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**Delay in post-ovariectomy estrogen-replacement negates estrogen-induced augmentation of
post-exercise muscle satellite cell proliferation**

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Short Title: Timing of Estrogen Return in Muscle

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Abstract:

Aim: This study examined the effects of a delay in post-ovariectomy replacement of 17β -estradiol (estrogen) on the post-exercise proliferation of muscle satellite cells.

Methods: Nine-week old, ovariectomized female Sprague-Dawley rats (n=64) were divided into eight groups based on: estrogen status (0.25 mg estrogen pellet or sham), exercise status (90 min run @ 17 m/min, -13.5° or unexercised) and estrogen replacement (“proximal”; estrogen replacement within two-weeks or “delayed”; estrogen replacement at eleven-weeks following ovariectomy).

Results: Significant increases in satellite cells were found in the soleus and white gastrocnemius muscle (immunofluorescent co-localization of nuclei with Pax7) 72 hrs following eccentric exercise ($p < 0.05$) in all exercised groups. “Proximal” E2 replacement resulted in a further augmentation of muscle satellite cells in exercised rats ($p < 0.05$) relative to the delayed estrogen replacement group. Expression of PI3K was unaltered and phosphorylation of Akt relative to total Akt increased following estrogen supplementation and exercise. Exercise alone did not alter the expression levels of Akt.

Conclusion: An 11 week delay in post-ovariectomy estrogen replacement negated the augmenting influence seen with “proximal” (2 week delay) post-ovariectomy estrogen replacement on post-exercise muscle satellite cell proliferation. This effect appears to be independent of the PI3K/Akt signaling pathway.

Key Words: Estrogen, Satellite Cells, Muscle, PI3K/Akt, Timing Hypothesis

Conflict of Interest: The authors report no conflicts of interest.

Introduction

Estrogen (17β -estradiol) has been reported to convey positive protective and regenerative benefits in numerous tissues following various forms of damage. Documented effects of estrogen include protective and/or regenerative benefits for vascular, neural, muscular and hepatic tissues following damage (Azcoitia et al. 2011, Manson et al. 2007, Sipilä et al. 2015, Hsu et al. 2015). When circulating estrogen levels are diminished, as seen in post-menopausal females, many of the beneficial effects of estrogen on these tissues are also lost. However a number of studies using human and animal models, have demonstrated that with estrogen replacement the protective and regenerative effects of estrogen on skeletal muscle, vascular, hepatic and neural tissues can be restored (Ahtiainen et al. 2012, Bourque et al. 2009, Dieli-Conwright et al. 2009, Pike et al. 2009, Pöllänen et al. 2010, Ronkainen et al. 2009, Suzuki et al. 2009, Velders and Diel, 2013, Hsu et al. 2015). Specifically in skeletal muscle, estrogen replacement soon after ovariectomy in rodents will also restore post-atrophy muscle regenerative capacity and diminish post-exercise muscle inflammation and damage indices (McClung et al. 2006, Iqbal et al. 2008).

Research using rodent models has noted that the timing of estrogen replacement following ovariectomy will affect its ability to influence tissue protection and repair following damage. Several studies have highlighted that a delay in estrogen replacement following ovariectomy will result in a loss of estrogen efficacy in restoring its regenerative and protective potential in neural and vascular tissues (Lenfant et al. 2011, Azcoitia et al. 2011, Suzuki et al. 2007). It has been suggested that some of the controversies regarding the potential adverse or beneficial effects of estrogen and hormone replacement in the health of post-menopausal women and specifically in muscle tissue may be related to the length of time that passed before post-menopause hormone replacement was initiated in long term studies examining health effects of hormone replacement (Schierbeck et al. 2012, Tiidus et al. 2013, Gurney et al. 2014).

Estrogen may protect neural tissue from ischemia-induced damage by reducing apoptosis and enhancing repair via facilitation of increased neurogenesis, dendritic and axonal regeneration and

myelination (Etgen et al. 2011). These estrogenic actions in neural tissues are mediated in part through α - and β -estrogen receptors and the PI3K/Akt signaling pathway (Azcoitia et al. 2011). However, the mechanisms as to why these protective effects are lost when post-ovariectomy estrogen return is delayed are not yet fully understood (Peng et al. 2010, Yu et al. 2004, Azcoitia et al. 2011).

