

ABSTRACT

Title of Thesis Defense: PHYSICAL ACTIVITY OR ESTRADIOL SUPPLEMENTATION CAN PREVENT THE METABOLIC SYNDROME IN OVARIECTOMIZED MICE

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Loss of ovarian hormones (ie. menopause) results in a significant increase in risk for developing the metabolic syndrome. Thus, the purpose of this study is to investigate the effectiveness of exercise in preventing the metabolic syndrome in a model of menopause. Female C57/BL6 mice were divided into three groups, ovariectomized (OVX), SHAM, and ovariectomized supplemented with 17 β -estradiol (OVX+E₂), with mice placed in either voluntary running wheel cages or standard cages for eight weeks. OVX animals ran significantly less than SHAM animals, with the OVX+E₂ group exhibiting a partial recovery in distance. Visceral fat mass and blood glucose levels were elevated in the OVX sedentary group as compared to SHAM and OVX+E₂ groups. Voluntary wheel running and 17- β estradiol supplementation attenuated increases in visceral fat mass, blood glucose levels, and ERK1/2 phosphorylation in OVX animals, thus preventing symptoms of the metabolic syndrome which accompany loss of ovarian hormones.

PHYSICAL ACTIVITY OR ESTRADIOL SUPPLEMENTATION CAN PREVENT
INCREASES IN FAT MASS IN OVARIECTOMIZED MICE

By

Lindsay Michelle Wohlers

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LIST OF ABBREVIATIONS

- 17- β – 17 beta hydroxysteroid dehydrogenase
- ACC - Acetyl CoA carboxylase
- AICAR - Aminoimidazole-4-carboxamide
- AMPK - 5'AMP-activated protein kinase
- BMI – Body mass index
- CAD – Coronary artery disease
- CaMKII - Calcium calmodulin dependent kinase II
- CPT-1 – Carnitine palmitoyl transferase-1
- CS – Citrate synthase
- CSA – Cross-sectional area
- CVD – Cardiovascular disease
- DAG – Diacylglycerol
- DMSO – Dimethylsulfoxide
- ERK – Extracellular signal-regulated kinase
- FAT/CD36 – Fatty acid translocase/cluster of differentiation 36
- FFA – Free fatty acid
- FSH – Follicle stimulating hormone
- GP – Glycogen phosphorylase
- HDL-C – High density lipoprotein cholesterol
- HRT – Hormone replacement therapy
- HSL - Hormone sensitive lipase
- IDF – International diabetes federation

IL-6 – Interleukin 6

IRS-1 – Insulin receptor substrate-1

IMTG – Intramyocellular triglyceride

LH – Lutenizing hormone

MAPK – Mitogen-activated protein kinase

NEFA – Non-esterified fatty acid

OVX – Ovariectomized

OVX+E₂ – Ovariectomized supplemented with 17 β -estradiol

PAS – Periodic acid Schiff

PKA – Protein kinase A

RER – Respiratory exchange ratio

TA – Tibialis anterior

TNF- α – Tumor necrosis factor alpha

WAT – White adipose tissue

WHI – Women’s health initiative

WHO – World health organization

INTRODUCTION

Over the past twenty years, the incidence of obesity among adults and children alike has risen dramatically (138). While most of the upward trend in obesity is attributed to decreased physical activity (201), other physiological factors have been linked to weight gain. For example, various metabolic disorders, such as menopause and hypothyroidism (214) have been associated with changes in body fat, body composition, and total body weight (50; 149; 175; 184). Menopause begins approximately in the fifth decade of life and currently it is estimated that 1 in 3 women are postmenopausal (36). Post-menopausal females are at greater risk of developing obesity than pre-menopausal women. Thus, a large percentage of the female population is at increased risk for developing obesity based on ovarian hormone status.

In studying the physiological changes associated with menopause, it is important to understand the function of estrogens. Estrogens are steroid hormones which operate as the primary sex hormones in females. Three major types exist: estradiol, estriol, and estrone, with 17 β -estradiol the predominant form present in pre-menopausal females (21). Estrogens play an important role in the development of female secondary sex characteristics, regulation of the menstrual cycle, and bone growth (129). During menopause, production of estrogens by the ovary declines and almost stops completely (49; 185; 206). Not only does this drop in production of estrogens result in the loss of menses, but it has also been associated with changes in metabolism, fat distribution, blood lipid profile, and blood glucose levels (129; 184). Thus, conditions such as the metabolic syndrome, cardiovascular disease, and osteoporosis may result as the level of estrogens declines (46; 216).

