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ISBN: 978-0-494-54223-1
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Canada

Effects of estrogen receptor activation on post-exercise muscle satellite cell proliferation

By

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THESIS

Submitted to Kinesiology and Physical Education, Faculty of Science
in partial fulfillment of the requirements for

Master of Science

Wilfrid Laurier University

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Abstract

This study investigated the role of estrogen receptor activation on total and proliferating satellite cells following running exercise in rats, by using an estrogen receptor-alpha (ER- α) specific agonist, 4,4,4 (4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT). PPT is the first ER- α specific agonist (*Stauffer et al 2000*) which binds the ER- α subtype with a 410-greater affinity than ER- β (*Stauffer et al 2000*). Previous studies have demonstrated that estrogen can augment muscle satellite cell numbers following exercise (*Enns and Tiidus, 2008*) and that this augmentation may be due to mechanisms activated via estrogen receptors that can influence total, proliferating, and differentiating satellite cell numbers (*Enns et al 2008*). Ovariectomised female rats (n = 64) were divided into four groups: sham, estrogen (0.25 mg estrogen), agonist (PPT), and estrogen plus PPT. Each group was further divided into exercisers and controls (non-exercisers). PPT administration commenced 2 days after estrogen implantation and continued for 6 days. After 7 days of estrogen exposure, exercisers ran intermittently for 90 minutes (17m/min, -13.5 degree grade). Soleus and white vastus muscles were removed 72 hours post-exercise and immunohistochemically stained for total (pax7) and activated (MyoD) satellite cells. Severity of muscle damage was assessed indirectly by measuring beta-glucuronidase activity. The results revealed that there were significant increases ($p < 0.05$) in fibers staining positive for total (pax7) and activated (MyoD) satellite cells when compared to shams in both the soleus and white vastus muscles. However, there was no significant difference ($p > 0.05$) observed between the treatment conditions supplemented with estrogen, PPT, and estrogen plus PPT. Augmented increases observed in muscle satellite cells post-exercise in the PPT supplemented group suggests that their activation is an estrogen receptor-mediated process. Due to no significant difference ($p > 0.05$) observed between PPT- and estrogen-mediated activation of ER- α receptors, ER- α is implicated as the receptor responsible for most, if not all, of the activation of satellite cell activity when induced by estrogen.

Acknowledgements

There are a few people who I would like to thank for aiding me on my journey towards the completion of my Masters:

First and foremost, I would like to thank my thesis advisor, Dr. Peter Tiidus, for his continual confidence and support. By way of his guidance and mentorship, I was able to realize and/or reaffirm aspects of both academia and life. I would also like to thank both Sobia Iqbal and Amy Thomas for which the completion of my thesis may not have become a reality. I will always cherish Sobia's lab training and the honour of working alongside Amy. Others who have contributed towards making my Masters a reality and cannot be excluded are: Eric Bombardier and Kelly Putzu.

As an individual who has been removed from the circles of academia for a number of years, I am grateful for the people who have entered, and positively influenced my life. But if I were to summarise the effects people, in particular friends and family, have had on me, it can be stated as follows:

Let your imagination run wild and be allowed to dream because, it is our dreams that allow us as individuals to have hope. With hope, the equation of life becomes simpler in that what is left is hard work, dedication, and courage that will transform our hopes and dreams into reality.

Kareem Bunyan

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Abbreviations

Ca ²⁺	Calcium
CK	Creatine Kinase
E2	Estradiol-17 β
EC	Excitation-Contraction coupling
ER α	Estrogen Receptor-Alpha
ER β	Estrogen Receptor-Beta
FGF	Fibroblast Growth Factor
HGF	Hepatocyte Growth Factor
HRT	Hormone Replacement Therapy
IGF-1	Insulin-Like Growth Factor-1
IL	Interleukin
I/R	Ischemia Reperfusion
NO	Nitric Oxide
TNF- α	Tumor Necrosis Factor-Alpha
EDL	Extensor Digitorum Longus
MPO	Myeloperoxidase
COX-2	Cyclo-Oxygenase-2
MAPK	Mitogen-Activated Protein Kinase
PI3K	Phosphatidylinositol-3-OH Kinase
EPR	Electron Paramagnetic Resonance
EGF	Epidermal Growth Factor
AF-1	Activation Factor-1
PKB (Akt)	Protein Kinase B
LBD	Ligand-Binding Domain
PPT	Propylpyrazole Triol
OVX	Ovariectomised

List of Definitions

Agonist: A chemical that binds and activates a receptor and could be considered as a full or partial agonist.

- A *full agonist* is highly efficient producing a full response while occupying a relatively low proportion of receptors.
- A *partial agonist* has lower efficiency than a full agonist. It produces sub-maximal activation even when occupying the total receptor population, therefore cannot produce the maximal response.

Antagonist: A chemical that attenuates the effect of an agonist, and can be either competitive or non-competitive.

- A *competitive antagonist* binds to the same site as the agonist but does not activate it, thus blocks the agonist's action.
- A *non-competitive antagonist* binds to an allosteric (non-agonist) site on the receptor to prevent activation of the receptor.

Antioxidant: An antioxidant is a molecule capable of neutralising the oxidation of molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent.

Apoptosis: Apoptosis is a form of programmed cell death in multicellular organisms.

Beta-Glucuronidase: Glucuronidases are enzymes that cleave molecules to produce glucuronic acid by cutting glycosidic bonds. Therefore they are classified as glycoside hydrolases that cleave glucuronides. In eukaryotes, glucuronidase is located in lysosomes and are important in recycling cellular components.

Chemotaxis: Chemotaxis is the organisation of movements according to certain chemicals/stimuli in the environment. Neutrophils, for example, undergo chemotaxis that allows them to migrate toward sites of infection or inflammation.

Concentric Contraction: The forces produced by the muscle are sufficient to overcome the resistance, and causes the muscle to shorten as it contracts.

Creatine Kinase: Creatine kinase (CK), also known as creatine phosphokinase (CPK) or phosphocreatine kinase, is an

enzyme that catalyses the conversion of creatine to phosphocreatine, utilising adenosine triphosphate (ATP) and generating adenosine diphosphate (ADP). In tissues such as skeletal muscle, that utilises ATP rapidly, phosphocreatine serves as an energy reserve for the rapid conversion to ATP.

Cytokines: Similar to hormones and neurotransmitters, cytokines are signalling proteins and glycoproteins used primarily for cellular communication.

Degranulation: Neutrophils release an assortment of proteins in three types of granules by a process called degranulation.

Eccentric Contraction: Forces generated by the muscle are insufficient to overcome the resistance resulting in muscle fibres lengthening as they contract. An eccentric contraction can also be considered as a means of decelerating a body part.

Fast Twitch Fibres: Fast-twitch (type II or white) fibres have fewer mitochondria, are capable of more powerful (but shorter) contractions, metabolize ATP more quickly, have a lower capillary to volume ratio, and are more likely to accumulate lactic acid. Type II fibres are distinguished by their primary sub-types, IIa, IIx, and IIb. Weightlifters and sprinters are examples of athletes who tend to have more type II fibres.

Fibroblasts: Fibroblasts synthesize and maintain the extracellular matrix. Fibroblasts provide a structural framework for many tissues, and play a critical role in wound healing.

Free Radical Species: Free radicals are atoms, molecules or ions with unpaired electrons on an open shell configuration. These unpaired electrons are typically highly reactive, making radicals likely to participate in chemical reactions.

Histology: Histology is the study of examining the microscopic anatomy of cells and tissues by viewing a thin slice under a light microscope.

Immunohistochemistry: Immunohistochemistry (IHC) is a process of localizing cell proteins in tissue sections relying on antibodies binding to antigens.

Inflammation: Inflammation is the response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. It is a

process that attempts to remove the injurious stimuli as well as initiate the healing process for the tissue.

Isometric Contraction: The muscle maintains the same length regardless of building tension.

Isotonic Contraction: Occurs when tension in the muscle remains constant despite a change in muscle length. This occurs when a muscle's maximal force of contraction exceeds the total load on the muscle.

Leukocytes: White blood cells, or leukocytes, are cells of the immune system that defends the body against infectious diseases and foreign materials.

Macrophages: Macrophages are cells that originate from white blood cells called monocytes. Monocytes and macrophages are phagocytes, acting in both non-specific as well as specific defense systems of vertebrate animals. Their role is to engulf and digest cellular debris and to stimulate lymphocytes and other immune cells to respond to the pathogen.

Monocytes: Monocytes are leukocytes, and have two main functions in the immune system: (1) replenish resident macrophages and dendritic cells under normal states, and (2) in response to inflammation signals, monocytes can move quickly (approximately 8-12 hours) to sites of infection and divide/differentiate into macrophages and dendritic cells to elicit an immune response.

Myoblasts: Myoblasts are a type of stem cell that exist in muscles. The fusing of myoblasts gives rise to new skeletal muscle fibres. Myoblasts that do not form muscle fibres differentiate into satellite cells.

MyoD: MyoD is a protein with a pivotal role in regulating muscle differentiation and is one of the earliest markers identifying myogenic commitment. MyoD is expressed in activated satellite cells, but not in quiescent satellite cells.

NADPH Oxidase: NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) is a membrane-bound enzyme complex found in the plasma membrane and membranes of phagosomes. This complex is normally latent but is activated to assemble in the membranes during a respiratory burst. It aids in the generation of superoxide, a highly-reactive free-radical, by transferring electrons from NADPH inside the cell

across the membrane and donating them to molecular oxygen.

Necrosis: Necrosis is the unnatural death of cells and living tissue. It commences with cell swelling, chromatin digestion, and finally, disruption of the plasma membrane and organelle membranes.

Neutrophils: Neutrophils are normally found in the blood stream. During the acute phases of inflammation, neutrophils migrate toward the site of inflammation, firstly initially through the blood vessels, then through interstitial tissue, following chemical signals.

Pax-7: P21(CDKN1A)-activated kinase 7 (Pax-7) is a human gene. The proteins encoded by this gene have been identified in the regulation of cytoskeletal dynamics, proliferation, and cell survival signalling. Pax-7 is expressed in all quiescent, activated and proliferating satellite cells.

Phagocytosis: The capability of ingesting microorganisms or particles.

Respiratory burst: Neutrophils are capable of ingesting microorganisms or particles. With each phagocytic event, reactive oxygen species and hydrolytic enzymes are secreted. The utilisation of oxygen during the generation of reactive oxygen species has been defined as the respiratory burst. The respiratory burst requires the activation of the enzyme NADPH oxidase, which produces superoxide, a reactive oxygen species.

Slow Twitch Fibres: Slow-twitch (type I or red) fibres have greater amounts of mitochondria, an increased capillary to volume ratio, store oxygen in myoglobin, rely on aerobic metabolism, produce ATP more slowly, and are relied upon for endurance events.

Superoxide: Superoxide is the anion O_2^- . It is produced as the product of the one-electron reduction of dioxygen. With one unpaired electron, the superoxide ion is a free radical.

Chapter One

Introduction

Skeletal muscle is one of the three major muscle types found within humans, the others being cardiac and smooth muscle. Skeletal muscle, which is held together by connective tissue, is comprised of muscle fibers that enable muscles to contract. Beside the ability of skeletal muscle to contract and generate force for locomotion, breathing and postural support, other functions include: fuel storage, temperature regulation, and acting as a force absorber. However, the proper functioning of skeletal muscle can be disrupted as a result of injury, disease condition, or overtraining (**Hawke & Garry 2001, Wazniak et al 2005**).

1.1 Skeletal Muscle Damage

Injuries to skeletal muscle fibers can occur in a number of ways such as sharp or blunt trauma, extreme temperatures, myotoxic agents, ischemia, muscle disease (such as dystrophy), inflammation and contractions of the muscles (**Faulkner et al 1993**). All of the aforementioned mechanisms by which skeletal muscles can be damaged results in some or all of the fibers undergoing necrosis (**Faulkner et al 1993**). Regardless of the contributing factor(s) that result in skeletal muscle fiber damage; the fiber damage and repair process follow a common pathway (**Faulkner et al 1993**). Injury induced as a result of shortening (concentric), Isometric, or lengthening (eccentric) contractions can occur however, there is a greater likelihood of injury resulting from eccentric

contractions. Data collected by **McCully & Faulkner (1985)** revealed that in comparison to concentric and isometric contractions, eccentric contractions elicited significantly greater injury to muscle fibers as illustrated by an increased number of fibers demonstrating histological signs of injury (**McCully & Faulkner 1985**). When taken into account that eccentric exercise is not as metabolically strenuous, as well as producing less lactic acid than that of concentric contractions (**Clarkson & Sayers 1999**); mechanical stresses imposed upon the structural elements of the muscle fibers from eccentric contractions provide an explanation as to the damage that occurs. Faulkner and colleagues demonstrated, using the extensor digitorum longus (EDL) muscle in mice, that there is a greater development of average forces during lengthening compared to shortening or isometric contractions (**Faulkner et al 1993**). They also state that: a) the force developed during eccentric contractions was approximately twofold greater in comparison to the force generated by maximum isometric contractions, and b) the number of strongly bound cross-bridges was approximately 10% greater than that of isometric contractions (**Faulkner et al 1993**). Therefore, increases in force generated during an eccentric contraction results in an increased strain on the individual cross-bridges. One reason for the greater damage incurred by eccentric contractions lies in the number of motor units recruited. Eccentric contractions recruit fewer motor units than concentric contractions and therefore provide a smaller cross-sectional area of muscle activated to handle the load (**Clarkson & Sayer 1999**).

With the use of electron micrographs, changes are observed in sarcomere lengths caused by lengthening contractions. During eccentric contractions, some sarcomeres tend to maintain their lengths while weaker sarcomeres are stretched to the point beyond overlap. If this process occurs on the descending limb of the length-tension curve, sarcomeres will progressively get weaker until there is no longer any overlap between myofilaments, and it is the rising passive tension in elastic elements that balances the tension in the remaining unstretched sarcomeres (**Proske & Allen 2005**). It is the inability of overstretched sarcomeres to reintegrate upon relaxation of the muscle that causes them to be disrupted and lie scattered randomly along the length of the sarcomere. As the number of disrupted sarcomeres increases, damage may spread longitudinally to adjacent sarcomeres and transversely to adjacent myofibrils (**Proske & Allen 2005**). A point will be reached when overstretched sarcomeres will lead to membrane damage, which includes the membranes of the sarcoplasmic reticulum, transverse tubules, or the sarcolemma which in turn is accompanied by an influx of calcium into the sarcoplasm.