While clear evidence is available regarding the loss of estrogen efficacy following delayed replacement in vascular and neural tissues, no such data is yet available for skeletal muscle. If indeed a delay in post-ovariectomy return of estrogen has negative effects on markers of post-damage muscle regenerative potential, then the mechanisms of these effects would also warrant further investigation. Work from our laboratory has previously demonstrated that estrogen will enhance post-exercise muscle satellite cell activation through estrogen receptor- α mediation and via activation of the PI3K/Akt pathway (Enns and Tiidus 2008; Enns et al. 2008, Mangan et al. 2014, Thomas et al. 2010). Hence, the purpose of this study was to determine whether a delay in post-ovariectomy return of estrogen would affect the ability of estrogen to augment post-exercise muscle satellite cell proliferation and to investigate whether this effect is mediated by the PI3K/Akt signaling pathway.

Methods

Animals

This study was approved by the Animal Care Committee at Wilfrid Laurier University and is in accordance with the Canada Council on Animal Care standards for animal research. A total of 64 ovariectomized female Sprague–Dawley rats (Charles River Laboratories, LaSalle, QC, Canada) were used in this study. The ovaries were surgically removed at 8 weeks of age at the source, and arrived at the laboratory one week later. Similar to our previous protocol (Mangan et al. 2014) animals were single-housed in a temperature-controlled environment with a standard 12:12 light/dark cycle and allowed ad libitum access to food (Tekland 22/5 Rodent Diet; Harlan-Tekland, Madison, WI) and water. Upon

arrival to the laboratory, the rats were randomly divided into eight groups based on three factors: estrogen delay, estrogen supplementation, and exercise level (Figure 1). The animals were acclimatized to the animal care facility for one week prior to commencement of any study protocols.

Estrogen Delay Protocol

The 64 ovariectomized rats were randomly divided into two equal groups based on when they underwent the estrogen supplementation protocol. The “proximal” group underwent the protocol immediately after the one-week acclimation period while the “delay” group began supplementation ten weeks following acclimation. Hence the “proximal” group received estrogen replacement within 2 weeks of ovariectomy and the “delay” group received estrogen replacement within 11 weeks of ovariectomy. This timing of proximal and delayed estrogen replacement is similar to that previously used in mice to demonstrate a loss of estrogen neuro-protective effects following delayed estrogen return (Suzuki et al. 2007).

Estrogen Supplementation Protocol

The ovariectomized rats were used as the control groups. The experimental groups were similar to the control groups except for the addition of an estrogen pellet that was administered subcutaneously through surgery. One or eleven weeks after arrival, rats were subjected to either an estrogen pellet implantation or a sham procedure (Enns and Tiidus 2008). Under aseptic conditions, the rats were anesthetized via inhalation of isoflurane. Excess hair on the incision site was removed using electric trimmers. Prior to making the incision, the analgesic Marcaine ($8 \text{ g} \cdot \text{kg}^{-1} \text{ BW}$; $2.5 \text{ mg} \cdot \text{ml}^{-1}$; Hospira Healthcare Corporation; Montreal) was injected subcutaneously into the skinfolds of the neck. A 1 cm long incision was made in the skin penetrating to the underlying fascia. For the proximal and delayed experimental groups, an estrogen pellet (0.25 mg 17β -estradiol, 21 day release, Innovative Research of America, Sarasota) was inserted. Each pellet releases on average $0.012 \text{ mg} \cdot \text{day}^{-1}$ of estradiol (Mangan et al., 2014). The control groups underwent similar sham surgery procedures albeit without the insertion of

the estrogen pellet. The incision site was then closed with a staple and the rat was taken off the isoflurane and placed into a separate cage to recover.

After the estrogen supplementation protocol, the rats were returned to their cages for the following seven days. Two five-minute treadmill acclimatization sessions took place on the motorized treadmill (Columbus Instruments, Columbus, OH) on days 3 and 4 following the estrogen pellet implantation prior to the commencement of the exercise protocol.

Exercise Protocol

Seven days following the estrogen pellet implantation protocol, the rats in the both the proximal and delayed estrogen replacement exercise groups were placed on a motorized rodent treadmill supplemented with an electric shock grid (Columbus Instruments, Columbus, OH, USA). The animals ran at $17 \text{ m} \cdot \text{min}^{-1}$ at a grade of -13.5° , for 5 minutes followed by 2 minutes of rest. This protocol was repeated until a total of ninety minutes had been completed. This intermittent protocol was designed to be non-fatiguing while still eliciting exercise-induced damage to muscles, that include the predominantly type I soleus (SOL) muscle and predominantly type IIb white gastrocnemius (WG) (Komulainen et al. 1994). The exercise-induced damage elicited by this protocol has previously been validated as demonstrated by significant increases in β -glucuronidase activity in both the SOL and WG (Enns et al. 2008, Enns and Tiidus 2008, Salminen and Kihlström 1985, Thomas et al. 2010). The sedentary control rats were placed on a stationary treadmill for the same time interval as the exercised rats. After the exercise protocol, the rats were returned to their cages.