One of the most visible changes associated with menopause is weight gain accompanied by a significant increase in visceral fat. This link between estrogens and weight gain has been demonstrated in both human and animal models (44; 117; 149; 188). Significant weight gain and increases in visceral fat mass have been attributed to decreased levels of estrogens in post-menopausal females (114; 116; 117; 169; 188). However, with the use of hormone replacement therapy (HRT), attenuation of weight gain and central obesity is possible (98; 138), suggesting that estrogens play an important role in weight maintenance in women. Unfortunately, studies recently performed as part of the Women's Health Initiative link use of HRT to increased risk of stroke, blood clots, heart disease, and cancer (206), suggesting that HRT may be contraindicated in post-menopausal women. In contrast, a number of investigations have questioned the validity of these findings and called for more extensive research of HRT use (for review see Wise 2003) (218). With the equivocal nature of HRT, further research is warranted in determining the mechanisms by which estrogens impact weight gain, particularly in the visceral region, in post-menopausal females (206).

Not only does estrogen status play a role in body composition, it has also been demonstrated to impact the onset of the metabolic syndrome (18; 30; 46; 48; 50; 184). Post-menopausal women have a 60% chance of developing metabolic syndrome, while in age-matched pre-menopausal women, the risk is only 20-30% (151). Presence of the metabolic syndrome is linked to an increased occurrence of coronary heart disease, type II diabetes, and other conditions resulting from arterial plaque build-up (55). According to the American Heart Association, metabolic syndrome is defined by the presence of three or more of the following criteria: elevated waist circumference, elevated

triglycerides, decreased HDL cholesterol, elevated blood pressure, and/or elevated blood glucose (71). The loss of estrogens has been linked to alterations in visceral adiposity, blood glucose regulation, and blood triglyceride levels among post-menopausal women (11; 18; 30; 50; 177; 184), with HRT reversing many of these negative changes (22; 27). Thus, HRT may be a way to prevent deleterious metabolic alterations in post-menopausal women. Furthermore, there are data which would suggest that estrogens provide women with a metabolic phenotype that favors lipid utilization over lipid storage (31; 172; 173).

While pharmaceutical treatment is often employed in treating many of the health problems associated with obesity and metabolic syndrome, the use of exercise as a treatment is an effective alternative for most individuals (1; 5; 52). Properly designed exercise programs, along with a balanced diet, can contribute to significant weight loss in obese individuals (7; 162). Furthermore, weight loss through diet and exercise often eliminates the presence of obesity-related diseases such as diabetes (92; 192) and cardiovascular disease (80; 81; 113). Regular exercise can also result in significant improvements in blood lipid profiles (81; 162) and blood pressure (169). Overall, exercise has been demonstrated as an effective treatment for many of the components of metabolic syndrome.

Although exercise has been utilized as an effective treatment for metabolic syndrome in males, or in pre-menopausal females, little is known about the potential for exercise treatment of weight gain and metabolic syndrome which occur in females following menopause. With the current recommendations indicating that females should discontinue use of HRT, the use of remedial therapies warrants further investigation. Unfortunately, few data exist demonstrating that exercise is an effective intervention in

post-menopausal women and it is unclear if exercise is effective at all without concurrent use of HRT. Therefore, the testing of exercise, and its potential interaction with estrogens, as a preventative measure in development of chronic conditions accompanying menopause, should be further investigated.

Purpose: The purpose of this study is three fold:

- 1) To explore the effectiveness of voluntary exercise in preventing metabolic syndrome associated with the surgical loss of estrogens.
- 2) To determine if estrogens are necessary for exercise induced benefits in female mice.
- 3) To elucidate if molecular and cellular mechanisms known to regulate lipid oxidation are up-regulated with exercise training when estrogen levels are decreased.

Specific Aim 1: To determine if voluntary exercise will attenuate the onset of the metabolic syndrome in ovariectomized mice and if this effect will be accentuated in mice supplemented with 17 β -estradiol.

H₁: Ovariectomized mice undergoing voluntary exercise training will have similar fat masses to female control mice, but higher masses than ovariectomized mice supplemented with 17 β -estradiol.

H₂: Ovariectomized mice undergoing voluntary exercise training will have similar levels of circulating free fatty acids to control mice, but higher levels than ovariectomized mice supplemented with 17 β -estradiol.

