Chapter One

Introduction

Skeletal muscle is a vital component of the body. Skeletal muscle is composed of muscle fibres that function together to perform contractions. The force generated through these contractions enables movement in the body. Skeletal muscle function can be impaired by trauma resulting from crush injury, lacerations, disuse and excessive exercise (1).

1.1 Skeletal Muscle Injury

Unaccustomed strenuous exercise, especially eccentric exercise can, cause muscle damage. Eccentric muscle contractions allow the muscle to develop force as it is being lengthened by an external load. McCully and Faulkner (2) investigated exercise-induced muscle injury proximal to the distal tendon of the extensor digitorum longus (EDL) muscle of anesthetized mice. The muscle was exposed to bouts of eccentric, concentric or isometric contractions, for which the muscle length, muscle velocity, level of muscle activation and the number of contractions were controlled. Following the contraction protocols the distal tendon was reattached and the animals were allowed to recover. Three days post-exercise, eccentric contractions in the muscle showed histological signs of damage and a reduction in maximum isometric muscle force. Exercise protocols of equivalent intensities involving isometric and concentric contractions did not result in as much muscle injury as those observed with the eccentric contractions (2). Eccentric exercise causes more damage than concentric exercise. This is thought to be because fewer motor units are recruited during eccentric exercise, resulting in a smaller cross-

sectional area that is available to handle the same load that would be handled in a concentric contraction (3).

Damage to muscle structures has been shown to occur following a bout of strenuous exercise. Muscle damage can be manifested as a loss in muscle function or mechanical disruptions in the muscle architecture. The functional implications of muscle damage include a reduced range of motion in joints, stiffness, altered fatigability, reduced muscle shortening velocity, and prolonged strength loss in muscles (4).

Eccentric exercises typically result in approximately 10-30% force loss immediately following exercise (5). High force eccentric exercise (exercise consisting of maximal eccentric actions) results in muscle strength loss of about 40-60% immediately after exercise, and strength is not fully recovered until about 10 days later (6; 7). Since muscle injury has an impact on performance in the workplace, home and on the athletic field, it is important to understand the mechanism behind muscle damage in order to devise means to prevent or reduce the detrimental effects.

Immediately following eccentric contractions many sarcomeres show signs of disruption and damage. Newhan *et al.* (8) found single disrupted sarcomeres and disrupted half-sarcomeres, surrounded by normal appearing sarcomeres under the electron microscope, following eccentric contractions of human quadriceps muscles. In the disrupted sarcomeres the myofilaments were disorganized and Z-band material was seen to be "streamed" across the sarcomeres or a widening of the Z-band was apparent (8). Friden *et al.* (9) supported the findings of Z-band disorganization following eccentrically induced muscle damage in human soleus muscles. Following injury, the Z-band material, which normally has a regular and discrete structure, was streamed and in

some cases there was a complete disruption, resulting in gaps in the lattice pattern, which peaked three days post exercise and decreased with time (9).

Damage to the membrane or sarcoplasmic reticulum (SR) would allow calcium (Ca^{2+}) to move down its concentration gradient from the extracellular space into the cytosol, potentially increasing the level of intracellular Ca^{2+} and resulting in a loss of calcium homeostasis. Duan *et al.* found that rat skeletal muscle had elevated mitochondrial Ca^{2+} levels following injury from downhill walking (10). High levels of Ca^{2+} have the potential to activate various molecular pathways in skeletal muscle, such as the calpain proteolytic pathways and the phospholipase A_2 pathway. Calpains are thought to play a role in muscle damage by being responsible for the initial breakdown of myofibrils and cytoskeletal proteins (eg. desmin, α-actinin) (11). Activation of phospholipase A_2 pathway may contribute to further post-exercise muscle damage of the sarcolemma, thus increasing the loss of intracellular molecules (12). To further support the role that elevated Ca^{2+} plays in the injury process, it has been observed that when muscle fibres are treated with calcium chelators (EDTA and EGTA) there is a reduced accumulation of Ca^{2+} in the mitochondria that is accompanied by an attenuation in muscle fibre injury (10). The inability of skeletal muscle to buffer the damage related increase in intracellular Ca^{2+} may lead to further damage in the muscle.

Structural alterations within the muscle are accompanied by the leakage of proteins such as myoglobin and creatine kinase (CK) out of the cell and into circulation. CK is a metabolic enzyme located intracellularly in muscles. Due to its location and solubility it is able to cross the muscle sarcolemma membrane if the membrane is damaged or disrupted. Therefore, measurements of CK release are an indirect indicator

of muscle membrane damage (13). Another common indicator of muscle damage is β-glucuronidase. Measurements of the activity of the lysosomal acid hydrolase β-glucuronidase have previously been shown to provide a quantitative indicator of exercise induced muscle damage (14).

Performance of an exercise that causes muscle damage results in an adaptation in the muscle. One bout of high force eccentric exercise is enough to elicit an adaptation response in humans, such that the muscle is more resistant to damage and repairs at a faster rate with subsequent bouts of exercise (15). The repeated bout effect has been demonstrated not only in human but also in animal models, using various types of activities employing different muscle groups (16). Several theories have been proposed to explain the repeated bout effect, such as neural, connective tissue, or cellular adaptations (16), but the specific mechanism has yet to be identified.

1.2 Skeletal Muscle Inflammation

Damage to muscle results in an inflammation response at the site of injury. During inflammation, leukocytes, primarily neutrophils and monocytes/macrophages, perform a wide range of functions preparing for repair and regeneration of the damaged muscle.

Neutrophils rapidly invade muscle when damage occurs, and are present at elevated levels when the injured fibres are being repaired. Neutrophils invade immediately following exercise and remain elevated for as long as 5 days post-exercise in humans (17). The time course varies with species but in general invasion of the

damaged tissue by neutrophils can persist for days to weeks. Neutrophils have been observed to peak 24 hrs following eccentric exercise in rats (18).

Neutrophils, along with monocytes and macrophages, play a critical role in inflammation through removal of necrotic tissue cellular debris (19). Neutrophils release cytokines that can attract and activate additional inflammatory cells (19). Neutrophils assist in the degradation of damaged tissue or foreign matter primarily through their phagocytic ability (20). Additionally, muscle proteins in the damaged areas may be degraded by proteolytic systems introduced by infiltrating phagocytic cells, or by proteases intrinsic to the muscle (21).

Neutrophils roll along the vessel wall searching the endothelium for signs of inflammation and chemoattractants that can activate and initiate their adhesion to the endothelium (22). At rest, more than half of the circulating neutrophils are located along the endothelial walls of blood vessels (22). In response to exercise, the increase in epinephrine, blood flow, and cell signaling molecules moves neutrophils away from the vessel walls and mobilize them into circulation, where they are redistributed to their required places in the body (23). In response to injury, the muscle cell communicates with the endothelium wall of the adjacent blood vessel, signaling the movement of neutrophils from circulation into the tissue. This communication is performed by a variety of cytokines.

Cytokines are a diverse class of compounds that are extensively used for cellular communication. All nucleated cells in the body produce cytokines and express cytokine receptors on their surface membrane. Cytokines bind to a specific cell surface receptor triggering a cascade of intracellular signals that are capable of altering cellular functions.

Skeletal muscles produce cytokines such as, interleukin (IL) 1, 6 and 8, and tumour necrosis factor (TNF) (24), in an effort to regulate function and maintain homeostasis. In response to injury there is an increased expression of IL-1 and TNF-α. These proinflammatory cytokines are responsible for upregulating the expression of endothelial leukocyte adhesion molecules and they play a role in initiating the breakdown of damaged muscle tissue (25). Activating the endothelium can cause the release of additional IL-1 and other proinflammatory cytokines, which recruit neutrophils to the site of injury (26).

Additionally, oxygen radical formation by neutrophil bursts are important in clearing away the damaged muscle tissue. The 50 to 100 fold increase in oxygen consumption by neutrophils results in an increased production of cytotoxic oxygen species, and possibly reactive nitrogen species (27). Nitric oxide (NO) was found to have a protective effect on muscle cells from neutrophil mediated cytolysis in vitro (28). Conversely, superoxide or superoxide derivatives have a cystolic function in neutrophil mediated lysis of muscle cells in vitro (28).

There may be secondary or collateral damage that occurs with neutrophil invasion of the muscle. When the generation of reactive radicals exceeds the ability of the tissue antioxidants to detoxify them, then the oxidative stress present can lead to further muscle damage (29). Release of high concentrations of cystolic and cytotoxic substances can damage healthy tissue (30). A fine balance has to be maintained since both overproduction and underproduction of superoxide by neutrophils can result in negative consequences.

Similar to neutrophils, macrophages are also capable of releasing reactive radicals and cytotoxic enzymes to aid in tissue degeneration. Macrophages are formed from undifferentiated monocytes circulating in the blood. In response to muscle damage macrophages invade the muscle and will concentrate near damaged fibres, and may even infiltrate the fibres (31).

Two main subpopulations of macrophages have been identified, $ED1^+$ and $ED2^+$. $ED1^+$ macrophages infiltrate muscle approximately 12-24 hours after exercise damage (32). It is thought that macrophages, especially $ED1^+$ macrophages, are responsible for the phagocytosis of injured tissue and secretion of inflammation regulatory cytokines. However $ED2^+$ macrophages are thought to contribute to satellite cell activation and proliferation in vivo (33). $ED2^+$ macrophages infiltrate muscle during later regeneration phase and induce satellite cell activation (34).

Activation of $ED1^+$ macrophages further contributes to inflammation by producing and releasing a variety of substances, including proinflammatory cytokines such as prostaglandins and IL-1 β (35). Not only is the macrophage response enhanced in the tissue by cell signaling, but additional neutrophils are also recruited (35). Unlike neutrophils, macrophages, in particular $ED2^+$, release growth factors necessary for muscle repair and regeneration. Most notable are the cell growth regulatory cytokines, such as insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), and transforming growth factor-β1 (TGF-β1). These cytokines activate and recruit fibroblasts (36). Fibroblasts provide the structural framework in muscles and play a critical role in the repair process. Additionally, fibroblasts continue to secrete their own

proinflammatory cytokines during the repair process, further contributing to the feedback loop.

1.3 Skeletal Muscle Repair

Skeletal muscle has the capacity to adapt to the physiological stress placed on it by growth, training and injury. As the removal of damaged tissue occurs, muscle fibre regeneration occurs by activation of satellite cells. Macrophages are not only critical for the removal of damaged and necrotic cellular debris, but they also attract myogenic satellite cells to the site of injury and release cytokines that regulate the myogenic satellite cell pool (37).

In undisturbed conditions, myogenic satellite cells remain in a non-proliferative, quiescent state. Myogenic satellite cells are undifferentiated stem cells. Quiescent myogenic satellite cells reside between the sarcolemma and the basal lamina. These cells contain little cytoplasm and few organelles (38). In response to muscle injury, satellite cells become activated and enter the cell cycle to provide the precursors required for new muscle formation during growth and repair (39). The ability of satellite cells to enhance muscle mass and repair muscle following injury makes them a vital component in skeletal muscle.

In response to cellular and extracellular signals associated with muscle damage satellite cells exit their quiescent state and migrate to the site of injury to repair or replace damaged muscle fibres. The signaling cascade for satellite cell activation is accomplished through the activating agents, nitric oxide (NO) released from the fibres or

the release of HGF from the extracellular matrix (40). Activated satellite cells have hypertrophied organelles and an expanded cytoplasm (38).

Following activation, satellite cells and myogenic precursor cells begin to proliferate (41). Committed myoblasts continue to proliferate and express muscle regulatory genes until the balance of protein expression drives the cells towards differentiation (42). Fibroblast growth factor stimulates an increase in the proliferation of satellite cells that have already been activated, however, it represses their ability to differentiate (43). Furthermore, insulin-like growth factor can activate proliferation and differentiation of satellite cells (43). Other factors that stimulate proliferation during muscle repair include HGF, LIF, GH, IL-6, insulin, leptin and testosterone (44).

The third stage in skeletal muscle regeneration is differentiation. It is the process whereby proliferating myoblasts formed from activated satellite cells, and other myogenic precursors, leave the cell cycle and fuse with existing myofibres to repair damaged segments or fuse together to form new myofibres during regeneration of damaged skeletal muscle (45). Differentiation is initiated in response to cellular and extracellular signals associated with muscle damage. Some factors that influence differentiation of satellite cells have previously been listed, but also include LIF, GH, insulin, IL-15, leptin and testosterone (44).

Following muscle regeneration the satellite cells return to a quiescent state. Myogenic satellite cells are self-renewing; the residual pool of satellite cells is re-established after each bout of injury. This beneficial process ensures that the pool of satellite cells is not depleted and is made available for further rounds of regeneration.

The distribution of satellite cells has been found to be distinctly different in the relative number of satellite cells within oxidative and glycolytic muscles in animals. The more oxidative fibres in muscle generally have a larger population of satellite cells (46).

Aged skeletal muscles are more prone than young ones to be susceptible to exercise-induced muscle damage and their regenerative capacity is also impaired (47; 48). In the slow twitch muscle of the soleus and the fast twitch muscle of the extensor digitorum longus muscles of rats there is a decline in satellite cell content with age (49). In rats, the proliferation potential of satellite cells also decreases with age (47). Human studies have also observed the same results. In the study by Kadi *et al.* (50), they demonstrated that the satellite cell proportion is reduced in older adults compared to younger women and men, implying that there is a reduction in the satellite cell pool as age increases.