Sacrifice, and Blood and Tissue Collection

Seventy-two hours after completion of the exercise protocol, the animals were injected with sodium pentobarbital ($55 \text{ mg} \cdot \text{kg BW}^{-1}$). Blood was collected from the right ventricle using a heparin-coated syringe and deposited into an Eppendorf tube. The blood was centrifuged at 4500 RCF for 10 min

at 4°C to fractionate the blood into erythrocytes and plasma. The plasma was collected, placed in a fresh Eppendorf tube, and frozen at -80°C until further analysis.

SOL and WG muscles were collected from both the right and left legs of the animals. The WG is located superficially on both the lateral and medial heads of the gastrocnemius (Matsakas et al. 2006). SOL has a fibre-type composition that is 96.6% Type I, while 88.1% of WG is comprised type IIB fibres (Bloemberg and Quadrilatero 2012). Muscle samples were placed in an ice-cold PMSF buffer (250mM sucrose, 5 mM HEPES, 10mM NaN₃ and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.5). Muscle samples intended for immunohistochemical analysis were coated in optimal cutting temperature medium (Tissue-Tek, Torrance, CA, USA), and placed in liquid nitrogen cooled isopentane in order to freeze. Samples intended from enzyme and protein analyses were flash frozen in liquid nitrogen and stored at minus 80°C until further analysis.

Muscle Homogenate Preparation and Western Blot Analysis

Frozen skeletal muscle was diluted 10:1 (vol:wt) in ice cold PMSF and manually homogenized using a glass mortar and pestle. The resulting homogenate was then frozen and stored at -80 °C. Protein extracts (25 µg) were separated using 10 - 12% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Membranes were then blocked (1 hr) with a 5% (w/v) non-fat dry milk or 5% (w/v) BSA (bovine serum albumin) in 1x TBST solution (Tris-buffered saline-Tween-20: 20mM Tris base, 137 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.5) at room temperature, followed by incubation in blocking solution with antibodies directed towards Akt (1:1000; Cell Signaling) and P-Akt (Ser 473, 1:500; Cell Signaling) and PI3K (P85, 1:500; AbCam overnight at 4°C. Subsequently, membranes were washed 3x 5-min with TBST, followed by incubation at room temperature (1 hr) with either goat anti-mouse (PI3K; 1:1000) or goat anti-rabbit (Akt, P-Akt; 1:1000) secondary antibodies conjugated to horseradish peroxidase, and washed again 3x for 5-min each with TBST. Antibody-antigen complexes were visualized after addition of sensitive chemiluminescent substrate, Luminata™ Forte Western HRP Substrate (Millipore, MA, USA) for P-Akt, and ECL Western Blot Substrate (BioVision, CA, USA) for

Akt and PI3K. Quantification occurred via densitometric analysis for the intensity of signals with SigmaScanPro software (version 5, Jandel Scientific, San Rafael, CA, USA).

Blood Plasma Analysis

Plasma estrogen levels were determined in duplicate using the Coat-a-Count radioimmunoassay kit (Inter Medico, Markham, ON, USA). This is a well-established procedure in our laboratory with a test–retest variability of 4–7% (Enns and Tiidus 2008).

Muscle Sectioning and Immunofluorescence

Mounted SOL and WG muscles (10 μ m) were cut in a cryostat maintained at -20°C . These muscle sections were transferred onto Vectabond-coated (Vector Laboratories, Burlington, ON, Canada) glass slides that were then dried at room temperature and subsequently stored at -80°C .