Excess calcium entering the sarcoplasm due to muscle membrane damage, can affect calcium homeostasis but also activate calpain (**Belcastro et al 1998**). Activation of calpain is believed to play a role in muscle damage by selectively degrading various contractile, metabolic and/or structural elements (**Belcastro et al 1998**). Calpain is a non-lysosomal protease located in the I and Z regions of muscle and cleaves a number of substrates which include desmin, α -actinin, vimentin, spectrin, integrin, and cadherin (**Belcastro et al 1998**).

Calpain is activated by elevated cytosolic calcium concentrations which in turn affects/cleaves specific muscle proteins and gives rise to the production of neutrophil chemoattractive products (*Tiidus et al 2001; Tiidus 2003*). Calpain is activated during exercise, and it is believed that calpain, or at least the peptide fragments it creates, are associated with some of the neutrophil chemotaxis observed immediately post-exercise (*Belcastro et al 1998*).

Electron micrographs allow for focal damage of the ultrastructure to be observed (displacement of the thick filament to one Z-line, crumpling of the interface between thick and thin filaments, and disorganization of the Z-lines) of single sarcomeres within specific fibers (*Faulkner et al 1993*). Histological abnormalities such as disarray of banding patterns in less than 5% of muscle fibers (*Armstrong et al 1983*) have been observed as a widening of the I-band in certain regions of myofibers in longitudinal sections and no clear differentiation in some regions between adjacent muscle cells (*Armstrong et al 1983*) which were not observed in the controls. The explanation provided of the aforementioned observation was a possible disruption of the sarcolemma (*Armstrong et al 1983*).

Researchers have also observed structural abnormalities such as disruption in the striation pattern of slow twitch fibers of the extensor muscles in rats following downhill running, Z-line disorganization in human soleus muscle two days post-exercise (*MacIntyre et al 1995*), extensive sarcomeric disruptions that occurred in the quadriceps muscle of humans immediately post-exercise, streaming and smearing of the z-lines, focal loss of Z-lines, and extension of the

Z-line into the A-band (**Lieber et al 1991**). Armstrong and colleagues (**MacIntyre et al 1995**) observed that Z-disk smearing was: multifocal within the same fiber, did not extend full the length of the fiber, infrequently spanned more than three sarcomeres, and in these areas, thick filaments overlapped each other and/or the I-bands (**Lieber et al 1991**).

Muscle proteins that are typically affected by eccentric contractions and contribute to the changes observed at the Z-lines are dystrophyn, desmin, fibronectin (diagram 1 depicts the sequence of events leading to Z-line changes).

Dystrophyn is a submembrane protein that interacts with actin and glycoproteins in the sarcolemma which, as a result, functions to stabilise the sarcolemma during contractions. Changes that occur in the dystrophyn-glycoprotein complex caused by eccentric contractions could affect other membrane proteins such as ion channels which in turn could alter calcium homeostasis (**Komulainen et al 1999**). Dystrophyn disruption can be used as a direct marker of myofiber membrane disruption which was found to increase during the early phase of recovery and dissipating towards the end of the first week of recovery (**McClung et al 2006**).

Desmin is a cytoskeletal protein specific to muscle which helps in the maintenance of muscle fibre integrity, synchronization of contractions, and is susceptible to proteolysis, particularly in the presence of calcium (**Komulainen et al 1999**).

Fibronectin, an extracellularly located matrix-organising glycoprotein, helps with the repair process by stimulating cell attachment and migration, as

well as forming components of the initial connective tissue matrix (**Komulainen et al 1999**).

Changes in the aforementioned proteins resulting from exercise become useful for estimating the early phases of myofiber damage prior to observable histopathological changes (**Komulainen et al 1999**).

Lysosomal acid hydrolase, β -glucuronidase, activity provides a useful, but indirect indicator/marker of the histopathological state and extent of exercise-induced damage of skeletal muscle (**Enns & Tiidus 2008; Komulainen et al 1999**). β -glucuronidase is a lysosomal enzyme comprised of four identical subunits and is involved in the stepwise degradation of glucuronic acid-containing glycosaminoglycans (**Shibley & et al 1993**). Lysosomal changes are associated with the muscle repair process and increases in total lysosomal acid hydrolases originate in invading inflammatory phagocytes as well as surviving muscle fibers (**Salminen & Kihlström 1985**). Increases in the levels of several lysosomal acid hydrolases were observed when the total activities of acid hydrolases was used as a quantitative measure of exercise-induced muscle injury revealing a high correlation between lysosomal enzyme activities and the histopathologic state of skeletal muscle (**Salminen & Kihlström 1985**). Increased β -glucuronidase activity was observed in both males and females 48 and 96 hours post-exercise, however, the increase was much smaller in females (**Komulainen et al 1999**). Increased β -glucuronidase activity tested 48 hours post-exercise was 70 and 300% for females and males respectively (**Komulainen et al 1999**). Komulainen and colleagues also observed female

soleus muscles that were undamaged (β -glucuronidase activity and histopathological changes were not observed) during the seven day period post-exercise after following a similar protocol proven to cause damage to the soleus muscle in male rats (*Komulainen et al 1999*).

Measurement of creatine kinase (CK) in the circulation, similar to β -glucuronidase activity, is useful for providing indirect evidence of post-exercise muscle damage. CK is a metabolic enzyme primarily located in the intracellular space of muscle. Due to its solubility characteristics, CK has the ability to cross the sarcolemma if damaged and enter the circulation (*Bar & Amelink 1997*). CK may not be an ideal marker to assess structural muscle damage however, changes in CK circulation can be used as a marker of sarcolemma disruptions from exercise or muscular activity (*Tiidus 2008*).

1.2 Skeletal Muscle Inflammation

Muscle damage induced by eccentric contractions elicits an inflammatory response. Inflammation causes the movement of fluid, plasma proteins, and leukocytes into the tissue when responding to injury, which in turn promotes the clearance of damaged tissue and prepares the tissue for repair. There are many aspects that comprise the inflammatory response, however, for the purposes of this paper, the roles of neutrophils and macrophages in the removal of damaged tissue as well as the repair process of muscle will be discussed.

One of the first steps of the inflammatory response is the accumulation of neutrophils in the area of muscle damage. Neutrophil infiltration into the area of

exercise-induced muscle damage begins prior to the cessation of exercise, continuing to increase rapidly over the following 12-24 hours and lasting for as long as 5 days post-exercise (**Tiidus et al 2001**). Because the invasion of macrophages into the damaged area typically occurs 12-24 hours post-exercise (**Tidball et al 1999; Tiidus et al 2001**), neutrophils would encompass the majority of leukocytes observed 1 hour post-exercise in rat skeletal muscle (**Tiidus et al 2001**).

Neutrophils contain and destroy damaged tissue or foreign matter primarily through phagocytosis; however, they can undergo a respiratory burst and degranulation (**Toumi et al 2006**). Neutrophils have the capability of generating hypochlorous acid via an MPO-mediated reaction and superoxide (O_2^-) via NADPH oxidase that could possibly contribute to further muscle damage during injury (**Toumi et al 2006**). Data obtained by Raj and colleagues indicated immediate post-exercise calpain activation and myeloperoxidase (MPO) activity in rodent skeletal muscle (**Raj et al 1998, Tiidus et al 2001**). They observed a correlation in calpain-like protease and MPO activities ($r=0.83$) and that their underlying processes were connected (**Raj et al 1998**). These reactions ultimately assist with breakdown and clearance of damaged tissue to help promote tissue repair (**Toumi et al 2006**).

Macrophages are similar to neutrophils in that they have the ability to produce oxygen free radicals and cytotoxic enzymes which lead to tissue degradation (**MacIntyre et al 1995**). Macrophage induced membrane lysis *in vitro* occurs via nitric oxide (NO)-dependant and superoxide-independent

processes (**Tidball & Wehling-Henricks 2007**). NO-mediated lysis of membranes is exacerbated in the presence of neutrophils, suggesting that interactions between myeloid cell populations contribute to their cytotoxicity while interactions of myeloid cells and muscle seem to be under the control of muscle-derived factors (*in vitro*), which in turn, promote NO release from macrophages (**Tidball & Wehling-Henricks 2007**). However, Tidball and Wehling-Henricks were unable to find supporting evidence of macrophage-mediated damage to muscle cell membranes during loading after a period of unloading (**Tidball & Wehling-Henricks 2007**). Tidball and Berchenko demonstrated that the majority of the influx of extracellular materials into the muscle via damage to the cell membrane occurred within the first 2-24 hours post-injury regardless of muscle loading during this period (**Tidball et al 1999**). Observations of ED1+ macrophages in the soleus muscle after reloading did not differ from controls indicating that membrane damage during 2-24 hours was independent of mechanical loading or ED1+ macrophage invasion during that period (**Tidball et al 1999**).

Macrophages are the most abundant inflammatory leukocytes 12 hours post-exercise and are the primary removers of cellular debris. ED1+ and ED2+ are subpopulations of macrophages that have been observed in animal tissue (**Clarkson & Sayer 1999**). ED1+ macrophages appear 12 hours post-exercise contributing to the removal of necrotic tissue, phagocytosing apoptic neutrophils (**McClung et al 2006**), and cytokine production, while ED2+ macrophages appear 24-48 hours post-exercise to help regulate the repair process as they

tend to appear after muscle necrosis is complete and muscle repair begins (*Clarkson & Sayer 1999; Kendall & Eston 2002; Tiidus 2003*). ED2+ macrophages contribute to the repair process by stimulating satellite cell activation and differentiation, but the repair of damaged muscle will not occur unless these macrophages are present (*Clarkson & Sayer 1999; Tiidus 2003*). It is suggested that macrophages are involved with the cell cycle regulation of myoblasts by the substances they secrete. Using MyoD as a marker of skeletal myoblasts, a doubling of the number of myonuclei was observed prior to myoblast fusion (*Massimo et al 1997*). The overall increase in nuclei observed in comparison to controls was attributed to myoblast proliferation and as a result, this evidence leads to supporting the hypothesis that macrophages possess a muscle specific proliferative effect (*Massimo et al 1997*).

In vitro findings indicate that macrophages can increase proliferation of myoblasts in culture and increase the proportion of myoblasts that express MyoD suggesting that macrophage-derived factors play a role in muscle growth and differentiation (*Tidball & Wehling-Henricks 2007*). Observations made after a single bout of downhill running, rats had elevated neutrophil and ED1+ macrophages while there were non-significant changes in ED2+ macrophages and MyoD+ cells (*Tsivitse et al 2003*). They felt that the elevations in neutrophil and ED1+ macrophages occur independently of changes in MyoD+ cells (*Tsivitse et al 2003*). ED2+ macrophages were observed not to be elevated 72 hours post-exercise which seems to contradict previously reported elevations in ED2+ macrophages 48 hours post-exercise encompassing lengthening

contractions (*Tsivitse et al 2003*). The explanation provided by the authors was that their downhill-running protocol may not have been sufficient to elicit enough injury in the soleus muscle as to observe increases in ED2 macrophages (*Tsivitse et al 2003*).

Macrophages, along with immune and non-immune cells, are important for producing/releasing cytokines, that aid in the inflammatory response during skeletal muscle repair (*St. Pierre Schneider et al 1999*) such as interleukin-1 (IL-1 β), IL-2, IL-6 and tumour necrosis factor- α (TNF- α) (*McClung et al 2006*). Interleukin-1 β is responsible for stimulating proteolytic enzyme synthesis, leucocyte adhesion and extravasion, activation of macrophages, and expression of other interleukins such as IL-2, IL-3, IL-6 and tumour necrosis factor- α (TNF- α) (*McClung et al 2006*). IL-6, a pro-inflammatory cytokine, provides an early stimulus for satellite cell activation and affects protein synthesis during skeletal muscle recovery (*McClung et al 2006*). Cytokines play an important role in inducing the synthesis of prostaglandins and are both involved in the mediation of the inflammatory process and inducing satellite cell activation (*McClung et al 2006*). For example, Cyclo-oxenase-2 (COX-2) is an enzyme crucial for successful and timely skeletal muscle regeneration (*McClung et al 2006*).

1.3 Skeletal Muscle Repair

During periods of growth and repair from injury, or in disease conditions, satellite cells have the ability to increase muscle mass and repair muscle after damage which makes them essential for the normal adaptive functions of skeletal muscle (*Wozniak et al 2005*). Satellite cells are muscle precursor cells that lie

between the external lamina and sarcolemma of skeletal muscle fibers. It is the commencement of damaged tissue removal that causes muscle fiber regeneration to begin under extensive control by the state of the external lamina and sarcolemma, growth factors, and mechanical events affecting fibers (**Wozniak et al 2005**). Muscle regeneration can be considered as undergoing four stages: 1) satellite-cell activation, 2) myoblast or precursor proliferation, 3) precursor differentiation, 4) return to quiescence (**Wozniak et al 2005**).

Satellite cells normally are in a state of quiescence, but as a result of myofiber injury and or hypertrophic signals they enter the cell-cycle where proliferation and differentiation occur to provide the necessary precursors involved in the skeletal muscle growth and repair process (**Enns & Tiidus 2008**).

Satellite cell activation and proliferation are under the control of various factors that arise due to tissue damage and inflammation, which include nitric oxide (NO) and insulin-like growth factor 1 (IGF-1) (**Machida & Booth 2004; Anderson 2000**). These factors are known to activate satellite cells post-injury and subsequently maybe affected by estrogen levels (**Stupka & Tiidus 2001**).

Insulin-like growth factors I and II (IGF-1 and IGF-2 respectively) for example, are secreted from skeletal muscle and contribute to the regulation of satellite cell proliferation and differentiation (**Hawke & Garry 2001**). Overload of skeletal muscle or eccentric exercise elicits an increase in IGF-1 levels, increased DNA content (indicating an increase in satellite cell proliferation), and hypertrophy of skeletal muscle (**Hawke & Garry 2001**). IGF-1 uses multiple signalling pathways such as calcineurin/NFAT, mitogen-activated protein kinase

(MAPK), and phosphatidylinositol-3-OH kinase (PI3K) pathways in the regulation of satellite cells (**Hawke & Garry 2001**). It has been proposed that IGF-1 stimulation of satellite cell differentiation seems to be controlled by way of the PI3K pathway (**Hawke & Garry 2001**).