1.4 Influence of Estrogen

Estrogen hormones play a vital role in developing and maintaining normal sexual and reproductive functions in both humans and animals. They also influence a variety of tissues, such as the uterus, prostate, bone, breast, brain, colon, and muscle (51). The term estrogen refers to three structurally similar steroid hormones: estrone, estriol, and estradiol 17β (E2). E2 is the primary estrogen found within the body.

The primary practical purpose of studying the effects of estrogen in response to skeletal muscle damage would be in order to gain further insight into the process in post-menopausal women. Post-menopausal women have reduced estrogen levels, and

therefore may be more susceptible to muscle damage and have a slower recovery time than pre-menopausal women.

In response to exercise-induced muscle damage, estrogen has been shown to reduce leukocyte derived inflammation (18). In particular, neutrophil infiltration into damaged muscle has been found to be reduced in response to estrogen supplementation (52). Recall that neutrophils increase inflammation post-exercise as they invade into the damaged muscles and produce oxidizing agents that are important in the clearance of damaged tissue debris (19-21; 53). It is postulated that a reduction in neutrophils is associated with a reduction in muscle damage, since neutrophils may exacerbate post-exercise damage by the generation of the oxidizing agents. It has been demonstrated that exercised female ovariectomized rats that received estrogen administration display an attenuation in muscle damage (CK activity), leukocyte activity, neutrophil infiltration and calpain-like activities as compared to exercised, unsupplemented animals (52). Additionally, Tiidus and Bombardier (18) compared sexually mature male and female rats and found a significant decrease in neutrophil infiltration post-exercise in female rats. When the male rats were supplemented with estrogen, neutrophil infiltration was attenuated to levels found in females. A reduction in neutrophils may decrease the severity and time course of inflammation, potentially enhancing the healing process.

Macrophages are also important in the removal of damaged muscle; additionally they are associated with the activation of muscle satellite cells and the repair process (39). St. Pierre-Schneider *et al.* (54) set forth to determine the time course of leukocyte invasion into damaged skeletal muscle of male and female mice. They reported a delay of macrophage migration to injured muscles of female mice compared to males by

several days following damage (54). McClung *et al.* (55) also found that a delay in macrophage invasion following injury was associated with differences in estrogen status. Ovariectomized female rats displayed an elevation in macrophage ($ED1^+$ and $ED2^+$) infiltration relative to intact female rats, seven days following injury (55). These studies demonstrate that macrophages are vital for a timely recovery from muscle damage and that estrogen plays a critical role in that process.

In terms of satellite cell response, it has been found that estrogen supplemented rats had an increase in muscle satellite cell content as compared to the rats that did not receive estrogen supplementation (56). Recent work by Enns & Tiidus (57) has found that estrogen supplemented animals had increased numbers of total, activated and proliferating satellite cells compared to estrogen naive animals following exercise induced muscle damage.

The mechanism by which estrogen influences the inflammation and repair process in damaged skeletal muscle is still uncertain. The proposed mechanisms for estrogen's effect may be through a direct nonspecific interaction with phospholipids, or by interactions with other membrane components, or even through an antioxidant effect. Understanding the mechanism behind estrogen's effects may allow us to optimize and manipulate this process.

It has been suggested that estrogen may have a membrane stabilizing effect. Estrogen is a fat-soluble hormone, capable of incorporating into the lipid bilayer of the cell and stabilizing the membrane (58). It is thought that this interaction could alter the fluidity and function of the membrane. Estrogen may incorporate into cell membranes, similar to how cholesterol integrates, and may act in a comparable nature to optimize

membrane fluidity and polyunsaturated fatty acid arrangement (59). Therefore, estrogen may have the capacity to limit membrane disruption in response to damage by stabilizing the membranes.

Additionally, estrogen may exert its effects through its antioxidant capacity. Antioxidants are molecules that have the ability to neutralize the unpaired electron from free radical species. Compounds with free radicals can cause chain reactions that damage cells. Antioxidants remove the free radical intermediates and terminate these potentially hazardous reactions. Estrogen possesses a hydroxyl group on its "A" (phenolic) ring, in the same position and configuration as found on the antioxidant vitamin E (60). Similar to vitamin E, estrogens may donate the hydrogen atoms from their phenolic hydroxyl group, halting the free radical peroxidation chain reactions (60). In this respect, exposure of skeletal muscle to estrogen may reduce muscle damage.

An alternative mechanism for estrogen's effect may be by direct receptor mediated mechanisms. Skeletal muscle is known to possess a small number of membrane bound estrogen receptors. Two estrogen receptors (ERs), ERα and ERβ, have been characterized and are expressed in mammalian skeletal muscle (61; 62). The specific roles that estrogen and its receptors serve in the muscle damage and repair processes remain elusive. The proliferative actions of estrogen seem to require EPα; whereas the differentiative and anti-proliferative effects of estrogen may be controlled by ERβ in ovaries (63). Therefore, the relative tissue distribution of the estrogen receptors may be a determinant in their form of action. Lemoine *et al.* (62) found that ERα levels in (untrained rats') skeletal muscle are distributed differently. Slow twitch muscle (soleus) had the greatest amount of ERα levels compared with an intermediate muscle

(gastrocnemius) and a fast twitch muscle (extensor digitorum longus) (62). These results are in agreement with those found in rabbits (64). Furthermore, it has been demonstrated that ERα and ERβ levels can be upregulated with exercise (65; 66).

Estrogen influences a variety of physiological and metabolic events within muscle. It has already been stated that estrogen contributes to the inflammatory and repair processes following skeletal muscle damage. Similar to the mechanisms behind estrogen's effects, the signaling processes also need further investigation. To date, it is known that estrogen influences the induction of heat shock proteins (HSPs). Heat shock proteins are highly conserved stress proteins that are induced in response to stressors, such as exercise and muscle damage (67). Their primary function is to act as chaperones, to mediate the proper folding of denatured proteins (68). Exercise induced muscle damage results in an increase in intracellular denatured proteins, signaling the induction of the HSP response. A relationship between the induction of the HSP response and a reduction in damage has been demonstrated in cardiac muscle. The induction of HSPs resulted in cardioprotection and delayed the onset of myocardial injury caused by ischemia (69). Paroo *et al.* (70) found a gender specific HSP response in rats. They found that males displayed a greater induction of HSP70 in skeletal muscle than females post-exercise (70). Moreover, ovariectomized rats supplemented with estrogen demonstrated a reduction in HSP70 levels compared to placebo treated ovariectomized rats, suggesting an estrogen mediated response (70). In support of these findings, it has been shown that male rats supplemented with estrogen resulted in a HSP response similar to those of females following exercise (71).

1.5 Influence of Progesterone

Progesterone receptors are distributed in numerous tissues. In rats they have been found to exist in the reproductive glands, certain regions of the oral mucosa and developing teeth, esophagus, larynx, skin, mammary gland, kidney, and skeletal muscle (72).

There has been limited work on the mechanism of progesterone's effect, but what is known is that there is a similar distribution of estrogen and progesterone receptors in most of the same cell types and tissues which may suggest that estrogen is involved in progesterone receptor induction (72). In the uterus, progesterone's effect is mediated by its receptors and estrogen increases progesterone receptor levels (73).

The interaction effects of progesterone and estrogen are limited, but it has been demonstrated that the combined supplementation influences skeletal muscle fatigue (74). In the study by St. Pierre-Schneider *et al.* (74) it was found that the time to fatigue was greatest in ovariectomized mice treated with progesterone alone, even more so than ovariectomized mice treated with placebo or a combination of estrogen and progesterone. Furthermore, it was found that the ovariectomized mice treated with progesterone alone had the greatest percent of isometric torque remaining immediately after eccentric contractions, as compared to the ovariectomized mice treated with placebo, estrogen, or a combination of estrogen and progesterone (74).

A related study done by Xing *et al.* (75) used the rat model to demonstrate the effects that hormones had on leukocyte content in response to endothelium vascular injury. They demonstrated that estrogen attenuated leukocyte populations and migration into injured tissue. The addition of progesterone and estrogen in combination negated the

protective effect observed by estrogen alone. Additionally, progesterone administration alone had no independent effect of leukocyte infiltration following damage (75).

The mechanism behind progesterone's actions has yet to be determined. Additionally, the interaction effects of progesterone and estrogen still need to be explored in order to determine their physiological significance. The practical implications of exploring this area may be for post-menopausal females that could be more prone to muscle injury. These women have altered hormone levels when they enter menopause and hormone intervention strategies involving estrogen and progesterone may be of importance. Additionally, further knowledge of the mechanisms behind muscle inflammation can be optimized (e.g., ER involvement and estrogen/progesterone interaction) to develop pharmaceutical or other intervention strategies to aid the skeletal muscle inflammatory process.

Chapter Two

Influence of Estrogen Receptor Blockage on Indices of Skeletal Muscle Inflammation

Statement of Problem

The purpose of this study was to investigate the role of estrogen receptors (ER) on muscle leukocyte (neutrophil and macrophage) infiltration following eccentric exercise through administration of the ER antagonist ICI 182, 780 to ovariectomized female rats with or without estrogen supplementation.

Subsidiary Problem

A secondary objective was to examine differences in leukocyte infiltration in skeletal muscle of different fibre types.

Hypotheses

1. It is hypothesized that leukocyte infiltration will be attenuated with estrogen supplementation.
2. ER blocker administration will negate the attenuation of leukocytes induced by estrogen supplementation.
3. Based on previous studies it is thought that both fast and slow muscle types will display similar trends in leukocyte infiltration.

Introduction

Unaccustomed strenuous exercise, especially eccentric exercise can cause muscle damage. Muscle damage can be manifested as a loss in muscle function or mechanical disruptions in the muscle architecture. Damage to muscle results in an inflammation response at the site of injury. During inflammation, leukocytes, primarily neutrophils and monocytes/macrophages perform a wide range of functions preparing for repair and regeneration of the damaged muscle.

Neutrophils rapidly invade muscle when damage occurs, and are present at elevated levels when the injured fibres are being repaired. Neutrophils invade immediately following exercise and remain elevated for as long as 5 days post-exercise in humans (76). Neutrophils have been observed to peak 24 hrs following eccentric exercise in rat (18). Neutrophils, along with monocytes and macrophages, play a critical role in inflammation through removal of necrotic tissue cellular debris (19). In response to muscle damage macrophages invade the muscle and will concentrate near damaged fibres, and may even infiltrate the fibres (77). Two main subpopulations of macrophages have been identified, $ED1^+$ and $ED2^+$. $ED1^+$ macrophages infiltrate muscle approximately 12-24 hr after exercise induced damage (19). It is thought that macrophages, especially $ED1^+$ macrophages, are responsible for the phagocytosis of injured tissue and secretion of inflammation regulatory cytokines. In contrast, $ED2^+$ macrophages are thought to contribute to satellite cell activation and proliferation in vivo (33).

In response to exercise-induced muscle damage, estrogen has been shown to reduce leukocyte derived inflammation (18). In particular, neutrophil infiltration into

damaged muscle has been found to be reduced in response to estrogen supplementation (52). It has been demonstrated that exercised female ovariectomized rats that received estrogen administration display an attenuation in muscle damage (CK activity), leukocyte activity, neutrophil infiltration and calpain-like activities as compared to exercised, unsupplemented animals (52). Additionally, Tiidus and Bombardier (18) compared sexually mature male and female rats and found a significant decrease in neutrophil infiltration post-exercise in female rats. When the male rats were supplemented with estrogen, neutrophil infiltration was attenuated to levels found in females. A reduction in neutrophils may decrease the severity and time course of inflammation, potentially enhancing the healing process. St. Pierre-Schneider *et al.* (54) set forth to determine the time course of leukocyte invasion into damaged skeletal muscle of male and female mice. They reported a delay in leukocyte migration to injured muscles of female mice compared to males by several days following damage (54). McClung *et al.* (55) also found that a delay in macrophage invasion following injury was associated with differences in estrogen status. Ovariectomized female rats displayed an elevation in macrophage ($ED1^+$ and $ED2^+$) infiltration relative to intact female rats, seven days following injury (55).

The mechanism by which estrogen influences the inflammation and repair process in damaged skeletal muscle is still uncertain. Estrogen may exert its effect via direct receptor mediated mechanisms. Skeletal muscle is known to process a small number of membrane bound estrogen receptors. Two estrogen receptors (ERs) ERα and ERβ have been characterized and are expressed in mammalian skeletal muscle (61; 62). The specific roles that estrogen and its receptors serve in the muscle damage process remain

elusive. Slow twitch muscle (soleus) had the greatest amount of ERα levels compared with an intermediate muscle (gastrocnemius) and a fast twitch muscle (extensor digitorum longus) (62). These results are in agreement with those found in rabbits (64). Furthermore, it has been demonstrated that ERα and ERβ levels can be upregulated with exercise (65; 66).