Immunofluorescent analysis was conducted in both SOL and WG of paired box 7 (Pax7), a transcription factor that is expressed in all satellite cell populations in the quiescent or activated state (Hawke & Garry 2001; Yin *et al.* 2013). Briefly, all samples were fixed in 4% formaldehyde solution for 5 min followed by 3 washes with 1 x PBS. Subsequently samples were submersed in the detergent, 0.5% Triton X-100, for 10 min allowing for permeabilization of cell membranes, and then washed 3 times in 1x PBS. The slides were then incubated for 1 hr in a blocking solution consisting of 10% normal goat serum in order to block nonspecific sites. Slides were then incubated with a primary antibody to Pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) for 1 hr at a dilution of 1:40. To detect Pax7, a goat anti-mouse AlexaFluor 488 secondary antibody (1:500) was used. The mounting medium, Prolong Gold antifade reagent, was added to each slide and subsequently mounted with a #1 cover slip. Slides were visualized using an Axio Observer Z1 structured-illumination fluorescent microscope equipped with an AxioCam HRm camera and associated AxioVision software (Carl Zeiss).

Satellite cells were identified and quantified from the immunostains in 5-8 muscle sections consisting of at least 400 myofibres. From these myofibres, the number of satellite cells (Pax7⁺ nuclei) per myofibre could be counted. This method of satellite cell marker identification is a well-established quantification method in our laboratory (Enns et al. 2008, Enns and Tiidus 2008, Thomas et al. 2010) and is similar to other quantification methods (Kurosaka et al. 2012). For example, Kurosaka et al. (2012) quantified satellite cell content using the approach of percentage of the sum of myonuclei and satellite cells and as satellite cells counts per fiber, and found consistent trends between both methods.

Statistical Analyses

All data were presented as means $\pm$ SD. For all analyses, differences between groups were measured using a three-way ANOVA with a level of significance of $p < 0.05$. If significance was found, differences between groups were evaluated using the Student-Newman-Keuls test.

Results

Exogenous supplementation of estrogen, via subcutaneous pellet insertion significantly altered circulating levels of estrogen ($\text{pg} \cdot \text{mg}^{-1}$) in estrogen-supplemented rats as compared with non-supplemented rats. Rats that received estrogen proximal to ovariectomy had greater circulating estrogen levels (237.30 ± 150.07) relative to sham control animals (9.41 ± 4.37). Rats that experienced a delay in receiving post-ovariectomy estrogen-supplementation (288.19 ± 204.59) also had greater circulating estrogen levels than non-supplemented rats (7.36 ± 3.37 , $p < 0.05$). The estrogen supplementation and ovariectomy induced circulating levels of estrogen seen in this study were similar to those reported in our previous studies (Mangan et al. 2014, Thomas et al. 2010). No significant difference was observed in the estrogen levels of animals that received estrogen-supplemented on either the proximal and delayed delivery schedules.

Estrogen supplementation improves satellite cell population when delivered proximal to loss of endogenous estrogen production.

The total number of satellite cells (number of Pax7⁺ nuclei per myofiber) followed a similar trend in both SOL and WG muscles (Figure 2). In both muscle types, estrogen supplementation, regardless of its post-ovariectomy time of return, resulted in a significant increase in post-exercise muscle satellite cell numbers relative to unsupplemented controls. Similar main effects ($p < 0.05$) for exercise and the timing of estrogen delivery were found. Further augmentation of post-exercise muscle satellite cell content was noted in animals with proximal post-ovariectomy estrogen replacement relative to those with delayed replacement in both soleus and white gastrocnemius muscles ($p < 0.05$). This augmentation effect of estrogen on post-exercise soleus and white gastrocnemius muscle satellite cell content was negated in the delayed estrogen replacement animals.

Phosphorylation of Akt is more pronounced following both estrogen supplementation and exercise while PI3K protein does not change.

Neither estrogen supplementation, exercise nor the timing of the estrogen delivery affected the expression of PI3K protein in either SOL or WG muscle (Figure 3). However, estrogen supplementation enhanced phosphorylation of Akt (p-Akt) relative to total Akt (t-Akt) following exercise in muscles from both the proximal and delayed estrogen replacement conditions without significant difference between groups. It was determined that estrogen supplementation significantly increased ($p < 0.05$) PI3K/Akt pathway activity as indicated by p-Akt in both SOL and WG muscle. Exercise significantly increased p-Akt/t-Akt in WG ($p < 0.05$) with a strong similar trend in SOL ($p = 0.057$). The timing of post-ovariectomy estrogen return did not appear to differentially affect post-exercise PI3K/Akt pathway activation.

Discussion

This study investigated whether the timing of estrogen supplementation following the loss of endogenous production via ovariectomy can influence the ability of estrogen to augment post-exercise muscle satellite cell proliferation and if so, whether this effect is manifested via the PI3K/Akt signalling pathway. To this end, the present study produced three main findings. First, we observed that estrogen supplementation, when occurring within 2 weeks of ovariectomy, is effective in augmenting the post-exercise increase in soleus and white gastrocnemius muscle satellite cell populations. This finding reaffirms previous reports of augmenting effects of estrogen on post-exercise muscle satellite cell numbers (eg. Enns and Tiidus 2008, Enns et al. 2008, Thomas et al. 2010). These previous studies also replaced estrogen within 2 weeks of ovariectomy.