Besides the roles that IGF-1 and IGF-2 have in regard to satellite cell stimulation and activation, there are other factors included in this process. Hepatocyte growth factor (HGF) is a cytokine that has been localized, along with its receptor c-Met, in satellite cells and adjacent myofibers but not in adjacent fibroblasts (**Hawke & Garry 2001**). HGF expression has been observed to be proportional to the extent of muscle injury, but has been implicated in a number of roles for satellite cell regulation such as a potent chemotactic factor, activator of satellite cells, and inhibitor of myoblast differentiation (**Hawke & Garry 2001**). C-met receptor activity is essential for satellite cell activation and is expressed by both quiescent and activated satellite cell populations. HGF binds to the satellite cell c-met receptor which initiates satellite cell activation (**Wozniak & Anderson 2007**).

Interleukin-6 (IL-6), a cytokine produced by a number of cells which include myoblasts and macrophages, has its importance in the degradation of necrotic tissue, synchronization of the satellite cell cycle, and inducing apoptosis of macrophages after muscle injury, however, IL-6 is not involved with increases in satellite cell proliferation (**Hawke & Garry 2001**).

It is worth mentioning that the migratory capabilities of satellite cells are dependant on the structural integrity of the basal lamina. A rupture or disruption

of the basal lamina resulting from myotrauma allows satellite cells to infiltrate adjoining myofibers using tissue bridges, whereas when the basal lamina remains intact, satellite cells migrate from the proximal portion of the myofiber to the area of injury (*Hawke & Garry 2001*).

What has been outlined to this point are some of the key aspects involved with the muscle damage and repair process and the remainder of this paper will be focusing on the roles of estrogen, and estrogen receptors. As a result, components of the inflammatory response and satellite cells will be readdressed and further elaborated upon.

1.4 Influence of Estrogen

Estrogens are 18-carbon steroid molecules secreted primarily by the ovaries as well as, but to a lesser extent, from the testes, and the adrenals in both males and females (*Kendall & Eston 2002*). Estrogen is the term used in reference to three structurally similar steroid hormones, estradiol-17 β (E2), estrone (E1), and estriol (E3) but E2 is the primary form of estrogen found in humans, as well as having the greatest estrogenic properties and as a result, E2 is the most commonly studied (*Kendall & Eston 2002*).

Estrogen is believed to act as an antioxidant or membrane stabilizer (*Komulainen et al 1999; Warren et al 2001; Tidball & Wehling-Henricks 2007*) and it is speculated based upon observations made with exercise-induced myofiber damage that estrogen may act to improve the structural integrity of the plasma membrane by a receptor-mediated effect and/or antioxidant property (*Komulainen et al 1999*).

An antioxidant is a molecule that has the capability to neutralise unpaired electrons from free radical species (**Kendall & Eston 2002**). A common feature of these molecules is that they have a carbon-ring structure originating from phenol species, which have one or multiple hydroxyl groups, allowing for their ability to reduce electrons (**Kendall & Eston 2002**). Lipid peroxidation, initiated by hydroxyl radicals attacking polyunsaturated fatty acids in membranes, can cause oxidative damage and possibly lead to membrane instability (**Kendall & Eston 2002**). Estrogen possesses a hydroxyl group on its A (phenolic) ring, which is in the same position and configuration observed with vitamin E, and thyroxine (**Kendall & Eston 2002**). Therefore, estrogen has been speculated as possessing antioxidant properties as it can potentially donate hydrogen atoms from the phenolic hydroxyl group and terminate reactions resulting from peroxidation (**Kendall & Eston 2002**).

Estrogen is a lipophilic structure and fat-soluble in nature which gives it membrane stabilization properties (**Kendall & Eston 2002; McClung et al 2006**). Binding of estrogen to the phospholipid bilayer allows for the possibility of altering membrane fluidity (**McClung et al 2006**). Neutrophil, macrophage, and inflammatory cytokine expression are components of the inflammatory response which are sensitive to ovarian hormones (**Komulainen et al 1999; Tiidus et al 2001; McClung et al 2006; Toumi et al 2006**).

It has been proposed that the mechanisms underlying estrogen's incorporation into cell membranes, would act in a similar fashion as cholesterol, and act to increase the efficiency of membrane fluidity and arrangement of

polyunsaturated fatty acids, thereby limiting disruptions to the muscle membrane after injury (**Tiidus 2003**). Evidence of estrogen's potential as a membrane stabilizer is the reduced CK leakage in females and estrogen treated muscle (**Tiidus et al 2001; Tiidus 2003**).

There is evidence suggesting estrogen possesses an inhibitory effect on muscle post-damage leukocyte infiltration (**St. Pierre Schneider et al 1999; Tiidus & Bombardier 1999; Stupka & Tiidus 2001; Tiidus et al 2001**). With the use of rats, females demonstrated lower levels of leukocyte infiltration than males. In fact, male rats supplemented with estrogen 14 days prior to exercise had similar results as females in that there is an attenuation of neutrophil infiltration (**Tiidus & Bombardier 1999**).

Testing for markers of neutrophil infiltration has commonly been conducted using measurements of MPO activity (**Tiidus et al 2001**) as MPO activity resides predominately in neutrophils (**Tiidus & Bombardier 1999, Tiidus et al 2001**). Female rats demonstrated reductions in skeletal muscle neutrophil infiltration as dictated by muscle myeloperoxidase (MPO) activity 24 hours post-exercise in comparison to males (**Tiidus & Bombardier 1999**). Comparatively, male rats that were administered estrogen for 2 weeks prior to exercise, demonstrated attenuated muscle MPO activity which resembled results observed with females (**Tiidus & Bombardier 1999**).

Attenuation of neutrophil infiltration 1-2 hours post-exercise can be influenced by estrogen acting as membrane stabilizer, influencing membrane fluidity and function either by: 1) Direct nonspecific interactions with

phospholipids, or 2) with other membrane components (*Tiidus et al 2001; Tiidus 2003*).

Limited neutrophil post-damage infiltration could be explained by estrogen's potential of inhibiting calpain activation, and therefore subsequently decreasing the creation of neutrophil chemoattractant peptides (*Tiidus et al 2001; Tiidus 2003*). As a result, reduced muscle membrane disruptions can limit imbalances in calcium homeostasis, and indirectly prevent the up-regulation of calpain (*Tiidus 2003*). Tiidus and colleagues demonstrated that estrogen supplementation did attenuate neutrophil infiltration, MPO, and calpain activity in the gastrocnemius and plantaris muscles of rats (*Tiidus et al 2001*).

Besides the attenuation of neutrophil infiltration, females also had a delayed infiltration of macrophages compared to males (7 and 5 days respectively) after running exercise (*St. Pierre Schneider 1999; Zai et al 2000; Tiidus 2003*).

With ovariectomised rats, macrophage concentrations remained increased at day 7 of recovery (*McClung et al 2006*). Phagocytic ED1+ macrophages in soleus muscle of ovariectomised females were still elevated at day 7 (*McClung et al 2006*). ED1+ macrophages do not decline until the completion of tissue necrosis during reloading recovery (*McClung et al 2006*). Therefore, prolonged elevation of ED1+ macrophages indicate delays in the removal of apoptotic neutrophils and/or continued removal of necrotic myofiber debris (*McClung et al 2006*). Delays in functions performed by ED1+ could influence the infiltration of ED2+ macrophages which occurs in the latter portion of ED1+ macrophage

accumulation in intact muscle and is affected by ovariectomy (**McClung et al 2006**). It is speculated that alterations in the phagocytosis of necrotic tissue and muscle regeneration stimulation by macrophage subpopulations may be related to altered inflammatory signalling (**McClung et al 2006**).

Estradiol has been demonstrated to play a role in skeletal muscle recovery following hindlimb suspension in female rats (**Moran et al 2006**). Failure of atrophied skeletal muscle to repair itself in ovariectomised rats is attributed to the reduced activation of Akt-p70^{S6k} signalling pathway, indicating that proteins involved in initiation of translation are affected by ovarian hormones (**Moran et al 2006**). Protein synthesis initiation in muscle is governed by the target of rapamycin (mTOR) of the Akt/mammalian signaling pathway (**Glass 2003**). Activating mTOR of p70^{S6k} causes the ribosomal protein S6 to be phosphorylated which causes an increase in translation of mRNA (**Bolster et al 2004**). It has been demonstrated that the Akt/mTOR pathway can be upregulated or downregulated during periods of muscle growth and muscle atrophy respectively (**Sitnick et al 2006**). Synergistic ablation, which is used as a hypertrophic model, causes increases in activation of Akt and p70^{S6k} however, hypertrophy can be blocked using rapamycin (a selective inhibitor of mTOR) (**Bodine et al 2001**). Overexpression of p70^{S6k} leads to increases in cell size (**Fingar et al 2002**) while removal of p70^{S6k} tends to lead to reductions in muscle mass (**Ohanna et al 2005**). Therefore, the Akt-mTOR pathway, more specifically p70^{S6k} is implicated as the possible target for skeletal muscle growth (**Sitnick et al 2006**) and circulating estrogens have demonstrated their ability to activate the Akt-mTOR

signaling pathway in a number of tissues (*Patten et al 2004; Pedram et al 2002*).

There are several satellite cell markers that are used to help identify satellite cells in the quiescent, activated, and/or proliferative stages of the cell cycle. Identification of protein products from muscle regulatory factor genes such as myf5, MyoD, myogenin, and MRF4 in the nuclei of activated satellite cells, as well as transcripts of muscle regulatory factors such as MyoD in perinuclear cytoplasm, can be used to identify satellite cells in both regenerating muscle and myogenic precursor populations between fibers (*Wozniak et al 2005*). However, quiescent satellite cells do not express MyoD and MEF2 myogenic regulatory factors or any known markers of terminal differentiation (*Hawke & Garry 2001*). Research has led to the identification of the paired boxed transcription factor pax-7 which is selectively expressed in quiescent and activated satellite cells (*Seale et al 2000; Hawke & Garry 2001*). Research focussing on skeletal muscle with the pax-7 mutation revealed complete absence of satellite cells which suggests, that pax-7 is essential for specification of satellite cell populations (*Seale et al 2000; Hawke & Garry 2001*).

The use of pax-7 provides the ability to identify satellite cell activation in response to tissue injury. Tiidus and colleagues demonstrated that there was an increase in the number of pax-7 positive satellite cells in soleus and white vastus muscles in estrogen supplemented male rats compared to unsupplemented male rats 72 hours post-exercise (*Tiidus et al 2005*). However, because pax-7, labels satellite cells that are quiescent, activated, and proliferating (*Hawke & Garry*

2001; *Enns & Tiidus 2008*), it is unclear as to which stage(s) of satellite cell activation is influenced by estrogen (*Enns & Tiidus 2008*).

A recent study conducted by Enns and Tiidus revealed increases in the number of fibers containing activated (MyoD-positive) and proliferating (BrdU-incorporated) satellite cells in the estrogen-supplemented groups compared to the sham group were observed (*Enns & Tiidus 2008*). The authors speculate that estrogen exerts its effects upstream of satellite cell activation as differences between the number of fibers containing activated and proliferating satellite cells were not observed (*Enns & Tiidus 2008*).

Even though demonstrated with disuse-induced muscle atrophy in rats, ovarian hormone loss delays muscle mass recovery in rat skeletal muscle and therefore implicates estrogen's importance for timely recovery of muscle mass following disuse atrophy in female rats (*McClung et al 2006*).

Observations of diminished skeletal muscle contractility following the loss of ovarian hormones were found in both the soleus and extensor digitorum longus (EDL) muscle of adult female mice (*Moran et al 2007*). Muscle function loss is not due to declines in the contractile protein content, but rather from alterations in myosin function (*McClung et al 2006*). More specifically the proportion of strong-binding myosin during contractions was 15% lower in muscles from ovariectomised mice compared to controls (*Moran et al 2007*).

Estradiol has the ability to reverse the effects of ovariectomy-induced contractile dysfunction indicating that this is a hormone involved in the regulation of skeletal muscle function in mice (*Moran et al 2007*). The proposed

mechanism by which estradiol restores contractility is by increasing the number of strongly-bound myosin as indicated by electron paramagnetic resonance (EPR) spectroscopy and active stiffness measurements (*Moran et al 2007*).

Besides contractile dysfunction, reductions in calcium-activated force have been observed in the soleus and EDL muscle fibers of ~20% following ovariectomy in rodents (*Moran et al 2007*). In conjunction, the proportion of strong-binding myosin heads during contraction was reduced in fibers from ovariectomised mice (*Moran et al 2007*). However, ovariectomised-induced reductions in strong-binding myosin can be reversed with estradiol administration as measured using EPR spectroscopy, site-directed spin labelling of myosin, and indirectly by active stiffness measurements (*Moran et al 2007*). As a result, it is speculated that myosin is the protein which is affected by estradiol and responsible for the ovariectomised-induced reduction in force-generation capacity (*Moran et al 2007*).

1.5 Skeletal Muscle Estrogen Receptors

Small amounts of estrogen receptor alpha and beta (ER- α and - β) (*Aizawa et al 2008*) are present in mouse, rat, and human skeletal muscle (*Harrington et al 2003*) suggesting that this tissue is sensitive to estrogen (*Moran et al 2007*).

It has been observed that there are certain domains of ER α and ER β showing a high degree of similarities, specifically the DNA- and ligand-binding domains (97% and 60% respectively) where as the similarities at the N terminus are quite small with only 18% homology (*Hall et al 2001*).

Four pathways have been identified for the mode of action undertaken by estrogen receptors (ER's) and are as follows: 1) classical ligand-dependant, 2) ligand-independent, 3) DNA binding-independent, and 4) cell-surface (non-genomic) signalling (**Hall et al 2001**). For the purpose of this review, only pathways 1 and 2 will be elaborated upon.

The classical ligand-dependant mechanism of ER action is the defining aspect of class I members of the nuclear steroid/thyroid receptor family for which ER α and ER β are members (**Hall et al 2001**). Binding of a ligand to the ER results in conformational changes of the ER causing high affinity binding to specific DNA response elements (ERE's) located in the regulatory regions of target genes to either stimulate or repress transcription (**Hall et al 2001; Harrington et al 2006**).

It has been accepted that ER's functions can be mediated through extracellular signals in the absence of estrogen (E2) in a ligand-independent fashion via phosphorylation of ER by cellular kinases (**Hall et al 2001**). Findings which have lent support to a ligand-independent pathway surround the ability of polypeptide growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) to activate ER and increase expression of ER target genes (**Hall et al 2001**). EGF and IGF-1 induce mitogen-activated protein kinase (MAPK) causing phosphorylation of serine 118 of ER α . Increasing activation factor-1 (AF-1) activity (**Campbell et al 2001**) by the (MAPK) pathway treated with EGF and IGF allows for the receptor to interact with ER α -specific coactivator p68 RNA helicase and activate target gene transcription (**Hall et al 2001**). It is

believed that this process may serve as a way in which to amplify growth factor pathways and enhance mitogenesis within ER-positive pathways. ER can alter transcription at certain sites via not binding directly to DNA (*Harrington et al 2003*). At these sites, ER imposes its effects by joining with transcription factors such as AP-1 or Sp1 (*Harrington et al 2003*). Conversely, ER's can transrepress gene expression by DNA inhibition or the activity of certain transcription factors such as NFkB which in turn is speculated to cause inhibition of the IL-6 gene (*Harrington et al 2003*).