In this study, we investigated the role of estrogen receptors (ER) on muscle leukocyte (neutrophil and macrophage) infiltration following eccentric exercise through administration of the pure ER antagonist ICI 182, 780 to ovariectomized female rats with or without estrogen supplementation. The pure ER blocker binds with an extremely high affinity and specificity to both ER α and β and possesses no estrogen agonistic properties (78). Alternatively, estrogen may act via non-receptor mediated mechanisms, such as direct interactions with other membrane components, or even through an antioxidant effect.

We quantified the effects of estrogen supplementation and ICI 182, 780 administration on neutrophil and macrophage infiltration in red and white fibre types of rat skeletal muscle following downhill running. The neutrophil marker His48 and the macrophage marker ED1 were quantified 24 hr post-exercise. In addition to measuring leukocyte infiltration, muscle damage was indirectly assessed by measuring β-glucuronidase activity.

Methods

Animals

The study was approved by the Animal Care Committee at Wilfrid Laurier University and all procedures were performed in accordance with the Canada Council on Animal Care. A total of 72 ovariectomized female Sprague-Dawley rats (Charles River Laboratories, LaSalle, QC) were used in this study. The ovaries were surgically removed at 9 weeks of age, and all animals were 10 weeks old upon arrival in our laboratory. Animals were housed two per cage in an environmentally controlled environment with a standard 12:12 light/dark cycle and allowed access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water *ad libitum.*

Experimental Design

Following one week of acclimation in the laboratory, animals were randomly subdivided into either the estrogen supplementation group or into the group receiving a sham procedure. Half of the estrogen supplemented animals received injections of the pure estrogen receptor blocker, ICI 182, 780, while the remaining animals received vehicle injections. Each of these groups were further subdivided into exercise (sacrificed 24 and 72 hr post-exercise) and a control (unexercised) group, as illustrated in Figure 1. Thus nine groups of rats (n = 7-9 rats per group) were used in this study.

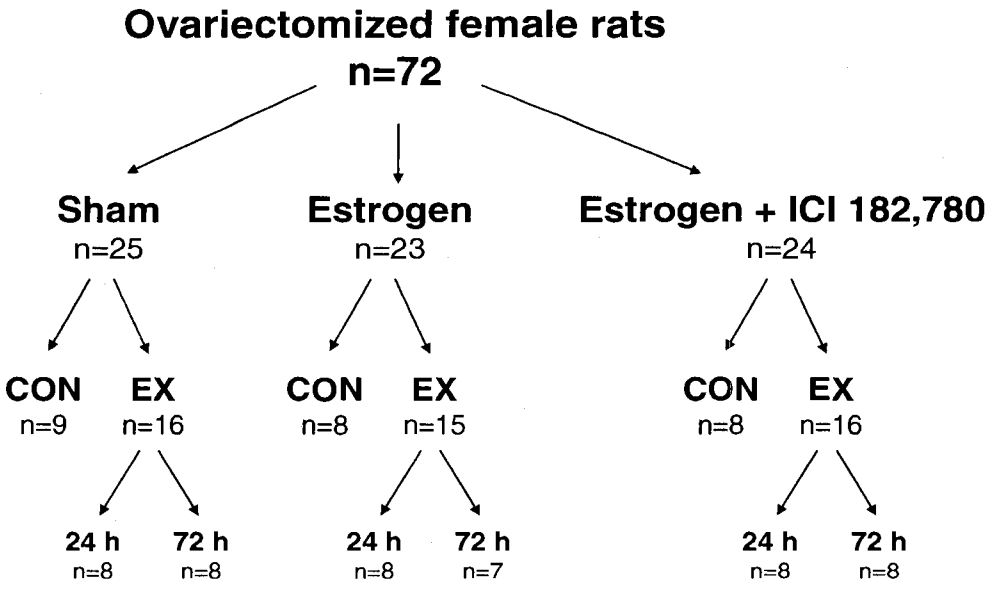

Figure 1: Schematic outline of the experimental protocol. CON = control (unexercised) condition; EX = exercised condition.

One day prior to and 6 days following estrogen pellet implantation (or sham procedure), animals were injected with either ICI 182, 780 (5 mg kg^{-1} s.c, dissolved in DMSO and diluted in corn oil, Cookson Inc., Ellisville, MO, USA) or a placebo (corn oil/DMSO) solution.

The estrogen pellet implantation and sham procedures were performed under aseptic conditions. Rats were anaesthetized via inhaled isoflurane, and a small incision was made in the skin folds of the neck. Blunt dissection was used to separate the skin from the underlying fascia. For the estrogen-supplemented animals, the estrogen pellet 0.25 mg 17β-estradiol, 21-day time release pellet (Innovative Research of America, Sarasota, FL) was inserted under the skin approximately 1 cm from the site of incision using forceps. For the sham procedure, blunt dissection and insertion of the forceps was performed similar to estrogen pellet procedure, but without a pellet insertion. The incision was sealed with Vetabond (3M St. Paul, MN). Animals were allowed to recover for 8 days prior to beginning the exercise protocol. This recovery time also allowed the pellet implanted groups to receive prolonged exposure to estrogen.

The animals being exercised were exposed to 5 minute familiarization sessions, one and two days prior to performing the exercise protocol. The animals ran on a motorized rodent treadmill with an electric shock grid (Columbus Instruments, Columbus, OH). The exercised animals ran on the treadmill at a –13.5° grade at a speed of 17 m/min. They ran for a total of 90 min using an intermittent protocol of 5 min running interspersed with 2 min of rest, as described elsewhere (79). This protocol is non-fatiguing and has been shown to elicit damage in the soleus and white vastus muscles in rodents (14; 80).

Tissue collection

All animals were sacrificed by an overdose of sodium pentobarbital (55 mg/kg i.p.). The exercised animals were sacrificed at 24 and 72 hours post-exercise. The control animals were euthanized at the same time as the exercised animals. Anesthesia was confirmed with the loss of withdrawal reflex from a toe web pinch. During sacrifice, the lower abdomen was opened and a blood sample (5 mL) was withdrawn from the descending aorta. Blood was allowed to clot, and centrifuged for 10 minutes at 3000 *g*. The serum was removed and stored at -20°C. The soleus and superficial (white) portion of the vastus muscle and uterus were removed. These muscle regions contain predominately Type I and Type IIb fibres, respectively (81). Tissue samples were thoroughly rinsed in physiological saline to remove excess blood, blotted dry and trimmed of visible connective tissue and tendons and also weighted in the case of the uteri to determine wet weight. The uteri were then dried in the oven to determine dry weight values. The fibres of the muscle were properly aligned and the muscle samples were mounted and coated in optimal cutting temperature (OCT) medium and quickly frozen in isopentane chilled to the temperature of liquid nitrogen, in preparation for immunohistochemical analysis. All tissue samples were immediately placed in liquid nitrogen, then stored at -20°C until analysis.

Serum Estrogen Assay

Serum estrogen levels were analyzed (in duplicate) using a commercially available radioimmunoassay (RIA) kit (Coat-a-Count TKE21, Inter Medico, Markham, ON).

β-Glucuronidase Activity Assay

β-glucuronidase activity was measured in the muscle samples using the method of Barrett (82) as described by Koskinen *et al.* (83). Samples were assayed (in triplicate) at 420 nm. Samples were expressed as the amount of substrate (5 mM *p*-nitrophenyl-β-D-glucuronide, Sigma-Aldrich) hydrolyzed per amount of protein and incubation time. To determine the total protein content in the samples the Lowry Protein Assay was performed (84).

Lowry Protein Assay to Determine Total Protein Content

Total protein content in muscle homogenates was measured using the Lowry Protein Assay (84). Each sample was assayed (in triplicate) at 650 nm. Measured protein values were corrected against a "blank" (water sample) and compared against a bovine serum albumin standard (Sigma-Aldrich, St. Louis, MO).

Immunohistochemistry to Determine Inflammatory Response

Neutrophil and macrophage cells in soleus and white vastus muscles were determined by sectioning the muscles onto Vectabond coated glass slides (Vector Laboratories, Burlington, ON). Transverse serial sections of soleus and white vastus muscles were cut at 7 μm using a cryostat (Leica CM3050S, Germany) at -20°C. The slides were stored at -20°C until staining. Sections were immunostained for the leukocyte markers: His48 (present primarily on neutrophils) (85) and ED1 (present primarily on macrophages) (86).

To quantify the cells, slides were placed under a light microscope (Leica DMLS) with the image projected onto a computer screen at a magnification of 400x (10x ocular

and 40x objective lens). Twenty random fields were selected and ten fibres from those fields were analyzed. This procedure was performed on two different cross sections of the same muscle, for a total of 400 fibres being analyzed from each sample. Cells on the outer edges of the cross section and those present in the blood were excluded during quantification. Figure 2 depicts representative positive cells that were used during the quantification of leukocytes.

Statistical Analysis

All data are presented as means ± SEM. Differences between groups were measured using a 2 (exercise) by 3 (conditions) factorial ANOVA at a level of significance of $P < 0.05$. Where significance was obtained, post-hoc multiple comparison testing was done using a Bonferroni test to determine where specific differences had occurred.

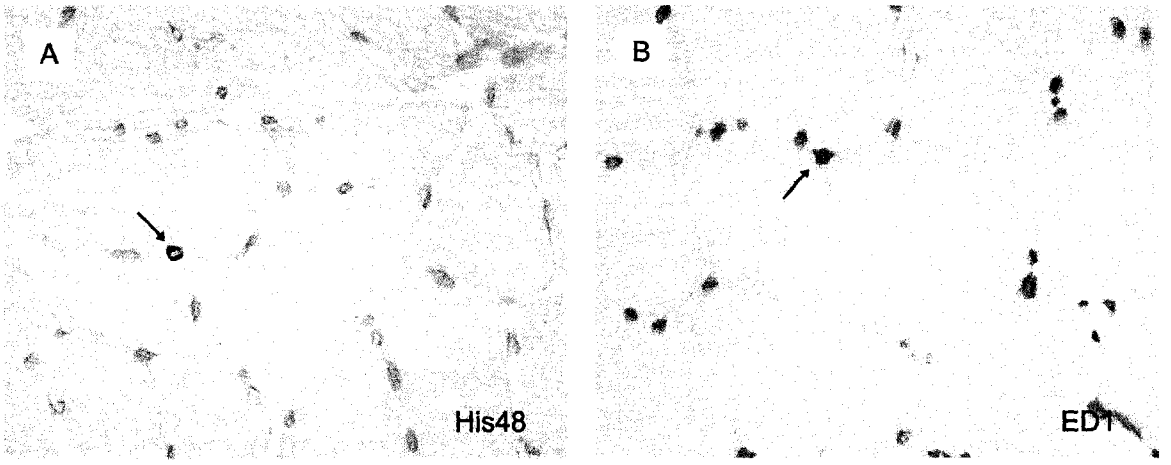

Figure 2: Representative images showing sections (7 μm) of rat skeletal muscles immunostained for (A) neutrophils (His48) and (B) macrophages (ED1) (400x magnification).

Results

Serum Estrogen Levels

Since exercise and time of sacrifice had no effect on serum estrogen levels, animals were pooled into three groups according to their supplementation status: sham, estrogen-supplemented and estrogen-supplemented plus blocker (ICI 182,780) groups. As depicted in Table 1, estrogen administered rats had significantly higher ($P < 0.05$) serum estrogen levels relative to sham (unsupplemented) rats. Furthermore, serum estrogen levels of estrogen-supplemented plus blocker (IC 182, 780) animals were significantly greater ($P < 0.05$) than both sham animals and those that received estrogen alone. There was no significant difference ($P > 0.05$) between estrogen-supplemented animals and those receiving both estrogen supplementation plus blocker (ICI 182,780).

Body Weights

All groups continued to gain weight throughout the experimental period. However, sham (unsupplemented) animals were significantly heavier than estrogen only treated animals and estrogen plus ER antagonist supplemented animals at the time of sacrifice ($P < 0.05$), as depicted in Table 2. There was no significant weight difference between estrogen supplemented and estrogen plus ER antagonist supplemented groups ($P > 0.05$) at sacrifice. These data are in line with previous work demonstrating that estrogen administration to estrogen naïve animals results in reduced body weight gain and lower body mass (52; 57) than sham (unsupplemented) animals. Age matched animals were chosen for the present study because weight matching between the groups would have involved using estrogen treated and estrogen plus ER antagonist supplemented animals of

higher age than sham animals. Since the present study was interested in using adult female rats, the experimental design avoids differences in age and age-related differences in hormonal status as confounding factors.

Uterus Weights

Estrogen binding to uterine estrogen receptors stimulates uterine growth; whereas binding of a receptor antagonist, such as ICI 182,780 to ERs will inhibit uterine growth (87). As depicted in Table 2, estrogen supplementation resulted in significantly greater ($P < 0.05$) uterus wet and dry weights compared to sham (unsupplemented) animals. Administration of blocker also resulted in significantly higher wet weight and dry weight relative to the sham animals ($P < 0.05$). Additionally, the blocker group had a 22% lower uterus wet weight and a 38% lower uterus dry weight compared to estrogen administration alone ($P < 0.05$). When the weights were normalized to overall body weights of the rats, these same trends were observed.