Interestingly, the second main finding of this study was the novel observation that when the post-ovariectomy return of estrogen was delayed for eleven weeks, this ability of estrogen to augment post-exercise skeletal muscle satellite cell proliferation was lost. This suggests that the means by which estrogen acts to augment post-exercise muscle satellite cell proliferation is impacted and lost by a significant delay in its post-ovariectomy replacement. These findings are similar to previous reports involving neurological tissue where similar delays in post-ovariectomy estrogen replacement also resulted in loss of the beneficial effects of estrogen on ischemia-induced damage reduction and augmented neural regeneration (Suzuki et al. 2007, Azcoitia et al. 2011). Delays in estrogen replacement following menopause in humans and ovariectomy in rodents have also been reported to negate the protective effects of estrogen on vascular tissues (Lenfant et al. 2011).

Our previous study found that blocking the PI3K/Akt signaling pathway in rodents that were estrogen replaced within two weeks of ovariectomy, negated the effects of estrogen in augmenting post-exercise muscle satellite cell proliferation (Mangan et al. 2014). Hence we hypothesized that the inability of estrogen to augment post-exercise muscle satellite cell proliferation due to an 11 week delay in estrogen replacement may be due to changes in activation of the PI3/Akt pathway consequent to loss of

estrogen exposure for this extended time period. Contrary to our hypothesis, expression of the PI3K protein was unaltered in any of the experimental conditions tested. Phosphorylated Akt, an active downstream effector of PI3K, was increased by estrogen supplementation and exercise status but this effect did not differ between the proximal and delayed estrogen return groups. While the increase in post-exercise phosphorylated Akt with estrogen supplementation supports our previous finding of a PI3K/Akt signaling pathway connection between estrogen and muscle satellite cell proliferation (Mangan et al. 2014), it does not appear that the activity of this signaling pathway is affected by an 11 week delay in post-ovariectomy estrogen replacement.

Based on these findings it appears that the inability of estrogen to augment post-exercise satellite cell proliferation, consequent to a delay in post-ovariectomy estrogen replacement, may not occur as a direct result from a change in function of the PI3K/Akt signaling pathway. Hence, this effect may be manifested through other mechanisms. While these other potential mechanisms are currently unknown, other signaling pathways may also be involved and/or differentially affected by a delay in post-ovariectomy estrogen replacement. Several other pathways involving estrogen related communication with muscle satellite cells have also been identified. These include estrogen induced increased hbEGF activity via GPER-1 which may stimulate bovine satellite cell proliferation, and also estrogen related activation of MAPK cascades which are involved in other myogenic activities (Kamanga-Sollo et al. 2014, Murray and Huss, 2011). Exploration of these and other signalling pathways with respect to the effects of timing of post-ovariectomy estrogen replacement are needed to further investigate the means by which estrogenic influence on muscle satellite cells may be manifested and ultimately lost due to estrogen replacement delay is necessary. MAPK signalling, has also been found to be an important factor in signaling estrogen related post-ischemia neuro-protection and augmented neuro-regeneration (Azcoitia et al. 2011). Similar interactions of estrogen and MAPK signalling have recently been reported in protection of hepatic tissues from hemorrhagic shock (Hsu et al. 2015). Various other factors may also be involved as estrogen induces protective effects in vascular ischemia through a number of mechanisms including

prevention of apoptosis and enhanced VEGF production (Lenfant et al. 2011). Whether these or other possible communication links between estrogen and skeletal muscle satellite cells may be negatively influenced by delayed post-ovariectomy estrogen return requires further investigation.