The estrogen/ER pathway has not been fully defined; however, there is evidence supporting the ER's binding to p85 subunit of Phosphatidylinositol-3-OH kinase (PI3K) in a ligand-dependant manner (*Lee et al 2005*). PI3K is necessary for estrogen/ER signalling as it activates serine-threonine kinase Akt by phosphorylation (*Lee et al 2005*). As Akt modulates the functions of numerous substrates involved with cell functions, the PI3K/Akt signalling pathway is responsible for functions such as cell proliferation, cell survival and cell cycle progression (*Lee et al 2005*).

Little is known concerning regulation and expression of protein kinase B (PKB), but it has been identified as a downstream target of PI3K activation (*Coffer et al 1998*). It appears that PKB is up-regulated as cells become more terminally differentiated (*Coffer et al 1998*).

Physiological effects of estrogen are mediated by the ER which is a ligand-inducible nuclear transcription factor (*Brzozowski et al 1997*). Hormone binding to the ligand-binding domain (LBD) of the ER initiates events leading to

activation or repression of target genes (*Brzowski et al 1997*). Agonists and antagonists bind the same site within core of the LBD but with different binding modes as demonstrated by the crystal structures of the ER LBD bound to either 17β -oestradiol or the antagonist raloxifene at resolutions of 3.1 and 2.6Å respectively (*Brzowski et al 1997*).

ER's have been shown to be upregulated during periods of exercise in animals and humans (*Lemoine et al 2002b; Wiik et al 2005*) while endurance elevates the expression of the ER- α gene as observed in the gastrocnemius muscle (*Aizawa et al 2008*). Wiik and colleagues also demonstrated that with a seven-week endurance program, levels of the ER α transcripts were increased significantly in female rat gastrocnemius muscle (*Wiik et al 2005*).

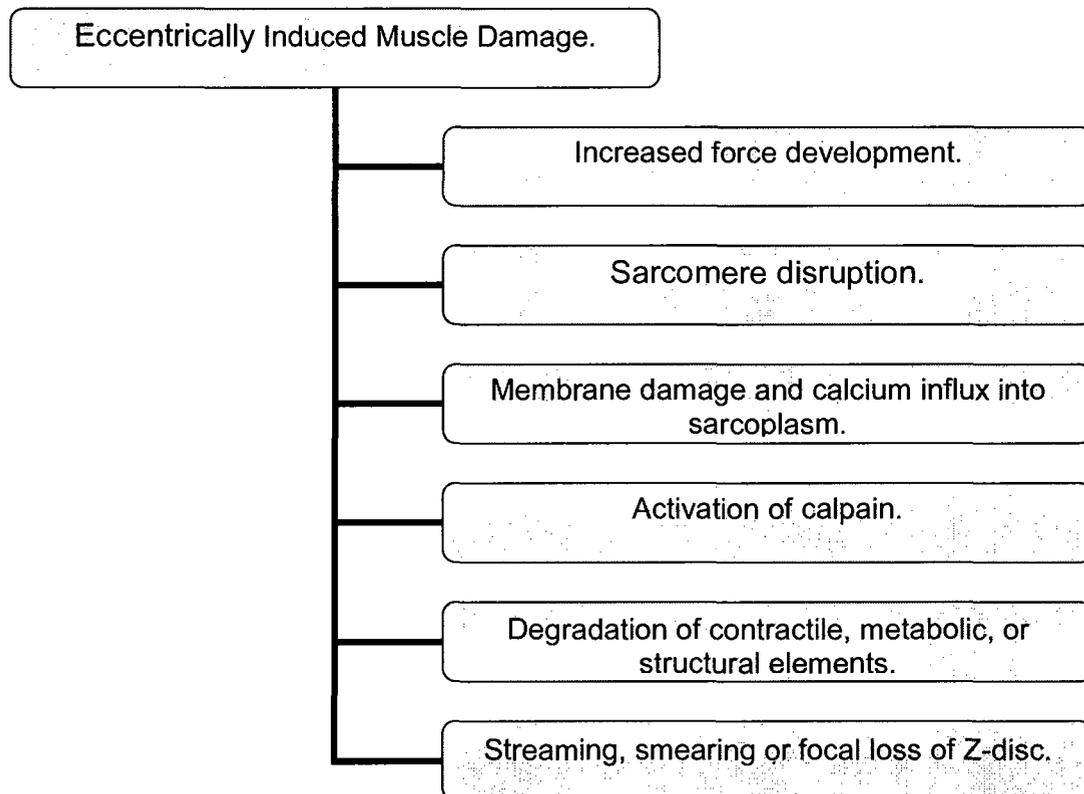


Diagram 1: Sequence of events that eventually lead to muscle damage.

Chapter Two

Effects of estrogen receptor activation on post-exercise muscle satellite cell proliferation

Purpose of Study

To determine if post-exercise satellite cell activation and proliferation is an estrogen receptor-mediated event in ovariectomised rats supplemented with and without estrogen and estrogen receptor agonist PPT.

Hypotheses

1. Estrogen supplementation will result in post-exercise increases in total/activated and proliferating satellite cells using Pax7 and MyoD as markers when compared to controls.
2. ER agonist (PPT) administration will yield similar post-exercise results in satellite cell increases as estrogen supplementation when compared to controls.

Introduction

It has been well documented that skeletal muscle can incur damage due to lengthening contractions (**Armstrong et al 1991, Frieden & Lieber 1992**) and as a result, a sequence of events occurs to aid in the repair process of muscle (**Armstrong et al 1991**). Membrane structures (sarcolemma, t-tubles, and

plasma membrane) are typically damaged as well as other structural damage during eccentric contractions, an inflammatory response is elicited. Neutrophils and macrophages are the primary removers of cellular debris from the area of damage, as well as aiding the initiation of the repair process. When the area of damage is cleared of cellular debris, the repair of damaged fibers can commence which is governed by satellite cells.

Satellite cells reside between the basal lamina and sarcolemma and are considered myogenic precursor cells (**Mauro et al 1961**). Unlike other tissues, adult skeletal muscles are terminally differentiated and as a result muscle growth and regeneration occur by way of the use of satellite cells (**Hawke & Garry 2001**). Satellite cells remain in a non-proliferative quiescent state until they are stimulated to enter the cell cycle (**Hawke & Garry 2001**). Upon stimulation, such as myotrauma or overload injury, satellite cells are activated, proliferate, and then differentiate to either fuse with existing damaged fibers or align and fuse to give rise to a new fiber (**Hawke & Garry 2001**). The mechanism(s) by which satellite cells enter their cell cycle are under the control of a number of factors which include nitric oxide (NO), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) (**Tatsumi et al 1998, Anderson 2000, Machida & Booth 2004**).

The influence of estrogen on indices of muscle damage, as well as the inflammatory response has been well documented; however, very little information regarding its influence(s) on the repair process is not as clear. Tiidus and colleagues attempted to address this question and revealed that in the presence of estrogen, there was a further marked increase in satellite cells post-

exercise in both the white vastus and soleus muscles which is significantly greater than the increase in shams (**Tiidus et al 2005**). A second study that was conducted by Enns and colleagues focussed upon satellite cell augmentation in the presence of estrogen and if muscle estrogen receptors mediate its effects by supplementing animals with an ER antagonist (ICI 182,780). Their results revealed observable increases in total, activated, and proliferating satellite cells in estrogen-supplemented animals compared to shams (**Enns & Tiidus 2008**). The aforementioned studies implicate estrogen as being able to influence the repair process; however, the mechanism(s) by which this is accomplished is still unclear. One possible explanation is that estrogen can stimulate activation via an estrogen receptor (ER) mediated event. Mammalian skeletal muscles contain two subtypes of estrogen receptors: estrogen receptor- α and $-\beta$ (**Lemoine et al 2002a, Wiik et al 2003, Kalbe et al 2007**) and are upregulated with exercise training (**Lemoine et al 2002b, Wiik et al 2005**). There a number of intracellular signalling pathways that estrogen receptors regulate which includes the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) pathway. The PI3K/Akt pathway is involved in the regulation of cell functions which include cell proliferation, cell survival, and cell cycle progression (**Coffer et al 1998, Lee et al 2005, Patten et al 2004, Sitnick et al 2006**).

Based on previous research (**Tiidus et al 2005, Enns & Tiidus 2008**) in conjunction with what is known regarding the PI3K/Akt pathway, it is possible that estrogen's ability to increase satellite cell populations is mediated via estrogen receptors.

The premise of the current study stems from a study conducted by Enns and colleagues whereby they investigated if post-exercise increases in satellite cells were mediated via estrogen receptors (**Enns et al 2008**). With the aid of an estrogen receptor antagonist (ICI 182,780), they revealed that in the presence of the antagonist, any effects exercise and/or estrogen had on increasing satellite cells was completely abolished (**Enns et al 2008**).

For the current study an estrogen receptor-alpha (ER- α) agonist, propylpyrazole triol (PPT), was employed to elucidate the role of estrogen receptors in the activation of satellite cells in ovariectomised rats supplemented with and without estrogen. PPT is an agonist which specifically binds the estrogen receptor-alpha (ER- α) subtype with a 410-fold greater affinity than that of ER- β (**Harris et al 2002, Stauffer et al 2000**). The effects of estrogen and PPT administration on satellite cell activation was studied using the soleus (primarily type-I fiber) and white vastus (primarily type-II fiber) muscles. Pax7 and MyoD were used as markers of satellite cell activation and proliferation respectively. Besides quantifying satellite cells, β -glucuronidase activity was measured to provide an indirect marker of muscle damage.

Methods & Materials

Animals: Ovariectomised female Sprague-Dawley rats are used with their ovaries surgically removed at approximately 9 weeks of age, and arrived at the lab approximately 10 weeks old. Rats were housed 2 per cage on a 12:12 light/night cycle while food and water are given freely in the Animal Care Facility (ACF).

Experimental Protocol: Rats were assigned randomly to treatment groups after one week of acclimation in the ACF. Surgeries performed were as follows: shams (sham procedure), estrogen (receive estrogen pellet), agonist (receive agonist injection), estrogen and agonist (receive estrogen pellet and agonist injection). Each group was then further subdivided into either exercise or non-exercise groups.

Estrogen supplementation was accomplished using subcutaneous pellet implantation. Estrogen pellets were 0.25mg of 17β -estradiol with a 21-day time release (Innovative Research of America, Sarasota, FL).

The ER- α agonist, 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), (Tocris Cookson Inc., Ellisville, MO, USA), was dissolved in dimethyl sulfoxide (DMSO) and injected subcutaneously. Animals were injected with PPT (0.5mg of PPT dissolved in 0.1ml of DMSO) or placebo (DMSO) commencing approximately 3 days post-surgery for a duration of 6 days.

Under aseptic conditions, estrogen pellet implantation and shams are performed on rats first anaesthetized by isoflurane inhalation. Estrogen

supplemented rats had a small incision in the skinfold of their necks, their skin separated from fascia with blunt dissection and pellet insertion ~ 1cm from incision site. Shams under went the same procedure performed without pellet insertion. Incisions were sealed using Vetbond (3M, St. Paul, MN). Recovery time was approximately 9 days from surgery to tissue collection. Recovery time allowed for the pellet-implanted group to receive prolonged exposure to estrogen.

Familiarization of the animals to the motorized rodent treadmill (Columbus Instruments, Columbus, OH) was conducted 3-4 days after surgery. Familiarization sessions were performed twice for five minutes (once on each of the succeeding 2 days before engaging in the full exercise protocol). The exercise protocol involved the animals running downhill intermittently (Run: 5mins, Rest: 2mins) for a total of 90 minutes of running time. The speed of the treadmill was set to 17m/min at an incline/decline of -13.5° grade (**Komulainen et al 1994**). This exercise protocol has been proven to be non-fatiguing, but able to elicit sufficient/significant damage in soleus and white vastus muscles (**Armstrong et al 1983, Han et al 1999**).

Tissue Collection: Animals were sacrificed 72 hours post-exercise by way of an overdose of sodium pentobarbital (55mg/kg). Blood samples, uterus, soleus and superficial (white) portions of vastus muscles were removed. Controls (non-exercisers) were sacrificed at the same time points as exercisers.

Blood samples were collected from the femoral artery, allowed to clot at room temperature, centrifuged at 3000g's for 10 minutes, and serum removed and stored at -20°C.

Muscle samples were rinsed in saline, blotted dry and trimmed of connective tissue and blood vessels. Samples used for immunohistochemical analysis were mounted on a specimen holder coated in Optimal Cutting Temperature (OCT) medium (Tissue-Tek, Torrance, CA) and frozen in isopentane (previously cooled in liquid Nitrogen). Samples used for enzyme and protein analyses were frozen immediately in liquid nitrogen and stored at minus 20°C until analysis. Timeline and sequence of experimental events (such as pellet implantation, running program, sacrifice, etc) are depicted in diagram 2.

Serum Analysis: Serum estrogen levels were determined in duplicate using a commercially produced radioimmunoassay kit (Coat-a-Count TKE21, Inter Medico, Markham, ON).

Beta-Glucuronidase Activity: Lysosomal acid hydrolase, beta-glucuronidase, activity was measured in the white vastus and soleus muscles using the method described by **Koskinen et al (2001)**. Samples were assayed in triplicate at 420nm and activities expressed as the amount of substrate (5mM p-nitrophenyl-beta-D-glucuronide, Sigma-Aldrich) hydrolyzed per incubation time and amount of protein, while the protein concentration was measured using the method of Lowry (**Lowry et al 1951**).

Immunohistochemical Procedures: Immunohistochemical procedures and quantification were performed as previously described (**Enns et al 2008, Enns & Tiidus 2008**). Transverse serial sections of soleus and white vastus were cut 10um in thickness using a cryostat (Leica CM3050S) at -20°C. Sections were mounted on glass slides treated with vectabond (Vectabond Laboratories, Burlington, ON), dried at room temperature and stored at -20°C. Sections were immunostained for markers of total (Pax7), and activated (MyoD). All samples were fixed with cold (4°C) acetone for 10 minutes while endogenous peroxidases were inactivated with 0.6% hydrogen peroxide in absolute methanol, followed by 3 washes in 15mM phosphate-buffered saline (PBS, pH 7.6). Nonspecific sites were blocked for 30 minutes using 5% normal goat serum in PBS (containing 5% nonfat milk powder).