β-Glucuronidase Activity

To determine whether the exercise protocol resulted in lower exercised induced muscle damage in estrogen supplemented animals relative to estrogen naïve animals, measurements of the activity of the lysosomal acid hydrolase, β-glucuronidase were assessed. Indeed, estrogen supplemented animals displayed significantly less ($P < 0.05$) β-glucuronidase activity 72 hours post-exercise than ovariectomized females in the soleus and white vastus muscles, as depicted in Table 3. In ovariectomized animals, administration of estrogen plus ICI 182, 780 significantly attenuated ($P < 0.05$) β-

glucuronidase activity 72 hours post-exercise in soleus and white vastus muscles, relative to sham (unsupplemented) animals; similar to the effects of estrogen only supplementation.

Leukocytes Infiltration

Leukocyte infiltration was measured by immunostaining for the neutrophil marker, His48, and the macrophage marker, ED1. A main effect with exercise was observed in neutrophils and macrophages, with control (unexercised) animals demonstrating significantly lower ($P<0.05$) leukocyte content compared to animals 24 hours after exercise in both muscle types. Increased neutrophil infiltration was observed 24 hours after exercise (Figures 3 and 4). A significant increase was observed in the soleus and white vastus muscle post-exercise ($P < 0.05$). In addition, macrophage infiltration was significantly increased ($P < 0.05$) post-exercise in both muscle types (Figures 5 and 6). Post-exercise, leukocyte infiltration was significantly reduced ($P < 0.05$) in estrogen supplemented animals compared to sham animals. Estrogen supplementation attenuated neutrophil infiltration by 33% in the white vastus muscle. Administration of estrogen reduced macrophage infiltration by 37 and 36% in the soleus and white vastus, respectively. The estrogen receptor antagonist, ICI 182, 780 did not significantly alter the attenuating affect of estrogen on neutrophil and macrophage infiltration in both muscle types.

Table 1: Animal body weights and serum estradiol levels for sham, estrogen and estrogen + ICI 182, 780 treatment groups. Values are pooled means + SEM. [a] $P < 0.05$ compared to sham treated. [b] $P < 0.05$ compared to estrogen-supplemented.

Treatment	Body Weight (g)	Serum Estrodiol (pg ml^{-1})
Sham	294 ± 4	12.5 ± 1
Estrogen	260 ± 3^{a}	79.3 ± 15^{a}
Estrogen + ICI 182, 780	260 ± 3^{a}	141.6 ± 28.6^{ab}

Table 2: Changes of uterus weights with estrogen status. Values are pooled means ± SEM. [a] $P < 0.05$ compared to sham treated. [b] $P < 0.05$ compared to estrogen-supplemented.

	Sham	**Estrogen**	**Estrogen + ICI 182,780**
Wet weight, g	0.098 ± 0.005	0.396 ± 0.021[a]	0.313 ± 0.016[a,b]
Wet weight, g per 100 g body weight	0.033 ± 0.002	0.154 ± 0.009[a]	0.120 ± 0.006[a,b]
Dry weight, g	0.027 ± 0.003	0.098 ± 0.009[a]	0.061 ± 0.003[a,b]
Dry weight, g per 100 g body weight	0.009 ± 0.001	0.038 ± 0.004[a]	0.024 ± 0.001[a,b]

Table 3: Changes in muscle β-Glucuronidase activity with downhill running. Values are means ± SEM. [a] $P < 0.05$ compared to unexercised controls. [b] $P < 0.05$ compared to sham treated.

		Sham	**Estrogen**	**Estrogen + ICI 182,780**
Soleus	Control	5.26 ± 0.27	4.55 ± 0.20	4.46 ± 0.29
	Exercise + 24 h	6.49 ± 0.40[a]	4.77 ± 0.33	4.94 ± 0.48
	Exercise + 72 h	8.04 ± 0.88[a]	5.11 ± 0.41[b]	4.97 ± 0.38[b]
White Vastus	Control	4.71 ± 0.39	4.04 ± 0.21	3.72 ± 0.40
	Exercise + 24 h	5.86 ± 0.45[a]	3.90 ± 0.20[b]	3.62 ± 0.28[b]
	Exercise + 72 h	6.19 ± 0.57[a]	3.83 ± 0.12[b]	3.54 ± 0.33[b]

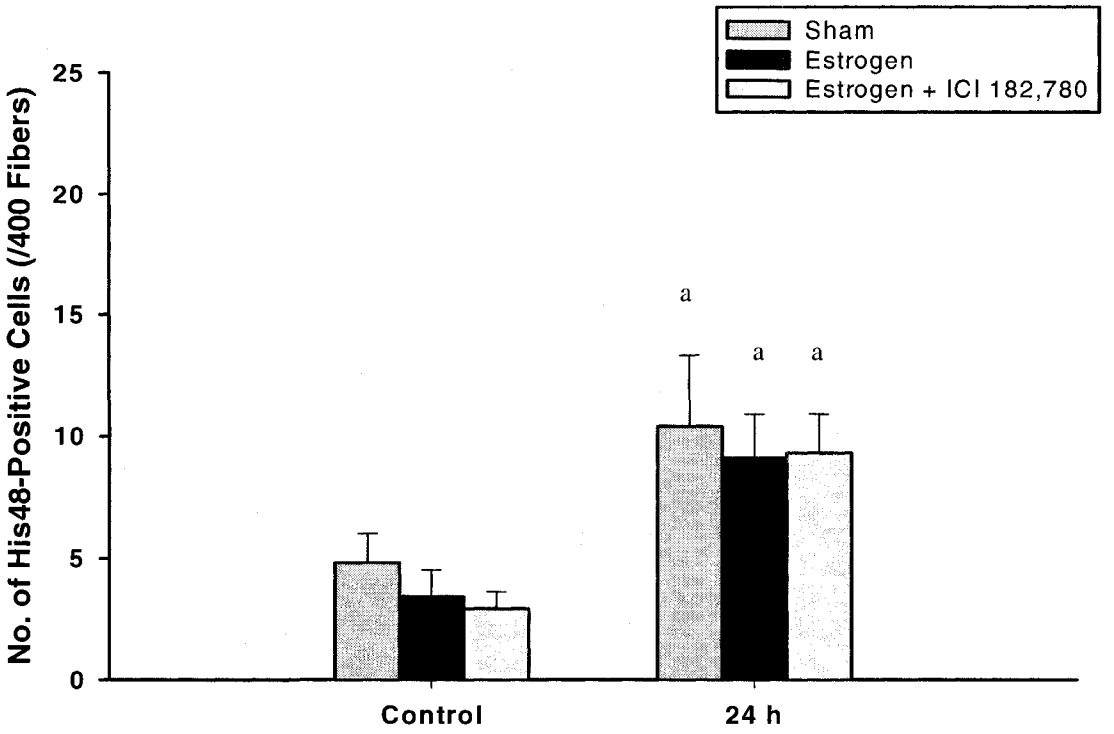

Figure 3: Effects of estrogen supplementation and ICI 182,780 administration on numbers of His48+ neutrophils in rat soleus (red) muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated

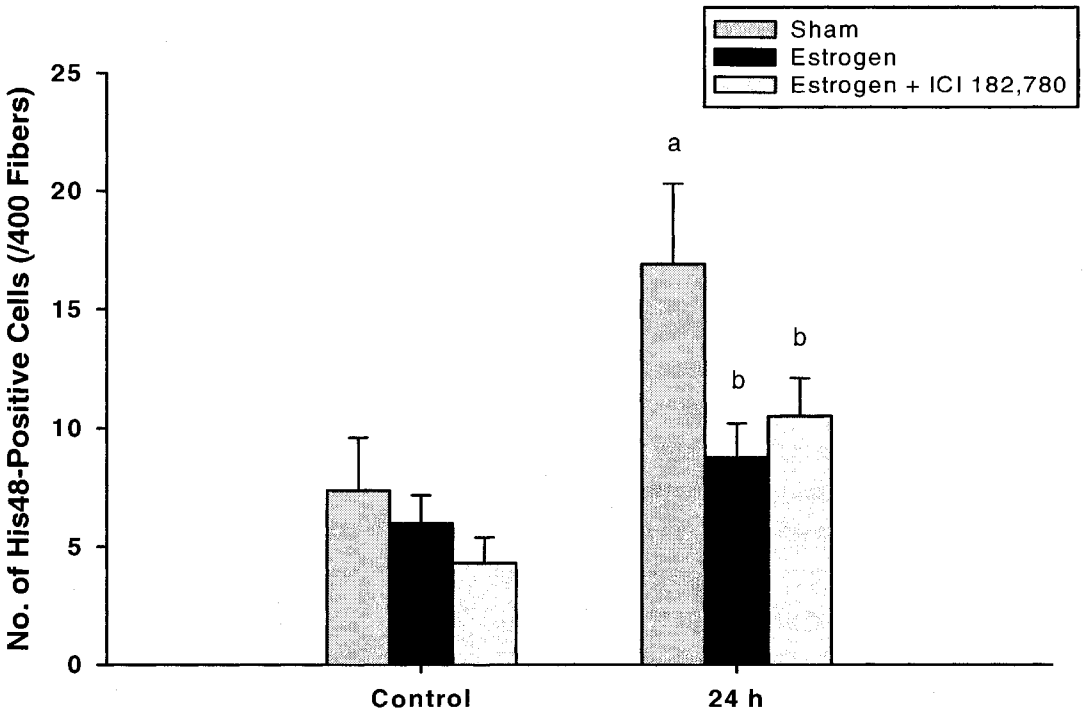

Figure 4: Effects of estrogen supplementation and ICI 182,780 administration on numbers of His48+ neutrophils in rat white vastus muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated

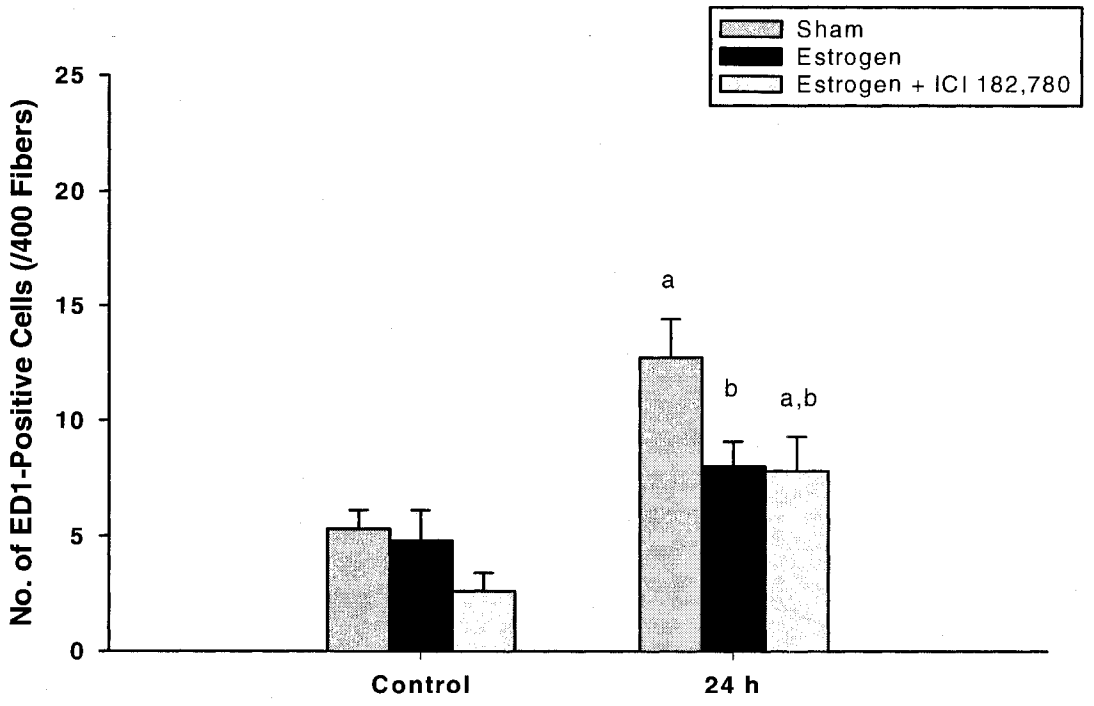

Figure 5: Effects of estrogen supplementation and ICI 182,780 administration on numbers of ED1+ macrophages in rat soleus (red) muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated.