Myogenic satellite cells are responsible for adaptation and regeneration of the myofiber in response to a variety of stimuli (Hawke and Garry, 2001, Yin et al., 2013). The loss of satellite cells has been related to a decreased regenerative capacity of skeletal muscle (Collins et al. 2005). Studies have identified a link between compromised muscular function, loss of muscle mass and the reduction of estrogen in postmenopausal women (Ahtiainen et al. 2012, Pöllänen et al. 2010, Ronkainen et al. 2009). Several studies have used ovariectomized female rodents as a proxy for postmenopausal women to examine the physiological mechanisms behind these relationships. These studies have shown an attenuation in post-exercise muscle satellite cell activation and proliferation following ovariectomy is recoverable with exogenous estrogen replacement within two weeks of ovariectomy (Enns and Tiidus 2008, Enns et al. 2008, Mangan et al. 2014, Thomas et al. 2010). The present study confirmed these previous findings and demonstrated the loss of this effect of estrogen when its post-exercise replacement is delayed for 11 weeks. Determination of those muscle specific changes and mal-adaptations that occur in the absence of estrogen is key to understanding the various potential benefits and hazards of postmenopausal hormone replacement on muscle health in aging females (Tiidus et al. 2013).

Controversy regarding the potential health benefits and hazards of estrogen replacement has continued since the publication of Women's Health Initiative study in 2004 (Anderson et al. 2004). It has been suggested that timing of post-menopausal estrogen replacement may be an important factor in determining whether estrogen has positive, neutral or negative effects on tissue regeneration and function (Suzuki et al. 2007, Azcoitia et al. 2011, Velders and Diel 2013, Tiidus et al. 2013). This study further demonstrates a time dependant effect of post-ovariectomy estrogen replacement such that the effects of estrogen in augmenting post-exercise enhancement of muscle satellite cell numbers are lost when estrogen replacement is significantly delayed. As more recent analyses suggest that estrogen replacement

proximal to menopause has overall positive health effects (Gurney et al. 2014, Hodis and Mack 2014), the use of estrogen replacement for the maintenance of muscle mass and function in post-menopausal women should also be considered.

There are many human studies that examine the effects of estrogen supplementation on various tissue specific and whole body effects in postmenopausal women, however little specific information is yet available as to the effects of timing of estrogen replacement, in skeletal muscle (Tiidus et al. 2013). Future studies could examine this cellular niche in both ovariectomized female rodents and postmenopausal women. This characterization would provide precise and valuable insight as to when estrogen replacement could begin to optimize any beneficial effects specifically in skeletal muscle and to potentially minimize health hazards. As this study was performed using young rats, its direct applicability to post-menopausal women also needs to be further validated. To this end, the use of an older rat cohort, particularly if ovariectomized at an older age could add further validity to these findings. A further potential limitation of the study is the possible increased stress of single housing of rats used in this study, which while similar in all groups might have added a further variable to the findings.

In summary, this study demonstrated, for the first time, that the augmenting effects of post-ovariectomy estrogen replacement on post-exercise muscle satellite cell proliferation are lost when replacement is delayed for 11 weeks. Although the PI3K/Akt pathway appears to be involved in estrogen-associated myogenic satellite proliferation, it does not appear to be the factor in the replacement delay related loss of estrogen augmentation of post-exercise muscle satellite cell proliferation.

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Figure Captions

Figure 1: Schematic of animal usage during study. Proximal = estrogen replacement at 2 weeks **post-ovariectomy**; Delayed = estrogen replacement at 11 weeks **post-ovariectomy**.

Figure 2: Effect of the timing of exogenous estrogen delivery, estrogen supplementation, exercise on total satellite cell markers (Pax7⁺ nuclei) in rat (a) soleus and (b) white gastrocnemius 72 h following downhill running.

n = 8 rats per group. Values are Means ± SE.

^a *P* < 0.05 elevated compared with sham–sedentary,

^b *P* < 0.05 elevated compared with estrogen–sedentary,

^c *P* < 0.05 elevated compared with sham–exercise.

Figure 3: Effect of the timing of exogenous estrogen delivery, estrogen supplementation, exercise on phosphorylation of Akt (p-Akt/ Total-Akt) in rat (a) soleus and (b) white gastrocnemius, and PI3K protein expression in rat (c) soleus and (d) white gastrocnemius 72 h following downhill running.

n = 4 rats per group. Values are Means ± SE.

^a *P* < 0.05 decreased compared with estrogen–exercise,

^b *P* < 0.05 elevated compared with sham–sedentary.

No differences *P* > 0.05 in PI3K phosphorylation between conditions or between proximal or delayed estrogen replacement groups

No differences *P* > 0.05 in effects of exercise on Akt phosphorylation between proximal and delayed estrogen replacement groups

Draft

Sham-Sedentary
Estrogen-Sedentary
Sham-Exercise
Estrogen-Exercise

Sham-Sedentary
Estrogen-Sedentary
Sham-Exercise
Estrogen-Exercise

A)

B)

C)

D)