Pax7 immunostaining was accomplished by the slides being incubated with an antibody specific to Pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) for 40 minutes at a dilution of 1:20, while with MyoD immunostaining, slides were incubated overnight at 4°C in an antibody specific for MyoD (Dako Canada, Mississauga, ON) at a 1:50 dilution.

After 3 rinses in PBS, slides were developed using commercially available kits (LSAB-2, Dako Canada) which contain universal biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. Satellite cells were visualized using NovaRed (Pax7) (Vector Laboratories) or diaminobenzidine (DAB) with nickel (MyoD) (Vector Laboratories). All slides were then

counterstained using hematoxylin QS (Vector Laboratories) and mounted using permanent mounting medium (Vector Laboratories).

Quantifying satellite cells encompassed the slides being put under a light microscope (Leica DMLS) and having the image projected onto a computer screen at a 400x magnification (10x ocular and 40x objective lens). Satellite cells were identified via oval-shaped dark blue bodies with red coloured edges located in the vicinity of the sarcolemma.

A total of 400 myofibers were counted from two individual sections from each muscle sample (200 fibers per section x 2 sections). The relative number of myofibers positive for each marker (Pax7 or MyoD) are calculated as the: [number of positive fibers / 400 x 100%].

Statistical Analysis: All data obtained is presented as means \pm SD. In order to assess any differences between groups, a one-way analysis of variance (ANOVA) at a level of significance of $p < 0.05$ was employed. Situations whereby significance is found, post-hoc testing by way of the LSD test was used to evaluate specific differences between groups.

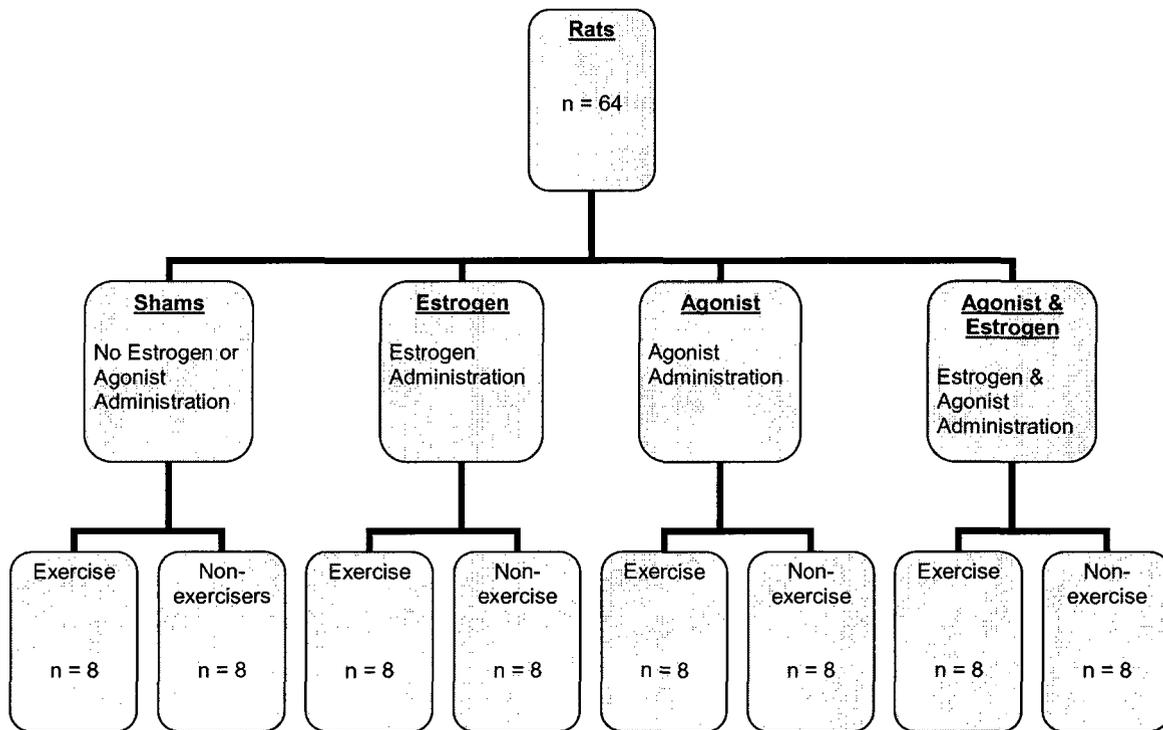


Figure 1: Outline of experimental design. Exercise = exercised condition; Non-exercise = unexercised (control) condition

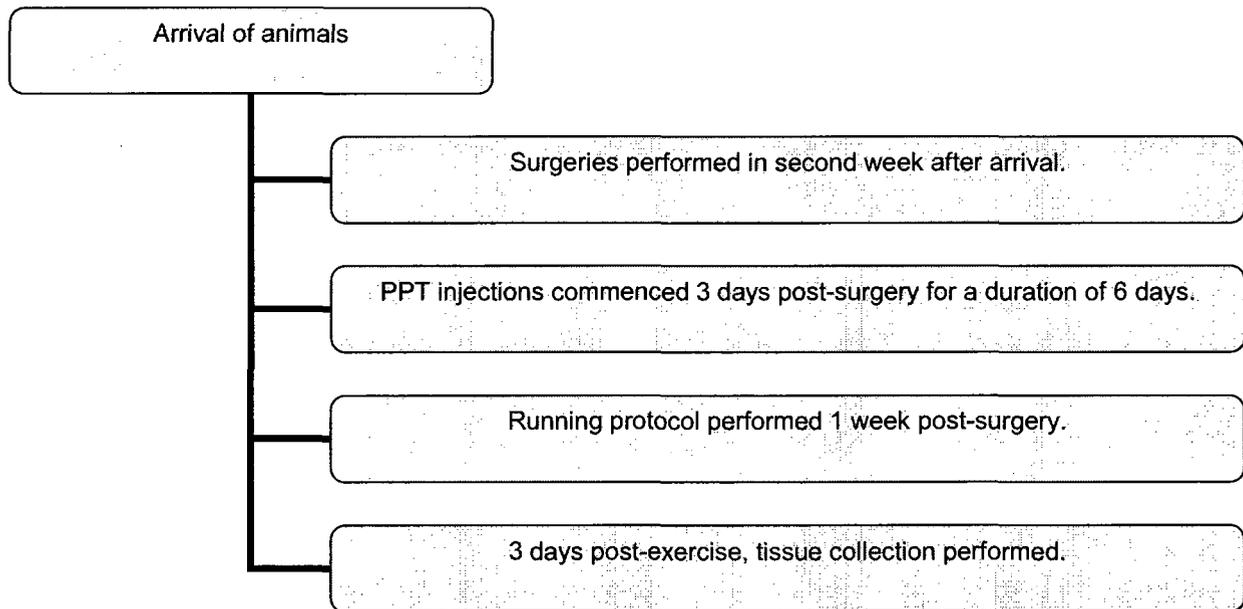


Diagram 2: Timeline of experimental protocol. Sequence and timing of events.

Results

Serum Estrogen Levels

Neither exercise nor time of sacrifice had any bearing on serum estrogen levels, therefore animals were grouped according to the treatment condition to which they were assigned (sham, estrogen-supplemented, PPT-supplemented, estrogen and PPT-supplemented).

As to be expected, figure 2 and table 1 illustrate that the group supplemented with estrogen had a significantly higher ($p < 0.05$) estrogen serum levels compared to both the sham and PPT-supplemented groups. However, there was not a significant difference ($p > 0.05$) between the groups supplemented with estrogen and both estrogen and PPT.

Body Weights

All animals progressively gained weight during the experimental time period. Upon the day of sacrifice, animals in the sham treatment group were significantly greater ($p < 0.05$) in weight than those treated with estrogen, PPT, or both estrogen and PPT. There was not a significant difference ($p > 0.05$) observed when comparing estrogen-supplemented animals with those supplemented with PPT or estrogen and PPT (Table 1 & Figure 3).

Uterine Weights

When binding of estrogen to uterine estrogen receptors occurs, this results in the stimulation of uterine growth. As a result, PPT (an ER-alpha

agonist) would mimic the effects of estrogen and cause increases in uterine weight when bound to uterine estrogen receptors.

Table 2 as well as figure 4 and 5 depicts that sham animals had significantly lower ($p < 0.05$) uterine wet weights in comparison to all other treatment groups (estrogen, PPT, estrogen and PPT). Conversely, animals supplemented with both estrogen and PPT not only had a significantly greater ($p < 0.05$) uterine weights than shams, but also with the groups supplemented with estrogen or PPT alone.

β -Glucuronidase Activity

Lysosomal acid hydrolase, beta-glucuronidase, activity was used as an indirect marker of muscle damage incurred during the downhill running protocol. The soleus muscle did reveal a significant difference ($p < 0.05$) for both exercise and treatment condition (**Table 3**). Exercised rats were observed to have a significantly greater ($p < 0.05$) β -glucuronidase activity compared to controls while the group treated with estrogen and PPT had a significantly lower ($p < 0.05$) post-exercise β -glucuronidase activity with respect to all other treatment conditions (shams, estrogen, PPT). Animals that were either estrogen- or PPT-supplemented also demonstrated a significantly lower ($p < 0.05$) post-exercise β -glucuronidase activity when compared to shams. These trends that were observed with the soleus muscle were not clearly demonstrated with the white vastus muscle. The data obtained for the white vastus muscle did not demonstrate significant increases in muscle damage with exercise or, reductions in β -glucuronidase activity when in the presence of estrogen and/or PPT.

Therefore, this discrepancy in the results obtained for the white vastus muscle poses as a limitation of the study due to the fact that, no muscle damage was observed while there were significant increases in satellite cell activation and proliferation. Previous studies (*Tiidus & Bombardier 2005; Enns & Tiidus 2008; Enns et al 2008*) using the same exercise protocol as was used for the current study, have clearly indicated the changes on post-exercise β -glucuronidase activities.

Satellite Cell Activation

The identification of satellite cells that have been stimulated to enter their cell cycle is accomplished by using Pax7 and MyoD as markers. The number of muscle fibers that stain positively for Pax7 (total/activated satellite cells) and MyoD (proliferating satellite cells) are depicted in **figures 6-9**.

The running protocol that the animals underwent elicited a significantly greater ($p < 0.05$) number of fibers staining positively for total/activated, and proliferating satellite cells in both the white vastus and soleus muscles compared to controls. These increases in satellite cells due to the exercise protocol were observed 72 hours post-exercise in all treatment conditions (sham, estrogen, PPT, estrogen & PPT).

It was observed that animals supplemented with estrogen and/or PPT had significantly greater ($p < 0.05$) number of fibers staining positively for Pax7 and MyoD in both the soleus and white vastus muscle when compared to shams. There were no significant differences ($p < 0.05$) between the estrogen, PPT, and estrogen & PPT post-exercise satellite cell counts.

Table 1: Animal body weights and serum estrogen levels for sham, estrogen, PPT, and estrogen & PPT treatment groups. Values were grouped as means \pm SD.
^ap < 0.05 compared to shams.

Treatment	Body Weight (g)	Serum Estrodiol (pg.mg ⁻¹)
Sham	310.59 \pm 13.07	11.3 \pm 3.6
Estrogen	267.69 \pm 11.51 ^a	92.1 \pm 70.8 ^a
PPT	277.72 \pm 9.50 ^a	12.7 \pm 3.7
Estrogen & PPT	259.12 \pm 9.66 ^a	78.2 \pm 42.7 ^a

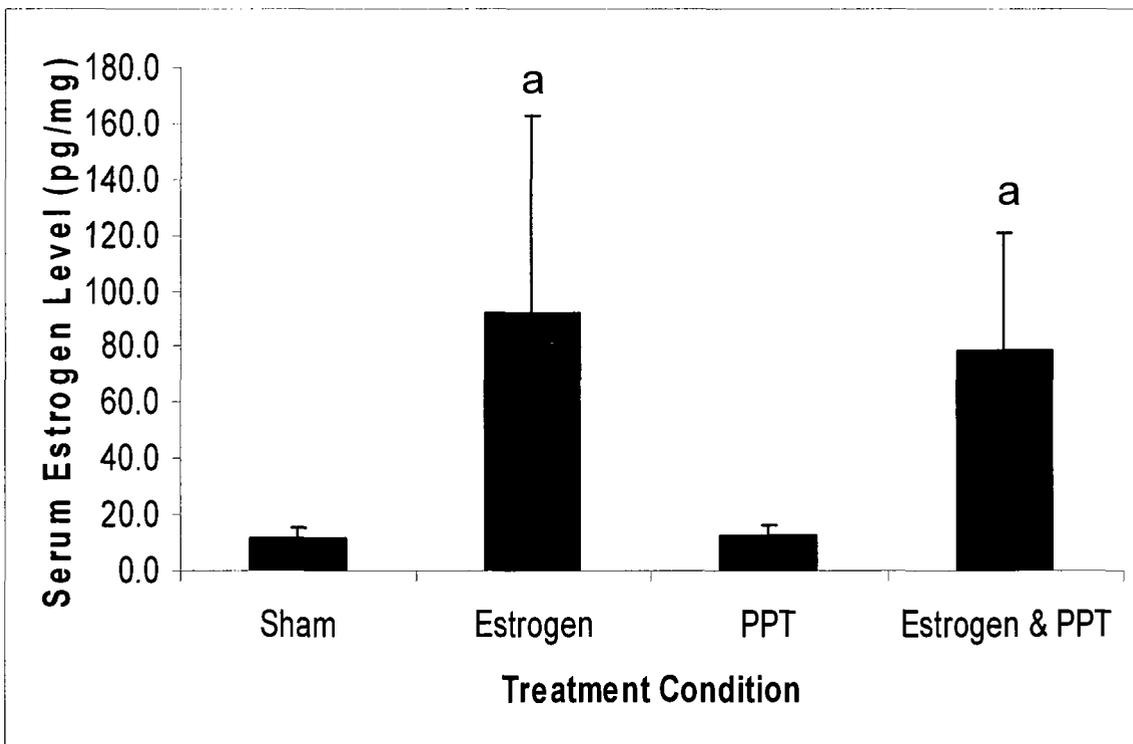


Figure 2: Serum estrogen levels for sham, estrogen, PPT, and estrogen & PPT treatment groups. Values were grouped as means \pm SD.
^ap < 0.05 compared to shams.