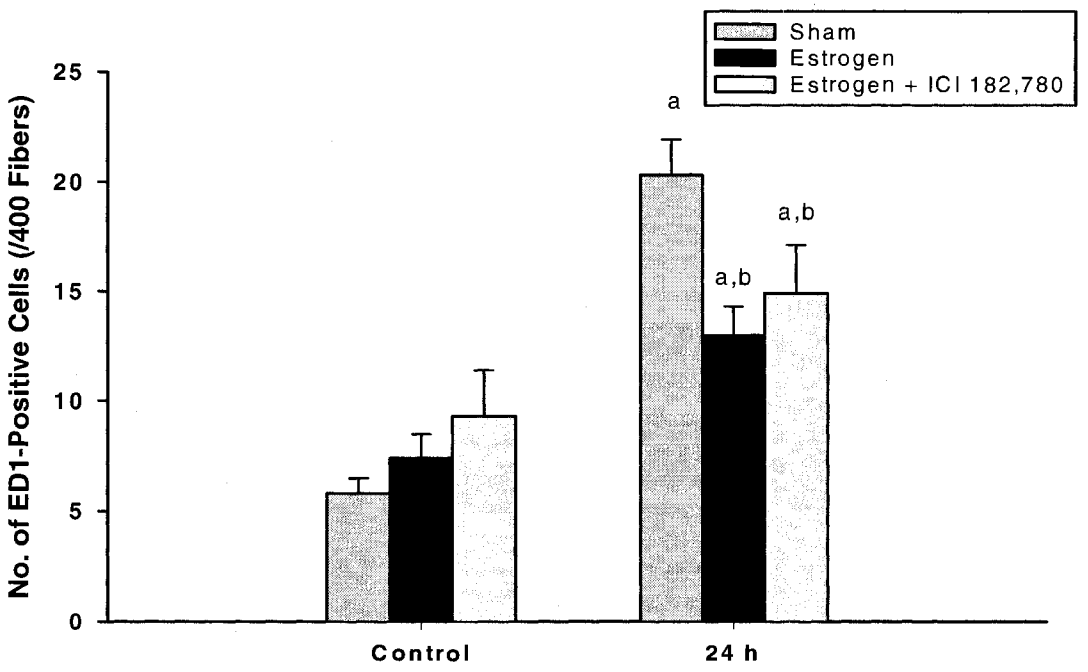

Figure 6: Effects of estrogen supplementation and ICI 182,780 administration on numbers of ED1+ macrophages in rat white vastus muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated.

Discussion

The primary goal of this study was to gain further insight into the mechanism by which estrogen exerts its effects. It has been well established that estrogen reduces post-exercise skeletal muscle damage. However, what is unknown is the mechanism behind estrogen's effects. We took the approach of examining the influence of estrogen receptors on estrogen effects on muscle leukocyte inflammation. We blocked the estrogen receptors with ICI 182, 780 to see if this altered estrogen's action on these parameters.

Previously, tamoxifen has been used as an estrogen receptor blocker, however the results from these experiments should be interpreted with caution due to tamoxifen's dual properties. Not only does tamoxifen have estrogen antagonistic properties but it also possesses agonistic characteristics (88). The use of a pure estrogen receptor blocker, such as ICI 182, 780 provides more precise answers. This pure ER blocker binds with an extremely high affinity and specificity to both ER α and ß and possesses no estrogen agonistic properties (78). The relative binding affinity of ICI 182, 780 to ERs is 0.89, whereas the affinity of estrogen for its receptors is 1.0 (78). To verify the ovariectomy was performed correctly and to quantify the biological response to treatment with estrogen and ICI 182, 780, the uteri were weighed. ICI 182, 780 effectively blocked ERs, as depicted by the significantly lower uterus wet and dry weights relative to the estrogen only supplemented group.

As would be expected, estrogen only supplemented animals and those receiving estrogen plus the blocker had significantly higher serum estradiol levels compared to the unsupplemented ovariectomized animals. These levels are considered to be on the upper

end of the physiological range and consistent with 17β-estradiol replacement values found in other laboratories and our own (52; 89). The range of circulating estradiol levels is quite large and variable. Age and stage of estrus cycle contribute to this variability. Reported estradiol levels range as high as 285 pg ml^{-1} (90) to values such as 15 pg ml^{-1} (91).

A major finding of the present study was the estrogen specific inflammatory response; with estrogen supplementation animals demonstrated an attenuation of leukocyte infiltration in skeletal muscle post-exercise compared to sham ovariectomized animals. Furthermore, this differential response also appears to be affecting the degree of muscle damage, as depicted by the lower β-glucuronidase activity in estrogen supplemented rats compared to sham ovariectomized rats. Moreover, the results suggest that the mechanism by which estrogen is influencing this response is through a non-estrogen receptor mediated process. Although the present study does not directly assess the involvement of the estrogen receptor as the mechanism behind estrogen's effects, if a casual relationship exists, the administration of the ER antagonist, ICI 182, 780 should block estrogen's effect. However, ICI 182, 780 did not alter the ability of estrogen to protect against skeletal muscle damage. There was no significant difference in β-glucuronidase activity between the animals that received estrogen supplementation alone and those that received estrogen plus the ER antagonist. Furthermore, treatment of the ovariectomized estrogen supplemented rats with ICI 182, 780 did not alter the leukocyte inflammatory response to exercise relative to those treated with estrogen alone, indicating that the hormonal mechanism is not primarily receptor mediated.

Alternatively, the results suggest a possible enhanced muscle sarcolemma stability with estrogen supplementation as the mechanism by which estrogen may influence leukocyte infiltration and damage. Duan *et al.* found that rat skeletal muscle had elevated mitochondrial Ca^{2+} levels following injury from downhill walking (10). High levels of Ca^{2+} have the potential to activate various molecular pathways in skeletal muscle, such as the calpain proteolytic pathways and the phospholipase A_2 pathway. Calpains are thought to play a role in muscle damage by being responsible for the initial breakdown of myofibrils and cytoskeletal proteins (eg. desmin, α-actinin) (11). Activation of phospholipase A_2 may contribute to the role of muscle damage by further damaging the sarcolemma and thus increasing the loss of intracellular molecules. To further support the role that elevated Ca^{2+} plays in the injury process, it has been observed that when muscle fibres are treated with chelators (EDTA and EGTA) there is a reduced accumulation of Ca^{2+} in the mitochondria, that is accompanied by an attenuation in muscle fibre injury (10).

Another possible mechanism for estrogen's effects may be through the induction of heat shock proteins. Exercise induced muscle damage results in an increase in intracellular denatured proteins, signaling the induction of the HSP response to mediate the proper folding of denatured proteins (68). A relationship between the induction of the HSP response and a reduction in damage has been demonstrated in cardiac muscle. The induction of HSPs resulted in cardioprotection and delayed the onset of myocardial injury caused by ischemia (69). There is an intrinsic gender HSP response in rats, with males displaying a greater induction of HSP70 in skeletal muscle than females post-exercise (70). Additionally, it has been shown that male rats supplemented with estrogen

resulted in a HSP response similar to those of females following exercise (71). Therefore, it is speculated that estrogen may exert its effect through one of the non-estrogen receptor mediated mechanisms, such as HSP induction or estrogenic stabilization of the sarcolemma.

In summary, estrogen administration affected post-exercise leukocyte inflammation. Estrogen administration attenuated post-exercise neutrophil and macrophage content compared to estrogen naïve animals. Combined estrogen plus ER antagonist administration did not significantly alter the attenuation observed by estrogen only administration. Hence, estrogen receptors may not be the primary mechanism by which estrogen affects leukocyte inflammation.

Chapter Three

The Effects of Progesterone on Estrogen Influence on the Indices of Skeletal Muscle Inflammation

Statement of Problem

The purpose of this study was to examine the specific and interaction effects of estrogen and progesterone on skeletal muscle in response to exercise induced damage.

Subsidiary Problem

A secondary objective was to examine differences in leukocyte infiltration in skeletal muscle of different fibre types.

Hypotheses

1. It is hypothesized that leukocyte infiltration will be attenuated with estrogen supplementation.
2. It is believed that the addition of progesterone and estrogen in combination will negate the attenuation of leukocyte infiltration by estrogen supplementation alone.
3. It is hypothesized that progesterone administration alone will have no independent effect on leukocyte infiltration.
4. Based on previous studies it is thought that both fast and slow muscle types will display similar trends in leukocyte infiltration.

Introduction

Muscle damage resulting from eccentric exercise can elicit leukocyte infiltration at the site of injury. Neutrophils invade immediately following exercise and assist in the degradation of damaged debris with their phagocytic and oxidative abilities (19; 92). Macrophages infiltrate muscle approximately 12-24 hours post-exercise (19). They are responsible for the removal of injured muscle debris, secretion of inflammation regulatory cytokines and satellite cell activation (19; 20; 33).

Estrogen can influence the degree of exercise-induced muscle injury. Previous work from our laboratory has demonstrated attenuating effects of estrogen on post-exercise leukocyte infiltration (52) and enhanced muscle satellite cell activation (57). It has been demonstrated that female ovariectomized rats that received estrogen administration demonstrated an attenuation in muscle damage (CK activity), leukocyte activity, neutrophil infiltration and calpain-like activities as compared to unsupplemented animals post-exercise (52).

There has been limited work on the mechanism of progesterone's effect, but what is known is that there is a similar distribution of estrogen and progesterone receptors in most of the same cell types and tissues which may suggest that estrogen is involved in the progesterone receptor induction (72). In the uterus, progesterone is mediated by its receptors and estrogen increases progesterone receptor levels (73). Progesterone receptors are distributed in numerous tissues. In rats they have been found to exist in the reproductive glands, certain regions of the oral mucosa and developing teeth, esophagus, larynx, skin, mammary gland, kidney, and skeletal muscle (72).

The interaction effects of progesterone and estrogen are limited, but it has been demonstrated that the combined supplementation influences skeletal muscle fatigue (74). In the study by St. Pierre Schneider et al (74), it was found that the time to fatigue was greatest in ovariectomized mice treated with progesterone alone, even more so than ovariectomized mice treated with placebo or a combination of estradiol and progesterone. Furthermore, it was found that the ovariectomized mice treated with progesterone alone had the greatest percent of isometric torque remaining immediately after eccentric contractions, as compared to the ovariectomized mice treated with placebo, estradiol, or a combination of estradiol and progesterone (74).

A related study by Xing et al. (75), used the rat model to demonstrate the effects that hormones had on leukocyte content in response to endothelium vascular injury. They demonstrated that estrogen attenuated leukocyte populations and migration into injured tissue. The addition of progesterone and estrogen in combination negated the protective effect observed by estrogen alone. Additionally, progesterone administration alone had no independent effect of leukocyte infiltration following damage (75).

In this study, we examined the specific and interaction effects of estrogen and progesterone on skeletal muscle in response to injury. We quantified the effects of progesterone and estrogen supplementation on leukocyte (neutrophil and macrophage) infiltration in red and white fibre types of rat skeletal muscle following downhill running. The neutrophil marker His48 and the macrophage marker ED1 were quantified 24 hr post-exercise. In addition to measuring leukocyte infiltration, muscle damage was indirectly assessed by measuring β-glucuronidase activity.

Methods

Animals

The study was approved by the Animal Care Committee at Wilfrid Laurier University and all procedures were performed in accordance with the Canada Council on Animal Care. A total of 63 ovariectomized Sprague-Dawley rats (Charles River Laboratories, LaSalle, QC) were be used. One animal was removed from the study due to illness. The animals had their ovaries removed at 9 weeks and be delivered at 10 weeks old. Animals were housed two per cage in an environmentally controlled environment with a standard 12:12 light/dark cycle and allowed access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water *ad libitum.*

Experimental Design

Following one week of acclimation in the laboratory, animals were being randomly subdivided into four groups: estrogen supplemented group, progesterone supplemented group, progesterone plus estrogen supplemented group or into the group receiving a sham procedure. The groups were further subdivided into exercise or control (unexercised) groups.

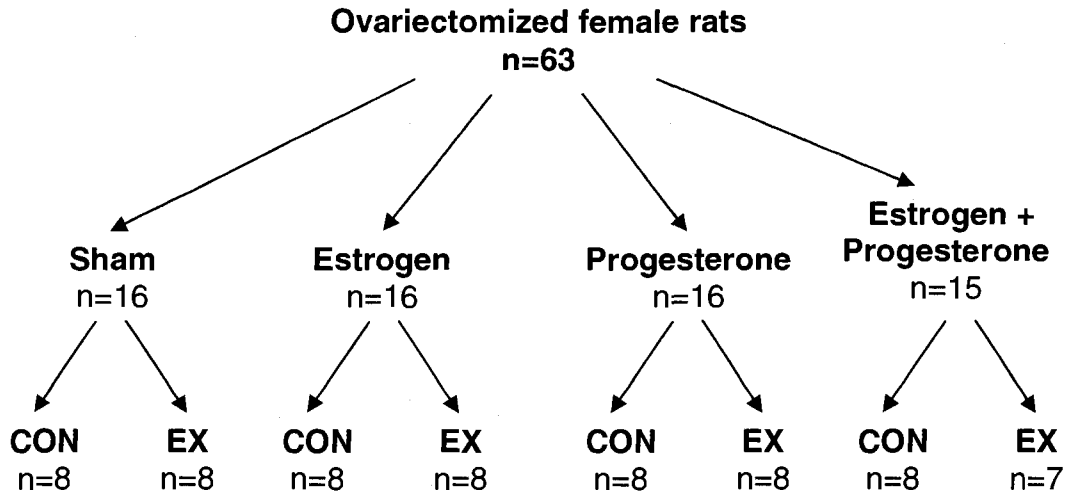

Figure 7: Schematic outline of the experimental protocol. CON = control (unexercised) condition; EX = exercised condition.