H₃: Ovariectomized mice undergoing voluntary exercise training will have similar fasting blood glucose levels to control mice, but impaired fasting blood glucose levels compared to ovariectomized mice supplemented with 17 β -estradiol.

Specific Aim 2: To determine if voluntary exercise will induce a metabolic phenotype in skeletal muscle that encourages lipid oxidation in ovariectomized mice, and if this effect will be accentuated with 17 β -estradiol supplementation.

H₄: The sedentary ovariectomized mice will have greater amounts of intramuscular triglycerides than the exercise-trained ovariectomized mice or 17 β -estradiol-supplemented mice.

H₅: The sedentary ovariectomized mice will have decreased expression levels of lipid oxidative genes as compared to the exercise-trained ovariectomized mice or 17 β -estradiol-supplemented mice.

H₆: The sedentary ovariectomized mice will have increased expression levels of genes promoting lipid storage as compared to the exercise-trained ovariectomized mice or 17 β -estradiol-supplemented mice.

H₇: The sedentary ovariectomized mice will have increased phosphorylation of ERK1/2 (Thr 202/Tyr 204) in adipose tissue at rest as compared to the sedentary SHAM mice and ovariectomized mice supplemented with 17 β -estradiol. The exercise-trained SHAM mice and 17 β -estradiol-supplemented mice will have increased phosphorylation ERK1/2 (Thr 202/Tyr 204) compared to their sedentary counterparts. The exercise-trained OVX mice will have phosphorylation levels of ERK1/2 (Thr 202/Tyr 204) similar to sedentary OVX mice.

METHODS

Animals: Thirty-two 8-week old C57/BL6 (Harlan) female mice were divided into three groups (SHAM, OVX, OVX+E₂). Due to limitations in the number of running wheels available, the total number of animals utilized was less than the total of 48 that was originally proposed. One group of mice (n=12) underwent a bi-lateral ovariectomy (OVX), a frequently used animal model of menopause (103). Our lab has previously shown that this model decreases the levels of circulating estrogens by 70% within 48 hours (191). A second group (n=10) was subjected to a SHAM surgery where they were anesthetized, but did not undergo ovariectomy. The third group (n=10) consisted of ovariectomized mice which underwent a second surgery one week after the ovariectomy where exogenous estrogen was introduced via implantation of a subcutaneous time-release pellet (Innovative Research, Sarasota, FL). This pellet results in the animals receiving ~40pg/mL of 17-β estradiol per day, which is similar to levels found in the mouse during estrous (142).

Mice were given ad libitum access to water and standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, 6% fiber) and were housed in a temperature-controlled room on a 12h light/dark cycle. The exercise training group was placed in cages with voluntary running wheels, while the sedentary group was placed in standard mouse cages. Running activity, including distance run, average speed, and running duration was monitored for 8 weeks with a photocell counter interfaced with a computer through customized software (Layfayette Instruments, Layfayette, IN). However, due to unforeseen circumstance involving the computer, only total running distance is reported. Following the 8-week training protocol, animals were sacrificed and

tissue (see table 1 below) was collected, snap frozen in liquid nitrogen, and stored at -80°C. Mice were removed from the wheel cages 24 hours prior to sacrifice to account for acute effects of exercise, followed by the removal of food 4-5 hours prior to sacrifice.

Table 1. Tissue samples collected from mice.

<i>Muscle Tissue:</i>			
L-Gastrocnemius	R-Gastrocnemius	L-Tibialis Anterior	R-Tibialis Anterior
L-Plantaris	R-Plantaris	L-Soleus	R-Soleus
L-Quadriceps	R-Quadriceps	L-Flexor Digitorum Brevis	R-Flexor Digitorum Brevis
<i>Other Tissues:</i>			
Visceral fat pad	Spleen	Pancreas	Brain
Heart	Blood	Liver	Hippocampus

In order to obtain serum samples, blood was removed from the chest cavity and placed in a microcentrifuge tube which was spun at 750g for 30 minutes at room temperature. The serum was drawn off, placed in a fresh tube, and stored at -80°C. All aspects of this study were approved by the University of Maryland Institutional Animal Care & Use Committee (IACUC) Review Board (protocol R-06-77).