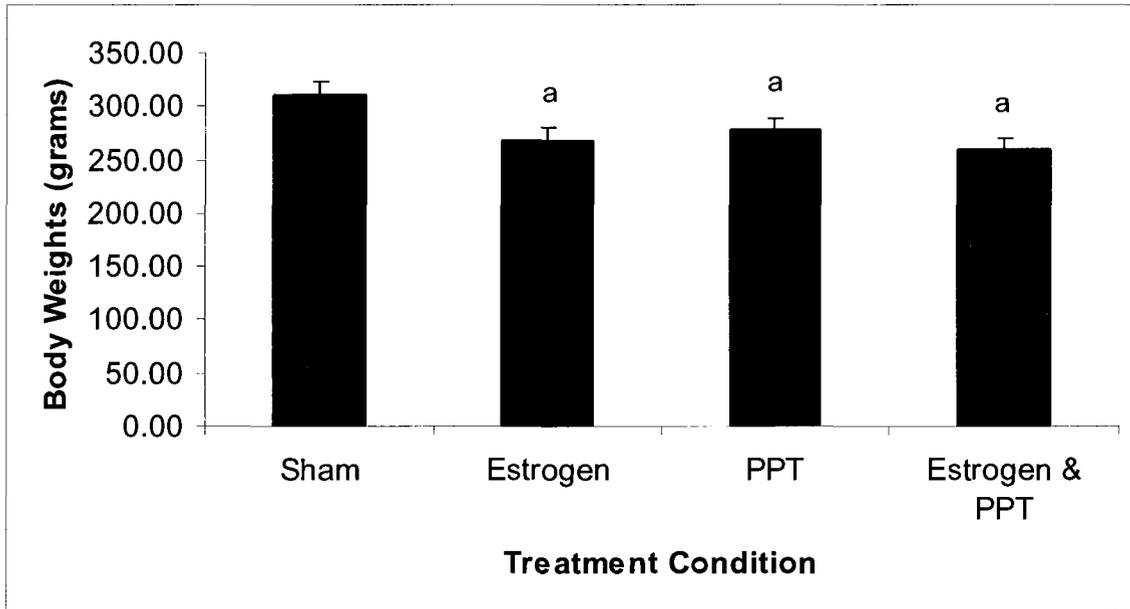


Figure 3: Animal body weights for sham, estrogen, PPT, and estrogen & PPT treatment groups. Values were grouped as means \pm SD.
^a $p < 0.05$ compared to shams.

Table 2: Changes in uterine weights. Values were grouped as means \pm SD.
^a $p < 0.05$ compared to shams.
^b $p < 0.05$ compared to estrogen-supplemented group.

	Sham	Estrogen	PPT	Estrogen & PPT
Wet Weight, g	0.1102 \pm 0.035	0.4087 \pm 0.064 ^a	0.3325 \pm 0.070 ^a	0.3254 \pm 0.084 ^{ab}
Wet Weight, g 100g⁻¹ body weight	0.0350 \pm 0.013	0.1481 \pm 0.041 ^a	0.1194 \pm 0.024 ^a	0.1875 \pm 0.057 ^{ab}

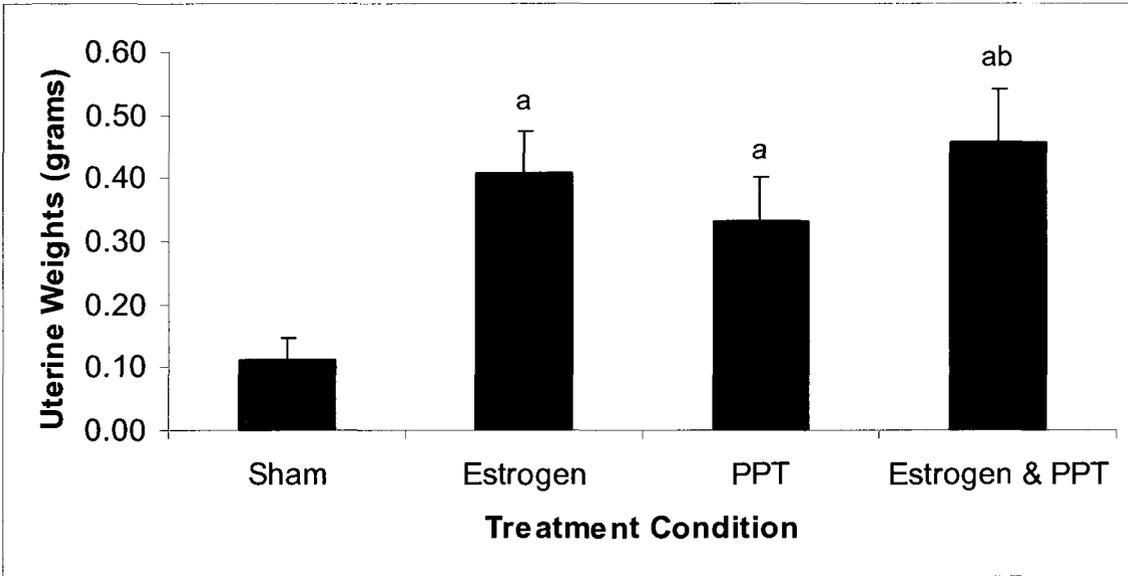


Figure 4: Uterine weights at time of sacrifice. Values were grouped as means \pm SD.

^ap < 0.05 compared to shams.

^bp < 0.05 compared to estrogen-supplemented.

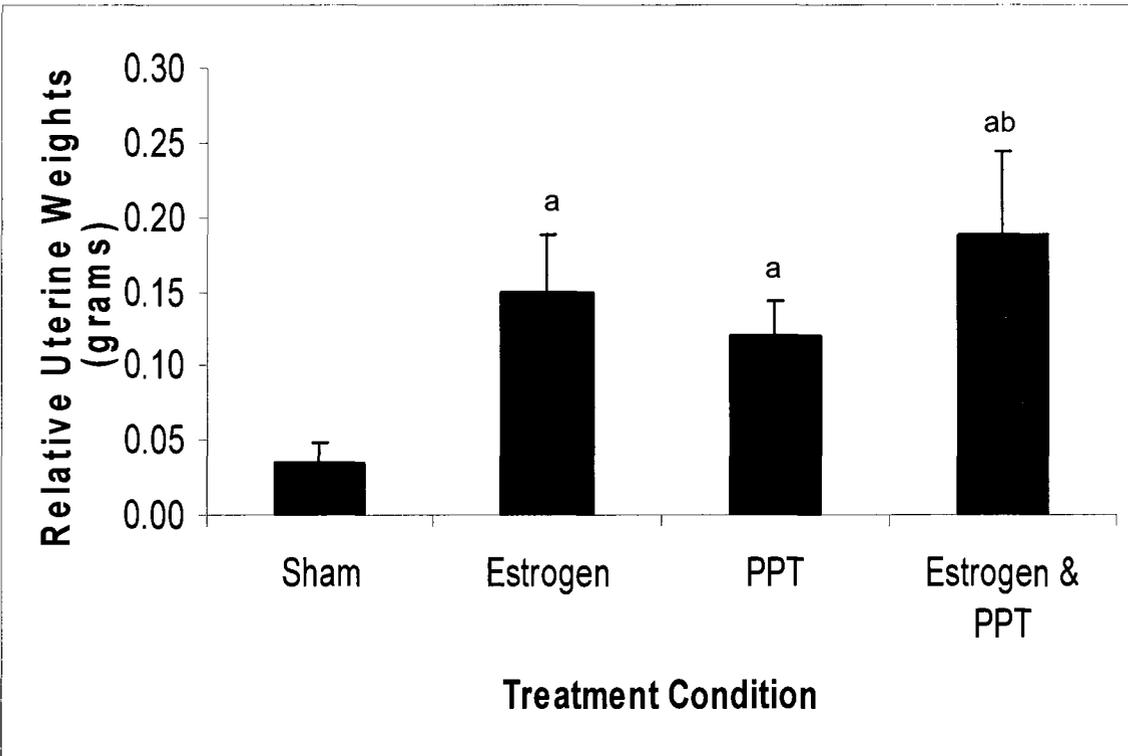


Figure 5: Relative uterine weights at time of Sacrifice. Values were grouped as means \pm SD.

^ap < 0.05 compared to shams.

^bp < 0.05 compared to estrogen-supplemented.

Table 3: β -glucuronidase activity in the white vastus and soleus. Values are listed as means \pm SD.

^ap < 0.05 compared to controls.

^bp < 0.05 compared to shams.

	Sham	Estrogen	PPT	Estrogen & PPT
White Vastus				
Control	2.44 \pm 0.95	2.10 \pm 0.95	3.39 \pm 1.31	2.70 \pm 1.08
Exercise	2.47 \pm 0.82	2.78 \pm 0.62	3.15 \pm 0.92	3.42 \pm 1.07
Soleus				
Control	4.70 \pm 0.97	4.56 \pm 0.58	5.21 \pm 0.50	3.99 \pm 0.83
Exercise	7.19 \pm 2.61 ^a	6.04 \pm 2.83 ^{ab}	5.55 \pm 1.01 ^{ab}	4.42 \pm 0.96 ^{ab}

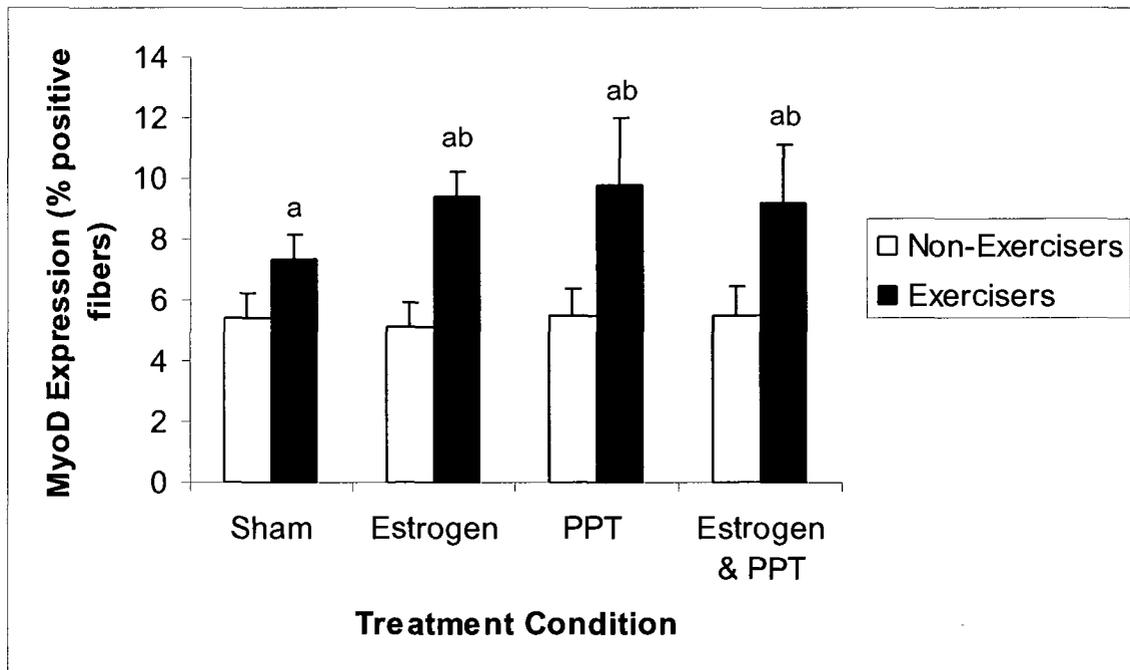


Figure 6: Effects of estrogen- and PPT-supplementation on expression of MyoD satellite cells in rat white vastus muscle 72 hours post-exercise.

Values are listed as means \pm SD.

^ap < 0.05 compared to controls.

^bp < 0.05 compared to shams.

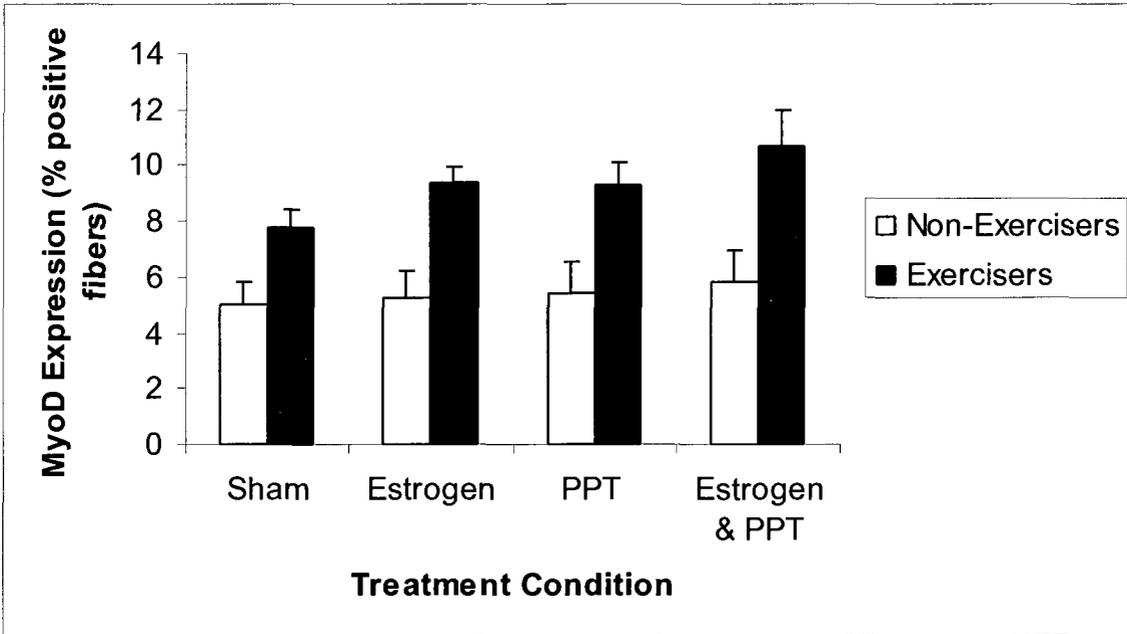


Figure 7: Effects of estrogen- and PPT-supplementation on expression of MyoD satellite cells in rat soleus muscle 72 hours post-exercise. Values are listed as means \pm SD.

^a $p < 0.05$ compared to controls.

^b $p < 0.05$ compared to shams.