Pellet implantation and sham procedures were performed under aseptic conditions. Rats were anaesthetized via inhaled isoflurane, and a small incision was made in the skin folds of the neck. Blunt dissection was used to separate the skin from the underlying fascia. For supplemented animals, the estrogen pellet (0.25 mg 17β-estradiol, 21-day time release pellet, Innovative Research of America, Sarasota, FL) and/or the progesterone pellet (25 mg progesterone, 21-day time release pellet, Innovative Research of America, Sarasota, FL) were inserted under the skin approximately 1-2 cm from the site of incision using forceps. For the sham procedure, blunt dissection and insertion of the forceps was performed similar to pellet procedure, but without a pellet insertion. The incision was sealed with Vetabond (3M St. Paul, MN) and skin staples (APPOSE ULC, USS DG, USA). Animals were allowed to recover for 7 days prior to beginning the exercise protocol. This recovery time also allowed the pellet implanted groups to receive prolonged exposure to hormones.

The animals being exercised were exposed to 5 minute familiarization sessions, one and two days prior to performing the exercise protocol. The animals ran on a motorized rodent treadmill with an electric shock grid (Columbus Instruments, Columbus, OH). The exercised animals were run on a motorized rodent treadmill downhill at a −13.5° grade at a speed of 17 m/min. They ran for a total of 90 min using an intermittent protocol of 5 min running interspersed with 2 min of rest, as described elsewhere (79). This protocol is non-fatiguing and has been shown to elicit damage in the soleus and white vastus muscles in rodents (14; 80).

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Serum Estrogen Assay

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Statistical Analysis

All data are presented as means ± SEM. Differences between groups were measured using a 2 (exercise) by 3 (conditions) factorial ANOVA at a level of significance of $P < 0.05$. Where significance was obtained, post-hoc multiple comparison testing was done using a Bonferroni test to determine where specific differences had occurred.

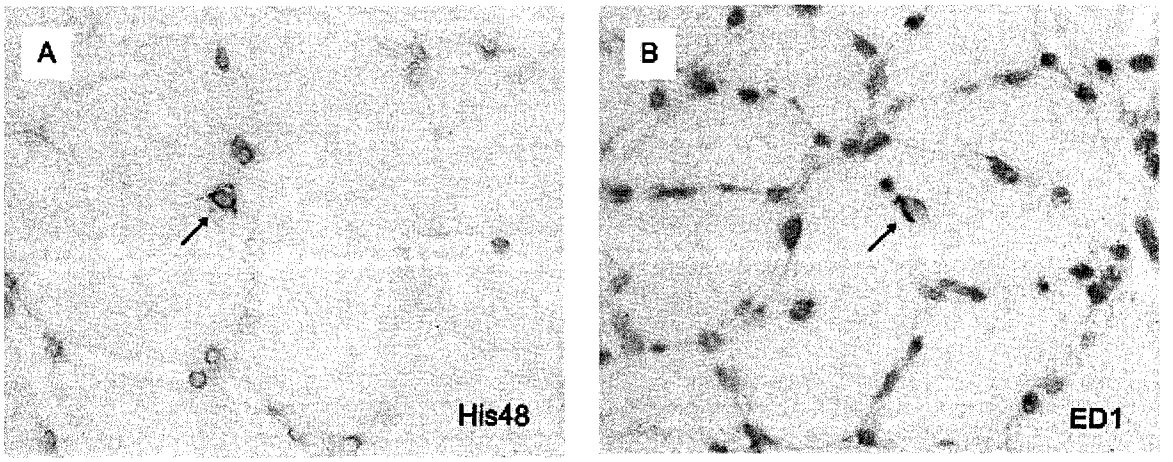

Figure 8: Representative images showing sections (10 μm) of rat skeletal muscles immunostained for (A) neutrophils (His48) and (B) macrophages (ED1) (400x magnification).

Results

Serum Estrogen Levels

Since exercise had no effect on serum estrogen levels, animals were pooled into four groups according to their supplementation status: sham, progesterone-supplemented, estrogen-supplemented and progesterone plus estrogen-supplemented groups. As depicted in Table 4, estrogen administered rats had significantly higher ($P < 0.05$) serum estrogen levels relative to sham (unsupplemented) and progesterone treated rats. Additionally, serum estrogen levels of progesterone plus estrogen-supplemented animals were significantly greater ($P < 0.05$) than both sham animals and those that received only progesterone treatment. There was no significant difference ($P > 0.05$) between estrogen-supplemented animals and those receiving both progesterone plus estrogen supplementation.

Serum progesterone levels

Since exercise had no effect on serum progesterone levels, animals were pooled into four groups: sham, progesterone-supplemented, estrogen-supplemented and progesterone plus estrogen-supplemented groups. As seen in Table 4, progesterone-supplemented animals had greater serum progesterone concentrations than sham (unsupplemented) rats ($P < 0.05$). Additionally, progesterone plus estrogen-supplemented animals had significantly greater serum progesterone levels relative to estrogen-supplemented and sham (unsupplemented) rats ($P > 0.05$). There was no significant difference ($P > 0.05$) between progesterone only supplemented animals and those receiving both progesterone plus estrogen supplementation.

Body Weights

All groups continued to gain weight throughout the experimental period. However, sham (unsupplemented) animals were significantly heavier than estrogen only treated animals and estrogen plus progesterone treated animals at the time of sacrifice ($P < 0.05$), as depicted in Table 4. There was no significant weight difference between progesterone supplemented animals and sham (unsupplemented) animals ($P > 0.05$) at sacrifice. These data are in line with previous work demonstrating that estrogen administration to estrogen naïve animals results in reduced body weight gain and lower body mass than sham (unsupplemented) animals (52; 57) and progesterone only supplemented animals (93). Age matched animals were chosen for the present study because weight matching between the groups would have involved using estrogen treated animals of higher age than sham and progesterone only treated animals. Since the present study was interested in using adult female rats, the experimental design avoids differences in age and age-related differences in hormonal status as confounding factors.

β-Glucuronidase Activity

To determine whether the exercise protocol resulted in lower exercised induced muscle damage in estrogen and/or progesterone supplemented animals relative to unsupplemented animals, measurements of the activity of the lysosomal acid hydrolase, β-glucuronidase were assessed. However, this assay detects muscle damage 72 hours post-exercise, as demonstrated by previous studies performed by our laboratory (57); as such, the present study did not display significantly differential responses in β-glucuronidase activity between groups 24 hours post-exercise ($P > 0.05$) .

Leukocyte Infiltration

Leukocyte infiltration was measured by immunostaining for the neutrophil marker, His48, and the macrophage marker, ED1. A main effect with exercise was observed in neutrophils and macrophages, with control (unexercised) animals demonstrating significantly lower ($P < 0.05$) leukocyte content compared to animals 24 hours after exercise in both muscle types. Also, a main effect with hormone administration was observed in neutrophils and macrophages, for both the soleus and white vastus muscles; the sham animals had significantly greater ($P < 0.05$) content than the estrogen treated and estrogen plus progesterone treated animals. Also, the progesterone only treated animals had significantly greater ($P < 0.05$) neutrophil and macrophage content than the estrogen treated and estrogen plus progesterone treated animals.

Significantly increased ($P < 0.05$) neutrophil infiltration was observed 24 hours post-exercise in both the soleus and white vastus muscles relative to control (unexercised) animals (Figures 9 and 10). Progesterone supplemented animals displayed significantly decreased ($P < 0.05$) neutrophil infiltration following exercise compared to control animals, in both muscle types. Estrogen supplementation significantly attenuated ($P < 0.05$) neutrophil infiltration compared to sham animals by 74 and 67% in the soleus and white vastus, respectively. The combination of progesterone and estrogen supplementation did not significantly alter the attenuating affect of estrogen in both muscle types.

The number of macrophage cells detected in sections of the soleus and white vastus muscles are depicted in Figure 11 and 12, respectively. There was a significant

increase ($P < 0.05$) in muscle macrophage infiltration detected between control (unexercised) and 24 hours post-exercise sham animals, for both muscle types. Post-exercise, macrophage infiltration was significantly reduced ($P < 0.05$) in progesterone supplemented animals compared to sham animals, in both muscle types. Additionally, exercised animals that received estrogen supplementation significantly attenuated ($P < 0.05$) macrophage infiltration in both the soleus and white vastus muscles. Estrogen and progesterone combined supplementation did not significantly alter the attenuation observed by estrogen alone.

Table 4: Animal body weights, serum estradiol and progesterone levels for various treatment groups. Values are pooled means + SEM. [a] $P < 0.05$ compared to sham (unsupplemented). [b] $P < 0.05$ compared to progesterone-supplemented. [c] $P < 0.05$ compared to estrogen-supplemented.

Treatment	Body Weight (g)	Serum Estrodiol (pg ml^{-1})	Serum Progesterone (ng ml^{-1})
Sham	290 ± 5	18.9 ± 2.2	1.07 ± 0.2
Progesterone	293 ± 5	16.4 ± 1.5	3.5 ± 1.39^{a}
Estrogen	262 ± 4^{ab}	153.0 ± 35.0^{ab}	1.41 ± 0.35
Progesterone + Estrogen	263 ± 5^{ab}	101.2 ± 15.6^{ab}	4.67 ± 0.73^{ac}

Table 5: Changes in Muscle β-glucuronidase Activity With Downhill Running. Values are means + SEM. [a] $P < 0.05$ compared to progesterone-supplemented animals. [b] $P < 0.05$ compared to estrogen-supplemented animals.

		Sham	Progesterone	Estrogen	Progesterone + Estrogen
Soleus	Control	5.32 ± 0.42	6.42 ± 0.44	4.21 ± 0.63[a]	3.68 ± 0.69[a]
	Exercise + 24 h	5.70 ± 0.54	5.09 ± 1.12	4.02 ± 0.64	3.84 ± 0.90
White Vastus	Control	2.92 ± 0.31	2.66 ± 0.25[b]	4.39 ± 0.53	3.70 ± 0.40
	Exercise + 24 h	3.23 ± 0.45	4.20 ± 0.47	3.29 ± 0.27	3.33 ± 0.55

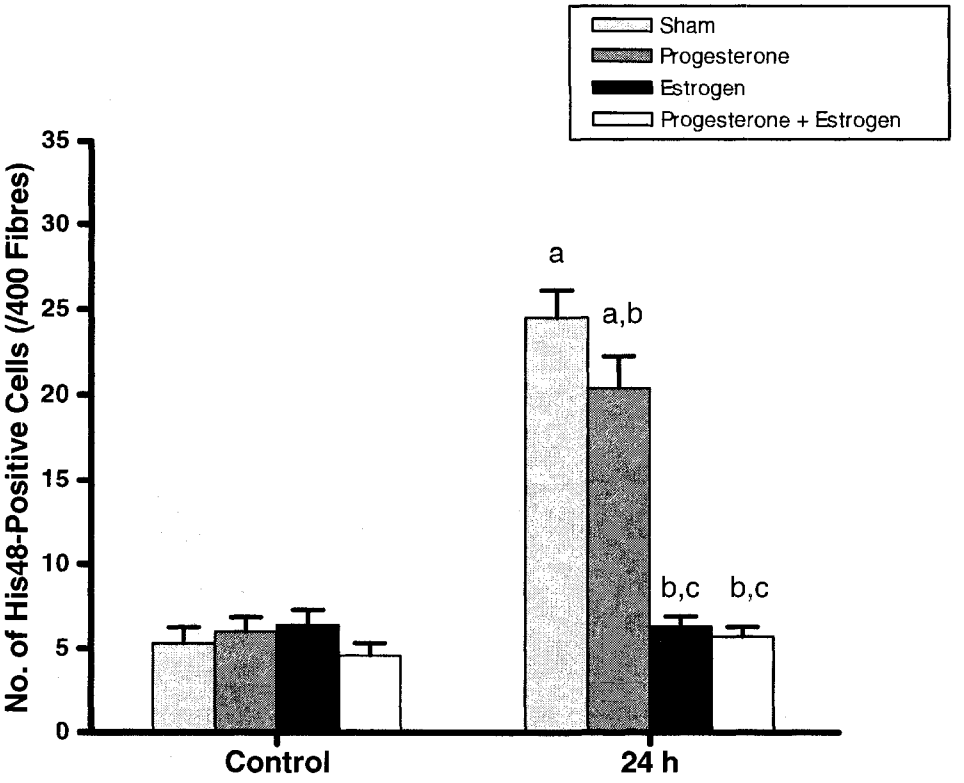

Figure 9: Effects of estrogen and progesterone supplementation on numbers of His48+ neutrophils in rat soleus (red) muscle 24 h following downhill running. Values are means + SEM . [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated.). [c] $P < 0.05$ compared to Progesterone treated.