PAS staining: A cryostat was used to cut a 10 µm thick section from each tibialis anterior (TA) sample. The muscle sections were attached to a glycine-coated glass slide, which was then placed in a staining dish. Carnoy's fixative was added to the dish for 10 minutes, followed by a gentle rinse with deionized water. The Periodic Acid solution was then added to the dish for 10 minutes, again followed by rinsing of the slides with deionized water. Next, Schiff Reagent was added for 5 minutes and deionized water was used to wash the samples three times after removal from reagent. Ascending alcohol

solutions (50%, 70%, 80%, 95% x 2, 100% x 2) placed in staining dishes were used to dehydrate the samples. After alcohol dehydration, samples were washed with neoclear (Harleco, Darmstadt, Germany) three to four times in a staining dish. Permount (Biomedex, Burlington, MA) was used to mount coverslips onto the glass slides. The slides were analyzed under a microscope, with fibers containing high levels of glycogen appearing pink, red, or purple in color. The numbers of positive, partially positive, and negative fibers were counted and expressed as a percentage of total fibers. A minimum of 50 fibers per muscle were counted.

Oil-Red-O: A cryostat was used to cut a 10 μm section of each frozen TA muscle sample. Samples were air dried onto a glass slide then fixed in formalin for 5 minutes followed by a 1-minute wash with tap water. The slides were then rinsed with 60% isopropanol, stained with Oil-Red-O solution for 30 minutes and rinsed with 60% isopropanol. Permount (Biomedex, Burlington, MA) was used to mount a coverslip onto the glass slide. The slides were analyzed under a microscope and representative pictures of triglyceride content were taken for each muscle from each animal.

Western Blotting of Adipose Tissue: Western blotting was performed in order to analyze protein content of total ERK1/2 and p-ERK1/2 (Thr 202/Tyr 204) in visceral fat. Fat was homogenized in Mueller buffer (50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $\text{Na}_4\text{P}_2\text{O}_7\cdot\text{H}_2\text{O}$, 100 mM β -glycerophosphate, 25 mM NaF, 50 $\mu\text{g}/\text{mL}$ leupeptin, 50 $\mu\text{g}/\text{mL}$ pepstatin, 40 $\mu\text{g}/\text{mL}$ aprotinin, 5 mM Na_3VO_4 , and 1 mM PMSF) using a mechanical homogenizer, then centrifuged for 10 minutes at 4°C and 13,000 rpm. The supernatant between the fat cake and the pellet was removed using a syringe and placed into a separate tube, which was spun once more under the same

conditions. The middle layer was drawn off again and placed in a fresh tube. Mueller buffer was used to create a 1:50 dilution of the sample for a Pierce protein assay where protein concentration was determined via absorbance at 562 nm as compared to a standard curve. Equal amounts of total protein were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes as previously described (32). Blots were incubated with Ponceau S (Sigma Chemical) to ensure equal loading of the lanes and then blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBS-T). Membranes were probed with antibodies for p-ERK1/2 (Thr 202/Tyr 204; 1:2000), or total ERK1/2 (1:2000) in a buffer of 5% BSA in TBS-T on a rocker in a cold room overnight. Following incubation with the primary antibody, membranes were washed in TBS-T (3 x 5 min) and then incubated for 1 hour with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (1:1000) in 5% nonfat dry milk in TBS-T. Next, membranes were washed in TBS-T (1 x 10 min, 3 x 5 min), followed by enhanced chemiluminescence reagent (ECL) (Pierce, Rockford, Ill). Following ECL exposure, bands were visualized and quantified with GeneSnap and the GeneTools program (Syngene, Frederick, Md).

RNA isolation: TRIzol reagent (Invitrogen Technologies, San Diego, CA) was used for RNA extraction from the quadriceps muscle samples by the guanidine isothiocyanide method previously described by Chomczynsky (35). First, each sample was homogenized with 1 mL of TRIzol and placed in a 1.7 mL microfuge tube. Homogenates were incubated for 10 minutes and vortexed periodically. Next, 0.2 mL of chloroform was added to each sample, which was then inverted until the sample became cloudy, followed by 10 minutes of gentle rotation. Tubes were centrifuged at 12,000 rpm for 12

minutes at 4°C. The upper aqueous phase was transferred to a new microfuge tube to which was added 0.5 mL of isopropyl alcohol. Following 10 minutes of gentle rotation, tubes were centrifuged at 12,000 rpm for 8 minutes at 4°C. The supernatant was then removed and discarded without disrupting the RNA pellet. Next, 1.5 mL of 75% ethanol was added to each tube, which was vortexed briefly, then incubated at room temperature for 5 minutes, then spun at 7500 rpm for 5 minutes at 4°C. The ethanol was removed without disrupting the RNA pellet. The pellet was air dried until it became semi-transparent, at which time 50 µL of TE buffer was added. A 1:50 dilution of each sample to TE buffer was placed in another tube. A UV spectrophotometer was used to measure absorbance at 260 and 280 nm. TE buffer was used to blank the spectrophotometer, then absorbance of the 1:50 dilutions was measured for each sample. The absorbance at 260 nm was used to calculate the volume of RNA necessary to reverse transcribe for 1 µg of RNA in the subsequent RT reaction.