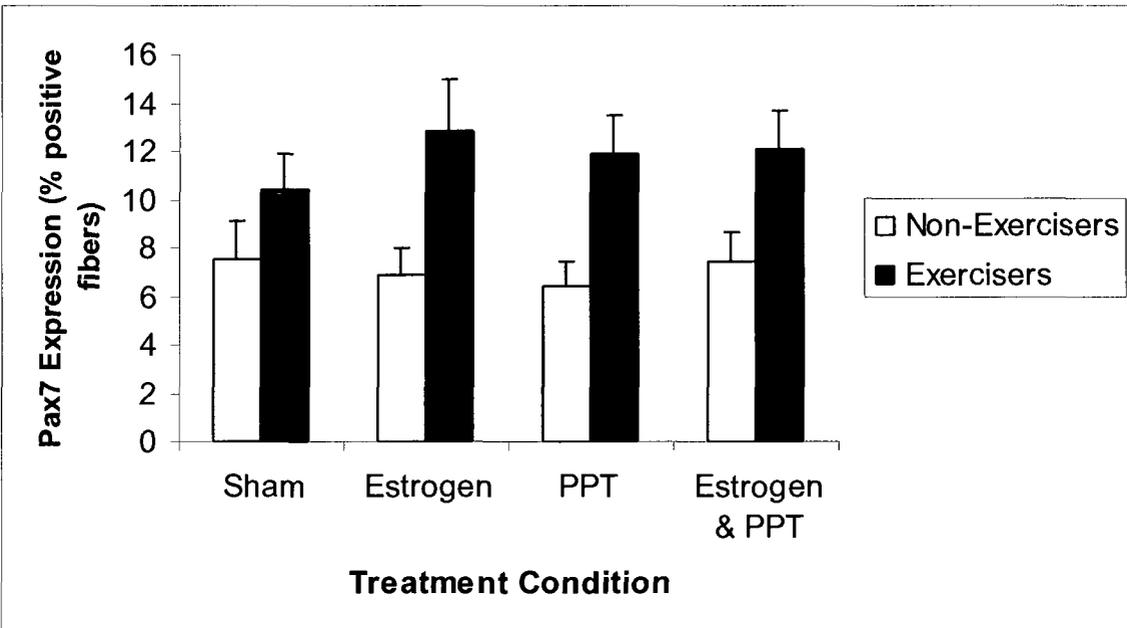


Figure 8: Effects of estrogen- and PPT-supplementation on expression of Pax7 satellite cells in rat white vastus muscle 72 hours post-exercise. Values are listed as means \pm SD.

^a $p < 0.05$ compared to controls.

^b $p < 0.05$ compared to shams.

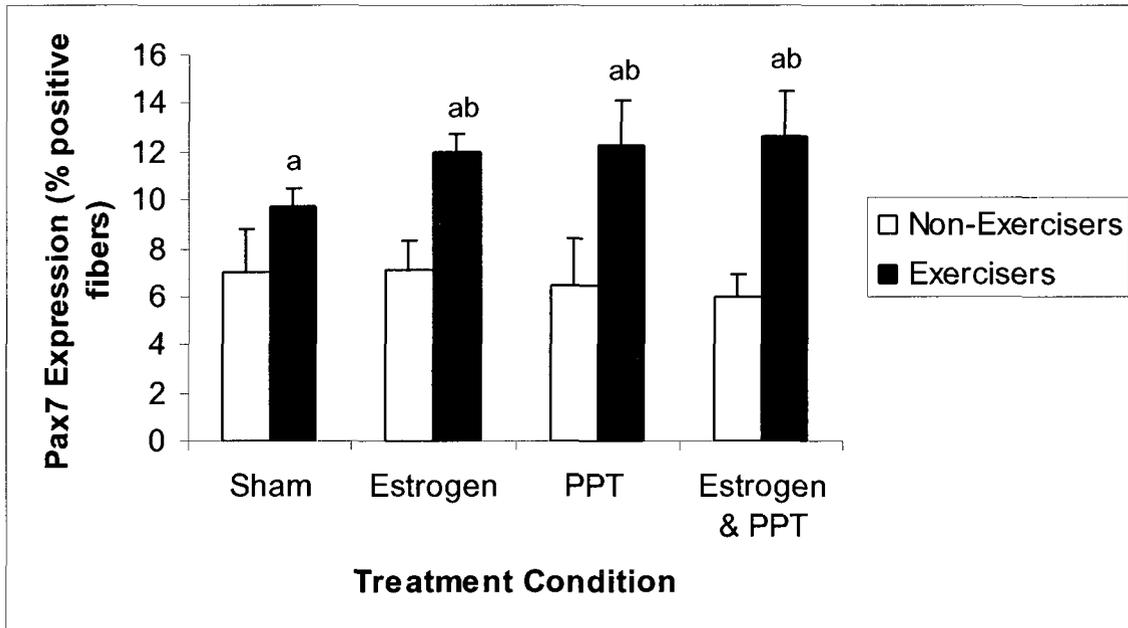


Figure 9: Effects of estrogen- and PPT-supplementation on expression of Pax7 satellite cells in rat soleus muscle 72 hours post-exercise. Values are listed as means \pm SD.

^a $p < 0.05$ compared to controls.

^b $p < 0.05$ compared to shams.

Discussion

Uterine & Body Weights

Propyl pyrazole triol (PPT) is a highly selective, synthetic ER- α agonist developed by ***Harris et al (2002)*** which has a 410-fold greater affinity for ER- α than ER- β (***Harris et al 2002***). Therefore, PPT's selectivity towards the ER- α subtype, renders it unable to activate ER- β . Results obtained by ***Harris et al (2002)*** seem to be consistent with the uterus predominantly expressing ER- α (***Harris et al 2002***) while with the use of ER α KO mice, there was an inability to stimulate uterine weight gain when in the presence of estrogen (***Couse et al 1995***). The aforementioned results leads to the idea that ER- α is necessary for the activation of a full uterotrophic response while ER- β is not required for these estrogenic effects (***Harris et al 2002***).

For the purpose of the current study, uterine wet weights were measured to verify ovariectomy was performed, as well as to provide an indirect measure of global ER saturation. PPT was able to effectively bind to the ER- α 's and cause significant increases in uterine wet weights in relation to shams.

In agreement with previous studies by ***Enns & Tiidus (2008)***; ***Enns et al (2008)***; and ***Iqbal et al (2008)*** estrogen- and/or PPT-supplemented animals had significantly lower body weights in comparison to shams while there was no significant difference observed between the estrogen- and PPT-supplemented groups. Reductions in body weight observed in the PPT-supplemented group was to be expected as illustrated by the idea that binding of the ER- α (in this

specific case, by PPT) is capable of decreasing food intake, meal size, and body weight in OVX rats (**Santollo et al 2007**).

Serum Estrogen Levels

Significant increases in serum estrogen levels were observed in the estrogen-supplemented groups compared to shams. These increases were seven- and eight-fold greater than that of the shams for the estrogen- and estrogen & PPT-supplemented treatment groups respectively while these groups showed no significant difference between each other.

A wide range of serum estrogen levels have been reported in the past in regards to ovariectomised rats receiving estrogen supplementation. Serum estrogen values range from as low as 15-20pg/ml (**Persky et al 2000**) and as high as 285pg/ml (**McNulty et al 2000**). However, even though a wide range of values have been reported regarding estrogen-supplementation of OVX rats, the values ascertained in the current study are similar to previous studies (**Enns et al 2008; Iqbal et al 2008**). The variability of serum estrogen levels reported could be influenced by age and stage of estrus cycle of the animals (**Enns et al 2008**).

Beta-Glucuronidase

Measurement of muscle beta-glucuronidase activity was performed to provide indirect evidence of post-exercise muscle damage incurred by our running protocol. Previous research (**Komulainen et al 1999; Salmien 1985**) has demonstrated that beta-glucuronidase to be a reliable marker of post-exercise muscle damage. Results of the current study are consistent with previous studies (**Enns & Tiidus 2008; Enns et al 2008**) in that there was an

attenuation of beta-glucuronidase activity in the groups supplemented with estrogen. There was no significant difference between the treatment groups supplemented with estrogen, PPT, and estrogen & PPT. In light of the aforementioned point and in conjunction with **Enns et al (2008)** observing no difference between estrogen- and estrogen & antagonist-supplemented groups, one might consider the idea that reduction in beta-glucuronidase activity is not an ER-mediated event.

Satellite Cells

The primary objective of this study was to determine if ER's play a role in the post-exercise activation/proliferation of satellite cells by using an ER- α agonist, PPT. Previous studies have demonstrated that estrogen does influence post-exercise satellite cell numbers (**Tiidus et al 2005; Enns & Tiidus 2008; Enns et al 2008**). However, the mechanism(s) by which estrogen stimulates satellite cells to become activated have yet to be elucidated.

The premise of the current study stems from results yielded by **Enns et al (2008)** whereby they blocked the activity of ER's using an ER antagonist ICI 182,780. They revealed that when in the presence of ICI 182,780, effects of estrogen and/or exercise on increases of satellite cell numbers were completely abolished. The data collected during the aforementioned study implicates satellite cell activation is an ER-mediated event. Therefore, in the presence of an ER- α agonist (PPT), it is speculated that satellite cell activation and proliferation would yield similar results to that observed with estrogen in the white vastus and soleus muscles.

The findings of the current study revealed that there were significant increases in fibers staining positively for markers of satellite cell activation (pax-7) and proliferation (MyoD) 72 hours post-exercise.

The groups supplemented with estrogen and/or PPT yielded similar results when compared to shams. The observance of a non-significant difference between groups supplemented with estrogen and/or PPT can be explained by the notion that mammalian skeletal muscle contains both ER- α and ER- β (*Wiik et al 2003*); however, skeletal muscle contain relatively fewer ER's than that of other tissue (*Katzenellenbogen et al 1995*). Therefore, with the small numbers of ER's present in skeletal muscle, saturation of the ER- α 's can be accomplished when in the presence of estrogen and/or PPT. The data obtained from this study, as well as in conjunction with what was observed by *Enns et al (2008)*, allude to the idea that ER's (more specifically, ER- α) plays a role upstream of the activation of satellite cells. Therefore, the mechanism(s) by which ER- α 's stimulate satellite cell activation is speculated to be under the control of the phosphatidylinositol 3-kinase (PI3K) pathway which is involved with ER signalling when ER's are bound by estrogen (*Campbell et al 2001*). ER's have the ability to bind the p85 regulatory subunit of the PI3K pathway in a ligand-dependant manner (*Simoncini et al 2000*) which causes this pathway to become activated and eventually leading to the phosphorylation of AKT (*Coffer et al 1998*). AKT is responsible for modulating a number of substrates that aid in the regulation of cell functions which include cell proliferation, cell survival, and cell cycle

progression, as depicted in diagram 3 (Vanhaesebroeck et al 2001; Coffey et al 1998; Gaven et al 2004).

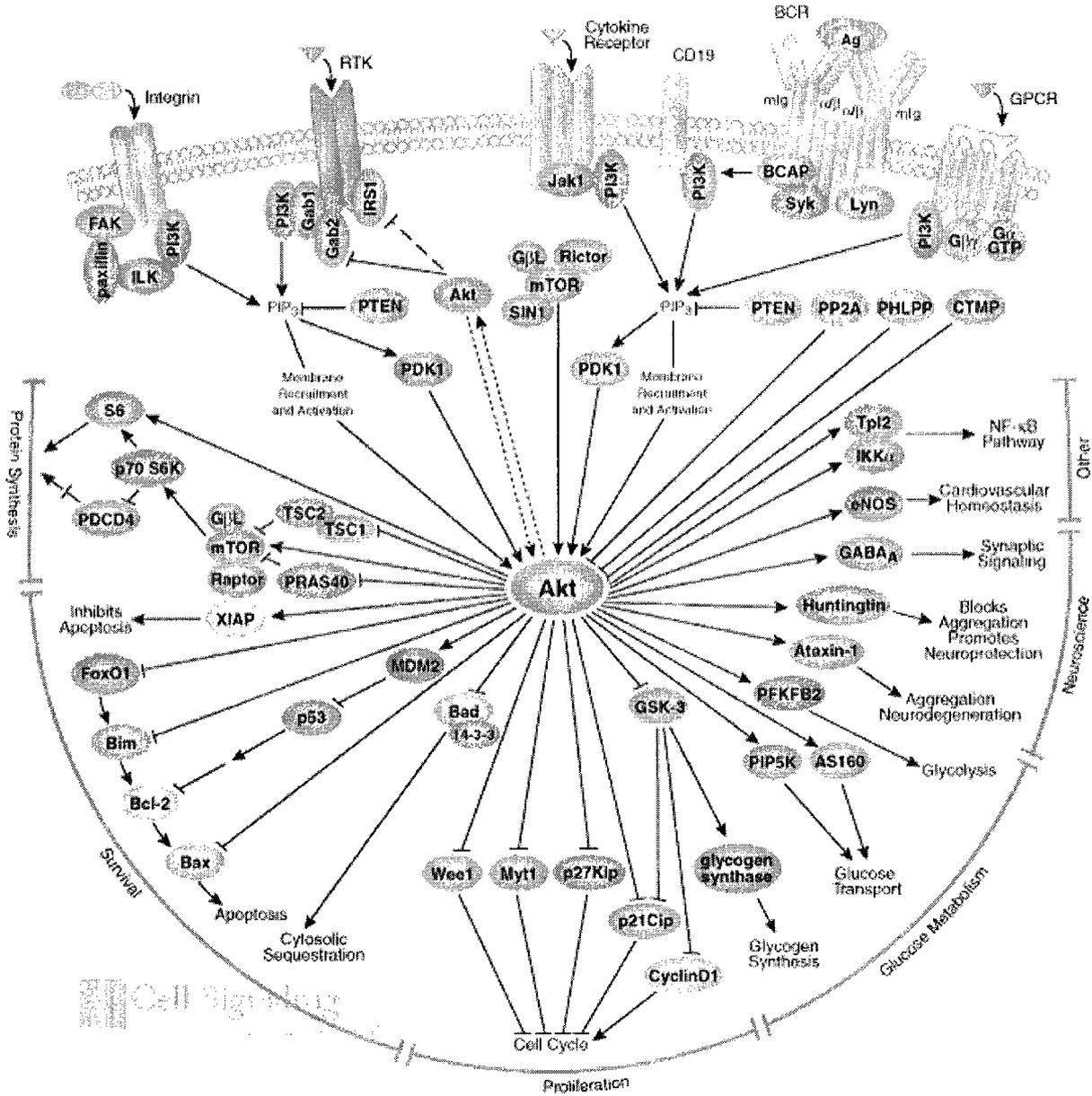


Diagram 3: Schematic of the PI3K/Akt pathway depicting the substrates Akt modulates which aid in the regulation of cell functions. (Cell Signaling Technology 2009).