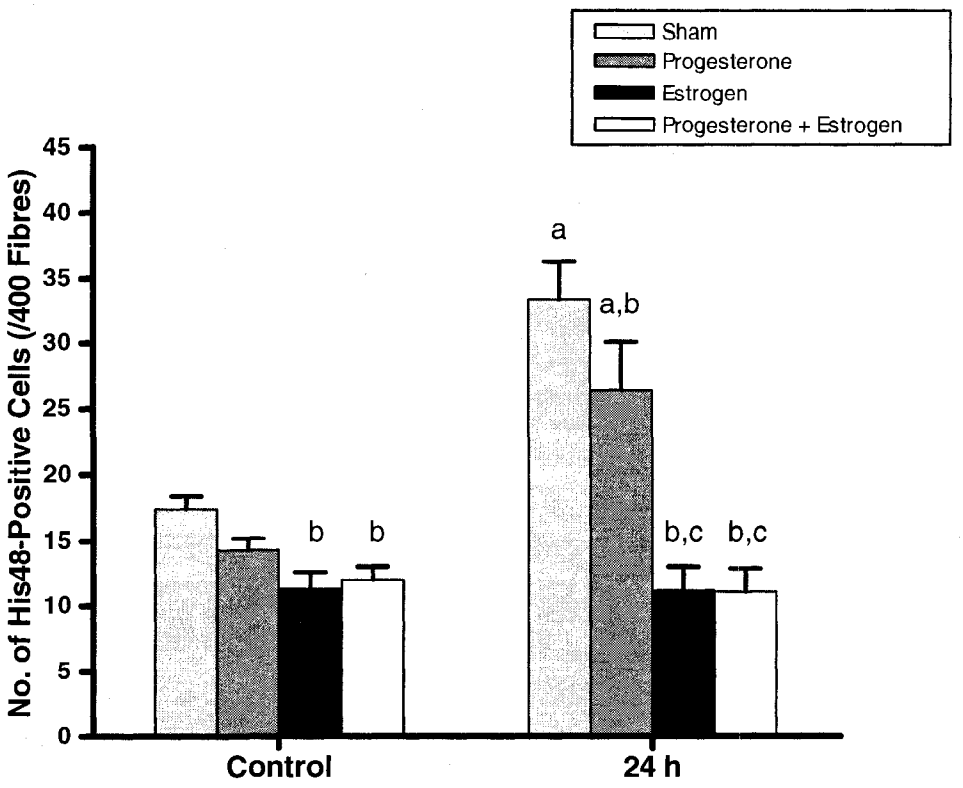

Figure 10: Effects of estrogen and progesterone supplementation on numbers of His48+ neutrophils in rat white vastus muscle 24 h following downhill running. Values are means + SEM . [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated. [c] $P < 0.05$ compared to Progesterone treated.

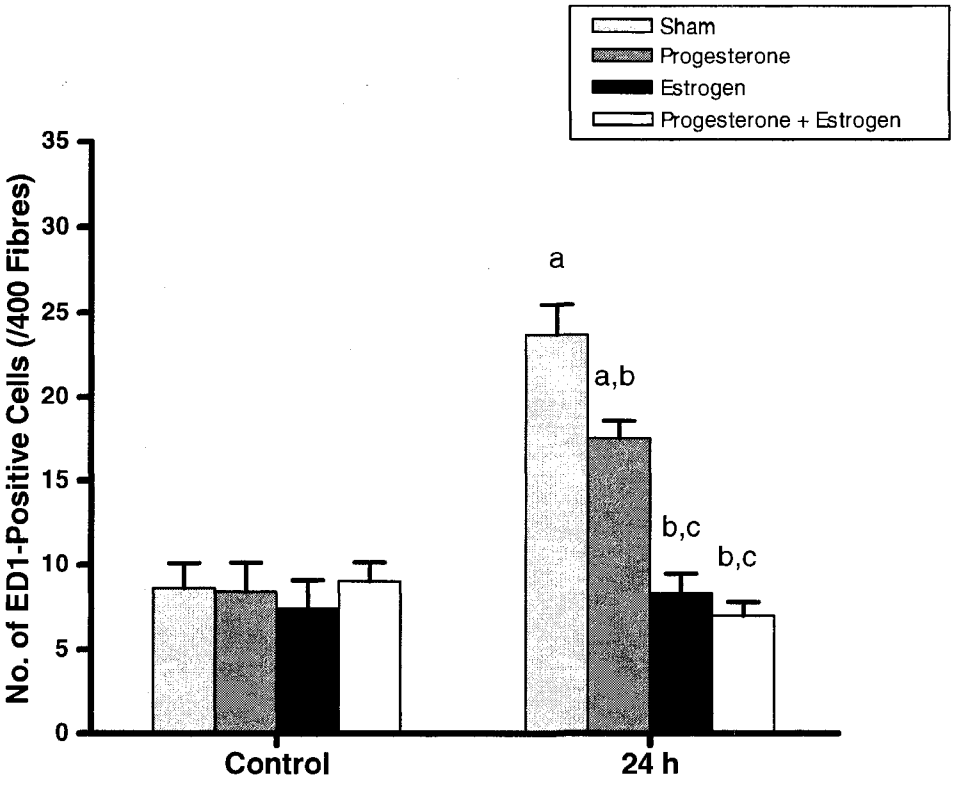

Figure 11: Effects of estrogen and progesterone supplementation on numbers of ED1+ macrophages in rat soleus (red) muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated. [c] $P < 0.05$ compared to Progesterone treated.

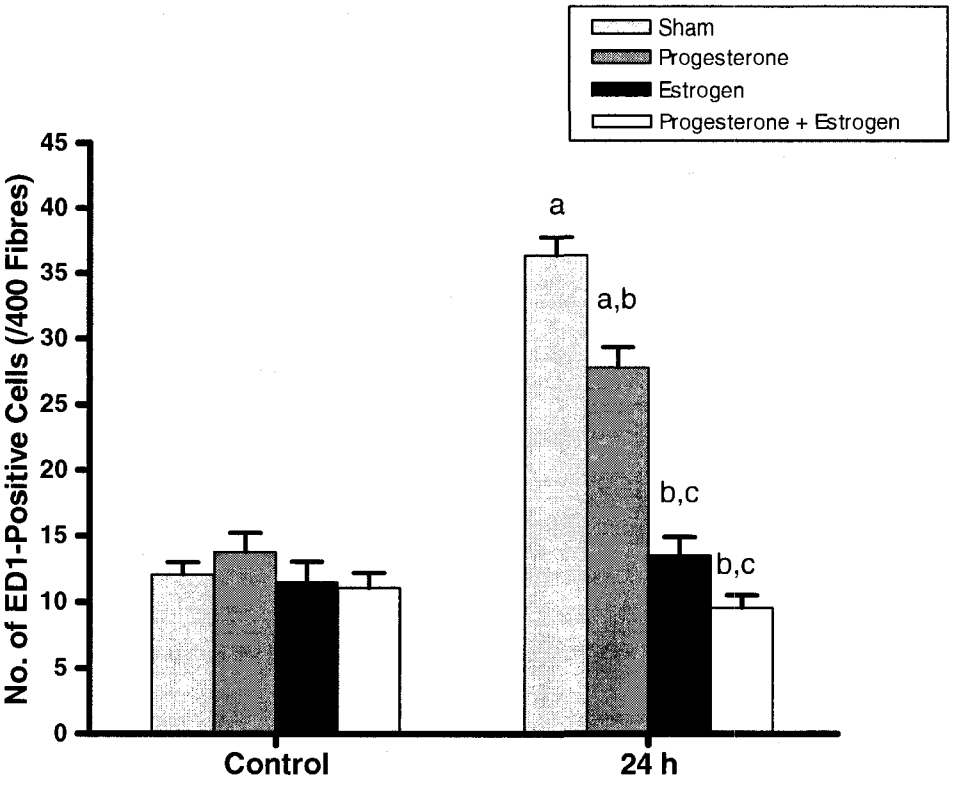

Figure 12: Effects of estrogen and progesterone supplementation on numbers of ED1+ macrophages in rat white vastus muscle 24 h following downhill running. Values are means + SEM. [a] $P < 0.05$ compared to Control (unexercised). [b] $P < 0.05$ compared to Sham treated. [c] $P < 0.05$ compared to Progesterone treated.

Discussion

We induced hormonal manipulation in the form of ovariectomized females to examine the specific and interaction effects of estrogen and progesterone on skeletal muscle in response to injury. It has been well established that estrogen reduces post-exercise skeletal muscle damage. However, the influence of progesterone plus estrogen in combination on skeletal muscle damage has not been extensively studied.

The major new findings of this study were that a) following exercise the percentage of leukocyte infiltration decreased significantly with estrogen supplementation in both muscle types, 67-69% and 63-74% in neutrophils and macrophages, respectfully; b) the combination of estrogen and progesterone supplementation did not significantly alter the attenuation observed by estrogen alone; c) leukocyte infiltration post-exercise in the soleus (red) and white vastus muscles were decreased in ovariectomized rats treated with progesterone alone relative to unsupplemented ovariectomized rats.

As expected, the estrogen only supplemented animals and those receiving estrogen plus progesterone had significantly higher serum estradiol levels compared to the unsupplemented ovariectomized and progesterone only supplemented animals. These levels are considered to be on the upper end of the physiological range and consistent with 17β-estradiol replacement values found in other laboratories and our own (52; 89). Reported estrogen levels range as high as 285 pg ml^{-1} (90) to values such as 15 pg ml^{-1} (91). Progesterone levels were considered to be at low physiological levels. The range of circulating estrogen and progesterone levels are quite large and variable. Age and stage of estrus cycle contribute to this variability. The rats in this study were exposed to

constant levels of estrogen and/or progesterone. Under physiological conditions concentrations are continuously fluctuating over a 3-4 day cycle. Therefore, a limitation of this study is that the findings might not represent leukocyte infiltration in response to damage that occurs when normal fluctuations occur in progesterone and estrogen levels.

This study demonstrated that skeletal muscle in female rats benefited from progesterone treatment alone and in combination with estrogen following injury. The protective effect of progesterone was depicted by its attenuation of leukocyte inflammation. A novel finding was seen with the concurrent administration of estrogen plus progesterone; progesterone and estrogen combined supplementation did not significantly alter the attenuation observed by estrogen treatment alone in skeletal muscle. Conversely, some studies have found that the addition of progesterone may counteract the beneficial actions of estrogen. For example, in rat arteries following vascular injury Xing *et al.* (75) observed that the addition of progesterone and estrogen in combination negated the protective inflammatory effect observed by estrogen alone. The estrogen dosage used in this study resulted in estrogen levels consistent with our study (75). The progesterone dosage used was approximately 3 mg day^{-1}, greater than our dosage of 1.2 mg day^{-1}.

In support of our findings, it has been shown that in the brain there is an upregulation of progesterone receptors with the combined treatment of progesterone and estrogen (94). Furthermore, Balaraman *et al.* (95) demonstrated that female rats with progesterone administration displayed a cardioprotective effect following myocardial ischemia/reperfusion (I/R) injury compared to males and ovariectomized females receiving progesterone supplementation. Progesterone treated ischemic females showed

significantly reduced muscle damage (serum CK levels), inflammation (neutrophil infiltration), lipid peroxidation levels (superoxide dismutase and glutathione levels), and apoptotic markers (DNA fragmentation) compared to vehicle treated I/R females (95). The progesterone dosage of approximately 0.6 mg i.p. following I/R injury, along with the endogenous circulating estrogen, was sufficient to elicit a response in the female rats. Moreover, these findings suggest that there is an interaction effect with progesterone and estrogen in female rats.

In conclusion, the present study not only demonstrates that progesterone independently influences skeletal muscle leukocyte infiltration due to injury, but in combination with estrogen, progesterone does not impede the attenuating influence of estrogen on post-damage leukocyte infiltration.

Chapter Four

Discussion & Conclusion

It is well known that estrogen influences muscle damage and repair processes. The movement of leukocytes (neutrophils and macrophages) into skeletal muscle is a critical response following injury. Our laboratory was the first to demonstrate that estrogen replacement in ovariectomized female rats and male-supplemented rats resulted in diminished leukocyte infiltration and inflammatory response in skeletal muscle (18). We have also demonstrated that muscle damage in response to exercise is attenuated with estrogen supplementation compared to sham (unsupplemented) animals (57). We have begun to examine the mechanisms behind estrogen's influence on skeletal muscle inflammation. Our laboratory's results demonstrated that estrogen administration to ovariectomized female rats induces elevations in cardiac neutrophils while suppressing calpain activity (96). Additionally, we have been examining the relationship between estrogen and the muscle repair process. Recently our laboratory has found greater numbers of total, activated and proliferating satellite cells post-exercise in estrogen supplemented animals compared to the non-estrogen supplemented group. The results demonstrate that estrogen may influence post-damage repair of skeletal muscle through activation of satellite cells (57).

The mechanism by which estrogen influences inflammation in damaged skeletal muscle needs to be examined further. Understanding the mechanism behind estrogen's effects may allow us to optimize and manipulate this process. In the first study, we investigated the role of estrogen receptors on muscle leukocyte infiltration following eccentric exercise in ovariectomized female rats with or without estrogen

supplementation. We blocked the estrogen receptors with ICI 182, 780 to see if this altered estrogen's action on these parameters.

The study found an estrogen specific inflammatory response; with estrogen supplemented animals demonstrating an attenuation of leukocyte infiltration in skeletal muscle post-exercise compared to sham ovariectomized animals. Furthermore, treatment of the ovariectomized estrogen supplemented rats with the ER antagonist, ICI 182, 780 did not alter the leukocyte inflammatory response to exercise relative to those treated with estrogen alone, indicating that the hormonal mechanism is not primarily receptor mediated.

Further work needs to be conducted in order to understand the mechanisms behind estrogen's effects during inflammation. Our study was successful in ruling out the estrogen receptors as the primary means by which estrogen exerts its effects. Perhaps estrogen is acting via non-receptor mediated mechanisms, such as direct interactions with other membrane components, induction of HSPs, or even through an antioxidant effect.