Reverse Transcription (RT): One µg of total RNA was reverse transcribed for each sample with the Applied Biosystems RT kit (Applied Biosystems, Foster City, CA) in a 20-µL reaction volume at 25°C for 10 min, followed by incubations at 37°C for 120 minutes and 85°C for 5 seconds. The samples were subsequently stored at 4°C for future analyses.

PCR: PCR was used to analyze mRNA expression levels of Citrate Synthase (CS), Glycogen Phosphorylase (GP), Carnitine Palmitoyl Transferase-1 (CPT-1) and Fatty Acid Translocase/Cluster of Differentiation 36 (FAT/CD36).

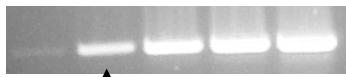
Table 2. Targets for mRNA expression, forward (F) and reverse (R) primers, annealing temperature, and cycle number for each target.

Target			Anneal. Temp(°C)	Cycle #
CS	F	CACAGCCCCTCACAGTGAAAGC	55	24
	R	GTAGTCTCGTAACTTCTCATCTGACA		
GP	F	GTCGCTTCTTCAAGGTCTGG	47	22
	R	AAGAAAGCAGCACGTTTCGAT		
CPT-1	F	GTCGCTTCTTCAAGGTCTGG	51	25
	R	AAGAAAGCAGCACGTTTCGAT		
CD36	F	GGCCAAGCTATTGCGACAT	51	25
	R	CAGATCCGAACACAGCGTAGA		
18S	F	GATCCATTGGAGGGCAAGTCT	55,47	25
	R	CCAAGATCCAACACTACGAGCTTTTT		

In order to optimize the PCR conditions for each target, PCR reactions were conducted at 20, 25, 30, 35, and 40 cycles. The PCR products were run out on a 2% agarose gel.

Optimal cycle number was determined by the cycle number that produced a band that was still in the linear range of detection, as shown below.

Cycle # 20 25 30 35 40



Selected cycle # = 25

Figure 1. An example of optimization of PCR conditions for GP.

A relative RT-PCR method using 18S, as an internal standard was used to measure mRNA expression of metabolic targets listed above by methods previously described (3).

Primers were diluted to a concentration of 10 μ M with TE buffer. A master mix was created with 2.5 μ L 10X Taq buffer, 1.5 μ L MgCl₂, 2.5 μ L dNTP, 0.4 μ L forward primer, 0.4 μ L reverse primer, 0.2 μ L Taq DNA polymerase, 14 μ L dH₂O, and 2 μ L

cDNA per reaction. However, each reaction for CPT-1 also contained 2.5 μ L dimethylsulfoxide (DMSO) and only 12 μ L dH₂O. This created a 25 μ L reaction for each sample. PCR was run in a PTC-100 Peltier thermal cycler (MJ Research, Waltham, MA) under the following conditions:

- 1) 75°C for 10 minutes
- 2) 95°C for 30 seconds
- 3) X°C for 30 seconds (annealing temp)
- 4) 75°C for 30 seconds
- 5) X cycles of steps 2-4 (specific for each target)
- 6) 75°C for 5 minutes

After removal from the thermal cyclers, 8 μ L of Orange G was added to each sample. Samples were loaded onto a 2% agarose gel (300 μ L SB, 6 g agarose, 15 μ L EtBr) along with a molecular weight marker, and run at 120V for one hour. Bands were visualized using a camera and Kodak ID software (Kodak, Rochester, NY). Quantification of bands was performed using ImageJ software (Image J, NIH Bethesda, MD). The ratio of GP to CS was used as an indication of energy utilization potential, while the ratio of FAT/CD36 to CPT-1 served as a measure of lipid storage.