Chapter Three

Discussion & Conclusion

It has been well documented that estrogen influences the muscle damage and repair process. Estrogen has been demonstrated to affect creatine kinase levels and β -glucuronidase activity which are typically used as indirect markers of post-exercise skeletal muscle damage. In the presence of estrogen, the aforementioned markers are considerably reduced when compared to controls (***Armstrong et al 1983; Komulainen et al 1999***). Therefore, the data lends itself to the idea that estrogen can act as a potential membrane stabiliser and/or antioxidant. In conjunction with affecting the magnitude of post-exercise muscle damage, estrogen plays a role in the alterations of leukocyte infiltration (namely neutrophils and macrophages) in response to skeletal muscle injury. Studies conducted in our lab have consistently demonstrated that when in the presence of estrogen, there is an attenuation in leukocyte infiltration (***Tiidus & Bombardier 1999; Enns et al 2008; Iqbal et al 2008***) and calpain (or at least calpain-like) activity (***Tiidus et al 2001***).

Besides influencing indices of post-exercise skeletal muscle damage (as determined by the markers CK and β -glucuronidase) as well as leukocyte infiltration, estrogen has been implicated in influencing the activity of cytokines such as NO, HGF, and IL-6. The aforementioned cytokines have been observed to contribute to the activation of satellite cells. IL-6, which is produced by a number of cells which include myoblasts and macrophages, aids in degrading necrotic tissue, synchronization of the satellite cell cycle, and macrophage

apoptosis after muscle injury (*Hawke & Garry 2001*). However, estrogen has been identified to down regulate IL-6 activity at the IL-6 promoter (*Pottratz et al 1994*). NO has the potential to be influenced by estrogen during periods of muscle injury (*Simoncini et al 2002; Prorock et al 2003*). Nitric oxide synthase (NOS) and NO levels are increased when in the presence of estrogen and may have an effect on the muscle damage and repair process (*Caulin-Glaser et al 1997; Node et al 1997*). NO has also been identified in influencing HGF, a known regulator of satellite cell regulation (*Tatsumi et al 1998*), and its release and localisation is a NO-dependant process during periods of muscle injury (*Tatsumi et al 1998*).

It has been demonstrated that estrogen may influence the skeletal muscle repair process. A recent study conducted in our lab revealed that estrogen supplemented rats had an elevated number of fibers containing total, activated, and proliferating satellite cells (*Enns & Tiidus 2008*). Therefore, estrogen seems to have a dual function in regard to attenuating indices of post-exercise skeletal muscle damage but, it also positively influences the repair process by activating satellite cells. To further understand and expand upon the research conducted by *Enns & Tiidus (2008)*, they attempted to explore the role estrogen receptors may have on facilitating the activation of satellite cells. *Enns et al (2008)* blocked the ER's using an ER antagonist (ICI 182,780) to aid in determining if ER's were an upstream contributor to satellite cell activation. It was observed that in the presence of ICI 182,720, any effect estrogen and/or exercise had on post-exercise satellite cell numbers was completely abolished (*Enns et al 2008*). This

novel finding was the first study to allude to the idea that satellite cell activation is an ER-mediated event (**Enns et al 2008**). The mechanism(s) of ER's ability to aid in satellite cell activation is still unclear however, the data collected by **Enns et al (2008)** allowed for the current study to attempt answering the question, "is satellite cell activation an ER-mediated event." The current study attempted to address the aforementioned question using an ER- α agonist (PPT) rather than the antagonist.

Propyl pyrazole triol (PPT) is a highly selective, synthetic ER- α agonist developed by **Harris et al (2002)** which has a 410-fold greater affinity for ER- α than ER- β (**Harris et al 2002**). Therefore, PPT's selectivity towards the ER- α subtype, renders it unable to activate ER- β . The current study revealed that when in the presence of the ER- α agonist, PPT, there was a significant increase in the number of fibers staining positive for activated and proliferating satellite cells when compared to shams. Due to observing no significant difference between the PPT- and estrogen-supplemented treatment conditions, there seems to be evidence to support the notion that ER- α is responsible for most, if not all of the activation of satellite cell activity when bound by estrogen.

There seems to be evidence suggesting estrogen and ER's may regulate biological processes via different pathways (**Hall et al 2001**). It is believed that ER's bound by estrogen can either bind directly to estrogen response elements (ERE's) which are located in the promoter regions of target genes or, can interact with other transcription factor complexes such as Fos/Jun (AP-1 responsive

elements) to initiate transcription of genes not containing ERE's in their promoter regions (**Heldring et al 2007**).

It has been speculated that a potential mechanism by which ER- α 's exert their effects is via the PI3-K pathway. The PI3-K pathway has been identified as being responsible for functions such as cell proliferation, cell survival, and cell cycle progression (**Lee et al 2005**).

Besides ER's exerting their effects when bound by estrogen, ER's have the ability to function when estrogen is not present. Epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) have been identified to stimulate ER's causing increases in the expression of ER target genes (**Hall et al 2001**). It is speculated that when levels of estrogen are low, as seen with males, that hormone-independent pathways can allow for ER activation (**Hall et al 2001**).

With what is known regarding the role ER- α 's may have on the skeletal muscle repair process, it can be speculated as to the potential applicability of this area of research. Studies have demonstrated that exercise alters satellite cell content, and satellite cell content varies between histochemically and functionally different muscles, as well as in individuals with differing physical activity levels and ages (**Kadi et al 2004**). Older individuals for example, typically have reduced satellite cell populations as a result of age (**Kadi et al 2005**). However, human studies have revealed that not only are satellite cells maintained with exercise training, but are enhanced (**Kadi et al 2005**). In conjunction with exercise, estrogen has demonstrated its ability to influence satellite cell pools (**Tiidus & Bombardier 2005; Enns & Tiidus 2008**) and now, in light of the current study as

well as the study conducted by **Enns et al (2008)**, it is revealed that estrogen's effects are mediated through the ER (more specifically ER- α). With this information, individuals who suffer from conditions such as sarcopenia, or who are postmenopausal can be the eventual target populations for this line of research. Sarcopenia is an age-related condition depicted by progressive declines in muscle mass and quality (**Lee et al 2007; Hameed et al 2002**). Therefore, understanding the mechanism(s) that lead to satellite cell renewal can aid in at least offsetting age-related declines in muscle mass, quality, and atrophy.

In regards to postmenopausal females, there is a reduction in the amount of circulating estrogen present. However, data collected from older females have indicated that higher serum estrogen concentrations are associated with increased muscle mass and strength (**Rokainen et al 2009**), while losses of 15-25% of total muscle strength (in addition to strength declines associated with ageing) seems to coincide with menopause (**Onambele et al 2006**).

The aforementioned data alludes to the idea that low levels of estrogen is potentially responsible for age-related muscle weakness and altered muscle composition (**Rokainen et al 2009**). There seems to be evidence of an age-gender interaction as postmenopausal women demonstrate significant increases in post-exercise muscle damage when compared with younger females and males, respectively (**Roth et al 2000**).

Ronkainen et al (2009) investigated the effects long-term hormone replacement therapy (HRT) would have on mobility and skeletal muscle mass,

composition, and function in postmenopausal women 54-62 years of age (**Ronkainen et al 2009**). They revealed that HRT was associated with better mobility and greater muscle power, while individuals in the HRT treatment group had a lower relative proportion of fat within the muscle, as well as having larger muscles when compared to controls (**Ronkainen et al 2009**).

The current study demonstrated that with the use of an ER- α agonist, similar results were yielded when compared to the estrogen-supplemented group in regards to satellite cell activation. The ER- α agonist used in this study (PPT) not only can influence satellite cell populations, but can also influence symptoms experienced by postmenopausal females.

Postmenopausal females tend to experience hot flashes, and as a result, may choose to undergo hormone replacement therapy (HRT). However, data collected by **Harris et al (2002)** revealed PPT's ability to attenuate experimentally induced hot flashes in rats (**Harris et al 2002**).

It can be summarized that ER- α is implicated as being responsible for most, if not all, of the activation of satellite cell activity when bound to estrogen. The applicability of the data collected in the current study may eventually lead to synthesizing estrogen analogs that can interact with ER- α 's to aid with post-exercise skeletal muscle repair process.

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Appendix A

PAX 7 Immunohistochemistry

Use frozen sections of rat muscle cut 7-10 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. Permeabilize 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% H₂O₂ (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: Pax 7 (1/20 diluted in blocking solution) 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 5 min (or until desired intensity is achieved).
16. Rinse in dH₂O 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
- * Pax 7 stock solution does not have anything in it to fight bacteria, so use sterile technique

Appendix B

MyoD Immunohistochemistry

Use frozen sections of rat muscle cut 7-10 μm 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. Permeabilize 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. Apply primary antibody: MyoD (DakoCytomation, 1/50 diluted in blocking solution) overnight at 4°C (in humidity chamber).
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 DakoLSAB-2 kit) for 10 min.
14. Wash in PBS for 3 x 5 min.
15. Apply DAB stain (Vector Laboratories) for 5 min (or until desired intensity is achieved).
16. Rinse in dH_2O liberally from wash bottle.
17. Counterstain using Vector Hematoxylin QS (add hematoxylin for 15 s and rinse off with running 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 C

β -Glucuronidase Enzyme Assay

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
 - turn on hot water bath to 37°C, to get it warmed up
2. Add 50 μ l of homogenate and water to appropriately labeled test tube
 - use 12x75mm glass culture tubes
 - freeze remaining homogenate and use for Lowry protein analysis
3. Add 450 μ l of Acetate buffer to tubes using repeater pipette
4. Preincubate tubes in 37°C bath for 5 minutes
 - place parafilm over rack so water doesn't drip in
5. Add 250 μ l of substrate using repeater pipette
6. Incubate for 16-18 hours
 - cover the rack with parafilm
 - must sit overnight . . . so make sure that you plan the timing
 - **record the exact time that the samples came out of the water bath, the total incubation time in seconds is needed in the calculation**
7. Add 1.5 ml of cold glycine buffer using repeater pipette
 - turn on centrifuge when first get in, in the morning so it can cool down
 - make up the day before and refrigerate because must be cold
8. Cool in ice water for 10 minutes
 - fill the sink with cold water, deep enough to cover the solution in the tubes, add a few ice cubes to keep cold
 - turn on computer, select CaryWinUV program and set up for **Simple Reads (make sure wavelength is 420 nm)**
9. Centrifuge at 3500 rpm for 10 minutes (temp 4°C)
 - samples must be poured into micro centrifuge tubes in order to go in the centrifuge
10. Read on spectrophotometer at 420 nm.
 - pipette approx 1 ml from tube, avoiding the pellet on the bottom, and put in a 2 sided clear, skinny cuvette
 - vacuum out cuvettes between samples
 - use the same cuvette throughout, but rinse with distilled water between the most concentrated standard and the first sample

Appendix D

Lowry's Protein Assay

1. Use **12*75 mm** culture tubes and do samples, standards, and blanks in triplicate.
2. Add 50 μ l of standards to appropriately labelled tubes.
3. Add 50 μ l of water to tubes labelled REAGENT BLANK.
4. Prepare a 20:1 dilution of homogenizing media (10 μ l media + 190 μ l water). Add 50 μ l of dilute media to tubes labelled SAMPLE BLANK.
5. Prepare a 20:1 dilution of EACH tissue sample (10 μ l sample + 190 μ l water). Add 50 μ l 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.
12. Plot standard curve to check linearity.

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**

Appendix E

Reagents

PBS, 0.015 M working solution (pH 7.6):

	<u>1x PBS</u>	<u>10x PBS</u>
Na ₂ HPO ₄ * 7H ₂ O (FW 268.1)	3.495 g	34.95 g
NaH ₂ PO ₄ * H ₂ O (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 dH₂O, pH to 7.6, and bring up to 1 L with dH₂O. Store at room temperature.

For 10x PBS solution (recommended):

- Measure reagents, add approx. 950 ml dH₂O, pH to 7.6, and bring up to 1 L with dH₂O. Store at room temperature. Each day: take out 100 ml of 10x stock PBS solution and add 900 ml dH₂O.

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% H₂O₂ solution (in MeOH):

- Add 10 ml of 30% stock H₂O₂ 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 1:20 dilution of Pax 7 antibody:

- Take 4.75 ml blocking solution and add 250 µl primary antibody (monoclonal mouse anti-Pax-7 antibody, purchase from Developmental Studies Hybridoma Bank)

For 5 ml of a 1:50 dilution of MyoD antibody:

- Take 4.9 ml blocking solution and add 100 µl primary antibody (monoclonal mouse anti-MyoD1, clone 5.8A, Dako)

DAKO LSAB-2 kit (HRP) for use on rat tissue, order catalogue #K0609
Website: <http://www.dakocytomation.ca>

Sodium acetate (anhydrous) (C₂H₃O₂Na) (0.1N) (mw 82.03)

- 0.82 g in 100 ml of H₂O pH 4.2

Glycine Buffer (0.1M) pH 10.8

- A) 0.375g glycine (C₂H₅NO₂, mw 75.07) + 0.2922g NaCl (mw 58.44) in 50 ml H₂O
B) 0.4 g of NaOH (mw 40) in 100 ml of H₂O

- Mix 52.2 ml of A and 47.8 ml of B and pH to 10.8

Substrate: p-nitrophenyl-beta-D-glucuronide (5mM) (sigma N-1627) (mw 315.2)

- 7.88 mg in 5 ml H₂O

Standard: p-nitrophenol (mw 139.1)

15.65 mg p-nitrophenol/10 ml of H₂O gives [11.25mM]
Need 50µl of [11.25mM] p-nitrophenol in 2.25 ml of solution gives a total [0.25mM]

Serial Dilution

0.25	Use above
0.125.1	1 ml 0.25 mM and 1 ml of H ₂ O
0.0625	1 ml 0.125 mM and 1 ml of H ₂ O
0.3125	1 ml 0.0625 mM and 1ml of H ₂ O
0.156	1 ml 0.03125 mM and 1 ml of H ₂ O

10 mM liquid p-NP solution was purchased from Sigma, and serial dilutions were made to the following concentrations:

Standard, mM	To make:
0.125	Take 563 µl of 10 mM p-NP stock and add 437 µl dH ₂ O
0.0625	Take 500 µl of 0.125 mM solution and add 500 µl dH ₂ O
0.03125	Take 500 µl of 0.0625 mM solution and add 500 µl dH ₂ O
0.015625	Take 500 µl of 0.03125 mM solution and add 500 µl dH ₂ O
0 (distilled water)	