It has been suggested that estrogen may have a membrane stabilizing effect. Estrogen is a fat-soluble hormone, capable of incorporating into the lipid bilayer of the cell and stabilizing the membrane (58). It is thought that this interaction could alter the fluidity and function of the membrane. Or perhaps, estrogen may possess antioxidant properties, donating the hydrogen atoms from their phenolic hydroxyl group, halting the free radical peroxidation chain reactions (60). In this respect, exposure of skeletal muscle to estrogen may reduce muscle damage. Another possible mechanism for estrogen's effects may be through the induction of heat shock proteins. Exercise induced muscle damage results in an increase in intracellular denatured proteins, signaling the induction

of the HSP response to mediate the proper folding of denatured proteins (68). A relationship has been demonstrated between the induction of the HSP response and a decrease in injury in cardiac muscle (69). Therefore, it is speculated that estrogen may exert its effect through one of the non-estrogen receptor mediated mechanisms, such as HSP induction, antioxidant effect or a membrane stabilization effect.

The vast majority of mammalian hormone based studies that examine inflammation in response to muscle injury, only examine the influence of estrogen. However, by only examining estrogen and not progesterone we are not gaining an accurate understanding of the effects of female sex hormones. Therefore, we used a more physiologically valid model in our second study to verify the effects of both estrogen and progesterone alone in combination on leukocyte inflammation post-exercise. Our main objective was to examine the effects that progesterone had on estrogen's ability to reduce exercised induced muscle damage.

The study demonstrated that female rats benefited from progesterone and estrogen treatment alone, as depicted by an attenuation in inflammation following injury. A novel finding was seen with the concurrent administration of estrogen plus progesterone; progesterone and estrogen combined supplementation did not alter the attenuation observed by estrogen treatment alone in skeletal muscle. Therefore, progesterone and estrogen in combination do not influence the leukocyte inflammatory response in an additive or negating manner relative to estrogen supplementation alone following exercise. Moreover, these results support earlier estrogen only studies. We now know that the addition of progesterone, to make the study more physiologically relevant, would not change these results.

There has been limited work on the mechanism of progesterone's effect, but what is known is that there is a similar distribution of estrogen and progesterone receptors in most of the same cell types and tissues which may suggest that estrogen is involved in progesterone receptor induction (72). In the uterus, progesterone's effect is mediated by its receptors and estrogen increases progesterone receptor levels (73).

The practical implications of exploring this area may be for post-menopausal females that could be more prone to muscle injury. These women have altered hormone levels when they enter menopause and hormone intervention strategies involving estrogen and progesterone may be of importance.

A limited number of estrogen replacement (in the form of hormonal replacement therapy) studies have been conducted on postmenopausal women to assess their effects on skeletal muscle damage. Even the examination of pre-menopausal and post-menopausal women's effects to skeletal muscle damage is scarce. Of the studies conducted, it has been found that estrogen replacement did not offer a protective effect to skeletal muscle from exercised induced damage (97; 98); since no significant differences in serum creatine kinase levels were found among the groups. The results from human based studies are not consistent with those of animal based studies. Perhaps this discrepancy is due to the variability that exists in humans or the small samples sizes for human based studies.

Additionally, further knowledge of the mechanisms behind muscle inflammation can be optimized (eg. ER involvement and estrogen/progesterone interaction) to develop pharmaceutical or other intervention strategies to aid the skeletal muscle inflammation process.

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Appendix A
β-Glucuronidase Enzyme Assay

REACTION

p-nitrophenyl-beta-D-glucuronide +H_2O ⟶ alcohol + D-glucuronide + p-nitrophenyl

PROCEDURE

1. Homogenize tissue (10-20 mg; optimal 15 mg) in glass pestle using a 33.33:1 (3%) dilution using distilled water.
   -use 16.66667 times the mass of tissue, do this 2 times
2. Add 50 µl of homogenate and water to appropriately labeled test tube
3. Add 450 µl of Acetate buffer to tubes using repeater pipette
4. Pre-incubate tubes in 37°C bath for 5 minutes
5. Add 250 µl of substrate using repeater pipette
6. Incubate for 16-18 hours tubes in 37°C bath
7. Add 1.5 ml of cold glycine buffer using repeater pipette
8. Cool in ice water for 10 minutes
9. Centrifuge at 3500 rpm for 10 minutes (temp 4°C)
10. Read on spectrophotometer at 420 nm.

REAGENT PREPARATION

1.Sodium acetate (anhydrous) ($C_2H_3O_2Na$) (0.1N) (mw 82.03)
0.82 g in 100 ml of H_2O pH 4.2

2.Glycine Buffer (0.1M) pH 10.8
A) 0.375g glycine ($C_2H_5NO_2$, mw 75.07) + 0.2922g NaCl (mw 58.44) in 50 ml H_2O
B) 0.4 g of NaOH (mw 40) in 100 ml of H_2O
Mix 52.2 ml of A and 47.8 ml of B and pH to 10.8

3) Substrate: p-nitrophenyl-beta-D-glucuronide (5mM) (sigma N-1627) (mw 315.2)
7.88 mg in 5 ml H_2O

STANDARD PREPARATION

Standard: p-nitrophenol (10 mM liquid p-NP solution was purchased from Sigma)

Serial Dilution, mM	To make:
0.125	Take 563 µl of 10 mM p-NP stock and add 437 µl dH_2O
0.0625	Take 500 µl of 0.125 mM solution and add 500 µl dH_2O
0.03125	Take 500 µl of 0.0625 mM solution and add 500 µl dH_2O
0.015625	Take 500 µl of 0.03125 mM solution and add 500 µl dH_2O
0 (distilled water)	

Appendix B
HIS48 Immunohistochemistry

Used frozen sections of rat muscle cut 10 microns thick.

1. Let slides air dry for 5 min or until warmed up to room temperature.
2. Fix sections with 100% cold acetone for 10 min.
3. Let slides air dry for 5-10 min.
4. Permeablize cells in 0.5% Triton X-100 solution (in PBS) for 5 min.
5. Wash in PBS for 3 x 5 min.
6. Apply 0.6% H_2O_2 (in absolute methanol) for 10 min.
7. Wash in PBS for 3 x 5 min.
8. Block with 5% normal goat serum in PBS (containing 5% nonfat milk powder) for 30 min. Tap off excess.
9. Incubate in primary antibody: HIS48 (1/50 diluted in blocking solution) (BD Biosciences, Mississauga, ON) for 3 h.
10. Wash in PBS for 3 x 5 min.
11. Apply bottle 3: GAM-Biotin (from Dako LSAB-2 kit) for 10 min.
12. Wash in PBS for 3 x 5 min.
13. Apply Bottle 4: Streptavidin-HRP (from Dako LSAB-2 kit) for 10 min.
14. Wash in PBS for 3 x 5 min.
15. Apply Vector NovaRed stain for 3 min (or until desired intensity is achieved).
16. Rinse in dH_2O liberally from wash bottle, then wash in bath for 2 x 5 min.
17. Counterstain using Vector Hematoxylin QS (add hematoxylin for 10-15 s and rinse off with running 37°C tap water for 30 s).
18. Place a drop of permanent mounting medium on cover slip and place slide upside down onto cover slip so that mounting medium covers section completely.
19. Invert slide and apply gentle pressure on cover slip to remove any air bubbles.

Notes:

* All kit reagents should be equilibrated to room temperature prior to procedures and all incubations are done at room temperature
* Place all buffer baths on rocker for gentle agitation

REAGENTS:

PBS, 0.015 M working solution (pH 7.6):

	1x PBS	**10x PBS**
$Na_2HPO_4 * 7H_20$ (FW 268.1)	3.495 g	34.95 g
$NaH_2PO_4 * H_20$ (FW 138.0)	0.27 g	2.7 g
NaCl (FW 58.44)	9.0 g	90 g

For 1x PBS solution: Measure reagents, add approx. 950 ml dH_2O, pH to 7.6, and bring up to 1 L with dH_2O. Store at room temperature.

For 10x PBS solution:
Measure reagents, add approx. 950 ml dH_2O, pH to 7.6, and bring up to 1 L with dH_2O. Store at room temperature. Each day: take out 100 ml of 10x stock PBS solution and add 900 ml dH_2O.

0.5% Triton X-100 solution:
Add 5 ml of Triton X-100 to 995 ml of 1x PBS. Store at room temperature.

0.6% H_2O_2 solution (in MeOH):
Add 10 ml of 30% stock H_2O_2 to 490 ml absolute methanol. Store at 4°C.

Blocking solution (5% normal goat serum in 5% nonfat milk powder/PBS):
For 10 ml:
Weigh out 0.5 g nonfat milk powder.
Add 9.5 ml 1x PBS.
Add 500 µl normal goat serum.
Store at 4°C.

For 5 ml of a 1:50 dilution of His48 antibody:
Take 4.90 ml blocking solution and add 100 µl primary antibody (BD Biosciences, Mississauga, ON).

Appendix C
ED1 Immunohistochemistry

Used frozen sections of rat muscle cut 10 microns thick.

1. Let slides air dry for 5 min or until warmed up to room temperature.
2. Fix sections with 100% cold acetone for 10 min.
3. Let slides air dry for 5-10 min.
4. Permeablize cells in 0.5% Triton X-100 solution (in PBS) for 5 min.
5. Wash in PBS for 3 x 5 min.
6. Apply 0.6% H_2O_2 (in absolute methanol) for 10 min.
7. Wash in PBS for 3 x 5 min.
8. Block with 5% normal goat serum in PBS (containing 5% nonfat milk powder) for 30 min. Tap off excess.
9. Incubate in primary antibody: ED1 (1/100 diluted in blocking solution) (AbD Serotec, Raleigh, NC) for 1 h.
10. Wash in PBS for 3 x 5 min.
11. Apply bottle 3: GAM-Biotin (from Dako LSAB-2 kit) for 10 min.
12. Wash in PBS for 3 x 5 min.
13. Apply Bottle 4: Streptavidin-HRP (from Dako LSAB-2 kit) for 10 min.
14. Wash in PBS for 3 x 5 min.
15. Apply Vector NovaRed stain for 4 min (or until desired intensity is achieved).
16. Rinse in dH_2O liberally from wash bottle, then wash in bath for 2 x 5 min.
17. Counterstain using Vector Hematoxylin QS (add hematoxylin for 10-15 s and rinse off with running 37°C tap water for 30 s).
18. Place a drop of permanent mounting medium on cover slip and place slide upside down onto cover slip so that mounting medium covers section completely.
19. Invert slide and apply gentle pressure on cover slip to remove any air bubbles.

Notes:

* All kit reagents should be equilibrated to room temperature prior to procedures and all incubations are done at room temperature
* Place all buffer baths on rocker for gentle agitation

Appendix D
Lowry Protein Assay

PROCEDURE:

1. Use 12*75 mm culture tubes and do samples, standards, and blanks in triplicate.

2. Add 50 µl of standards to appropriately labelled tubes.

3. Add 50 µl of water to tubes labelled REAGENT BLANK.

4. Prepare a 20:1 dilution of homogenizing media (10 µl media + 190 µl water). Add 50 µl of dilute media to tubes labelled SAMPLE BLANK.

5. Prepare a 20:1 dilution of EACH tissue sample (10 µl sample + 190 µl water). Add 50 µl of dilute sample to tubes labelled SAMPLE.

6. Add 0.5 ml of alkaline copper reagent to all tubes.

7. Mix well and let stand for 10 minutes at 25°C (room temperature).

8. Add 2.0 ml of phenol reagent to each tube. Mix each tube individually IMMEDIATELY after adding phenol reagent.

9. Incubate for 5 minutes at 55°C.

10. Cool in tap water for 1 minute.

11. Read on spectrophotometer at 650 nm.

{PRIVATE }REAGENTS:

1. ALKALINE COPPER REAGENT:

   0.05 g $CuSO_4.5H_2O$
   10.00 g Na_2CO_3
   0.10 g POTASSIUM SODIUM TARTRATE
   2.00 g NaOH

   TO 100 ml WATER ADD THE $CuSO_4$ AND DISSOLVE COMPLETELY. ADD THE REMAINING REAGENTS IN ORDER. STORE AT 20°C FOR 2 WEEKS.

2. FOLIN-CIOCALTEU PHENOL REAGENT: (Sigma-Aldrich, St. Louis, MO)

   TO 80 ml WATER ADD 5.0 ml of 2 N PHENOL REAGENT. MAKE FRESH DAILY.

3. BOVINE SERUM ALBUMIN STANDARD, 1.0 mg/ml: (BSA,Sigma-Aldrich, St. Louis, MO)

   ADD 10 mg of BSA TO 10 ml WATER. MIX BY INVERSION. SERIAL DILUTE TO OBTAIN SOLUTIONS AS FOLLOWS:

   1.0 mg/ml
   0.5 mg/ml
   0.25 mg/ml
   0.125 mg/ml
   0.0 mg/ml

CALCULATION:

$$\frac{(A_{650} \text{ of sample} - A_{650} \text{ of sample blank})}{(A_{650} \text{ of standard} - A_{650} \text{ of reagent blank})}$$

Multiply by

Concentration of Standard (mg/ml) * 20 (dilution factor) = Protein concentration, mg/ml