NEFA Assay:

In order to determine levels of non-esterified fatty acid (NEFA) in mouse serum, a colorimetric assay was performed (Wako Diagnostics, Richmond, VA). A standard curve was made using supplied NEFA stock solutions. Tubes were labeled for each sample and for all concentrations of the standard curve. To each tube, 25 μ L of sample was added, followed by the addition of 1 mL of reagent A. Contents were mixed well and then

incubated for 5 minutes at 37°C. Following incubation, absorbance was measured at both 550 nm and 660 nm. Next, 0.5 mL of reagent B was added to each tube. Tubes were mixed well and incubated for another 5 minutes at 37°C. After the second incubation, absorbance was measured at 550 nm and 660 nm. Absorbance values at 660 nm were subtracted from absorbance values at 550 nm to get final absorbance values. Absorbance value versus concentration was plotted for the standard curve, which resulted in a linear relationship. The linear equation was used with the final absorbance values for each sample in order to calculate NEFA levels.

Blood Glucose Measurement:

When animals were sacrificed, 100 µL of blood was placed into a microcentrifuge tube and stored at -80°C until analyses were made. Blood was thawed on ice and vortexed just prior to conducting measurements. Blood glucose was measured using a YSI Glucose Analyzer (YSI Life Sciences, Yellow Springs, OH). The machine was calibrated and samples were measured in manual mode. For each sample, the sipper aspirated 25 µL of blood. In order to get readings in duplicate, measures were taken from two separate probes. Measures were made for each sample with the two probes and recorded in mg/dL.

Statistical Analysis:

All data are expressed as means ± SEM. Statistical significance was determined using t-tests (one-tail) with Sigma Stat statistical analysis software (Systat Software Inc., San Jose, CA). All comparisons that were analyzed were based on the specific, directional hypotheses described in the proposal. A p-value ≤ 0.05 was considered significant.

RESULTS

The effects of OVX, exercise training, and 17 β -estradiol supplementation on anatomical characteristics and markers of metabolic syndrome in 16-week-old C57/Bl6 mice. The average body and tissue weights for animals in each group are summarized in Figure 2 and Table 3(A-B). The visceral fat mass in the sedentary OVX group was significantly greater compared to all the other groups, with a significant 313% higher value compared to the sedentary SHAM animals ($p=0.004$) (Fig. 2). Further, voluntary wheel running was effective at preventing the increase in visceral fat mass in the OVX mice as compared to the sedentary OVX mice ($p=0.027$). Supplementation with 17 β -estradiol prevented any increase in the visceral fat mass of the OVX animals when compared to the SHAM animals, with no additional effect of exercise in conjunction with 17- β estradiol supplementation. Body mass of sedentary OVX animals was significantly greater than that of sedentary SHAM animals ($p=0.044$) (Table 3A). Heart and liver weights in the OVX+E₂ ex group were significantly greater than those of the SHAM sed group ($p=0.011$ and $p=0.012$, respectively) (Table 3B).

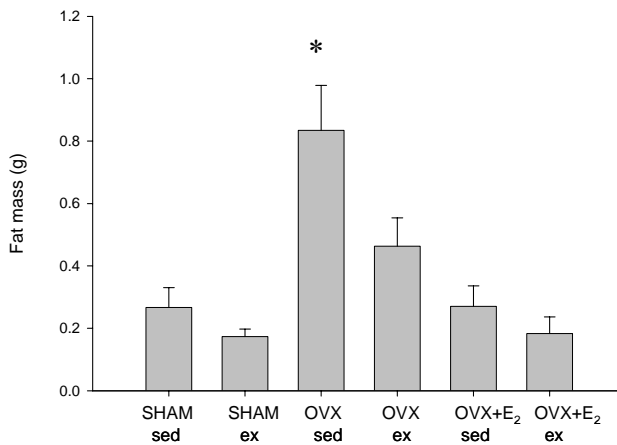


Figure 2. Visceral fat mass for each group. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means \pm SEM. *Indicates significantly greater than all other groups ($p<0.05$).

Table 3 (A-B). Anatomical characteristics. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

A) Body weight and muscle mass

Group	Body Weight (g)	TA-R (g)	TA-L (g)	Quad-R (g)	Quad-L (g)
SHAM sed	22.94± 0.57	0.038± 0.001	0.039± 0.001	0.152± 0.008	0.145± 0.007
SHAM ex	23.35± 0.49	0.043± 0.001	0.039± 0.002	0.146± 0.011	0.153± 0.011
OVX sed	25.20± * 0.96	0.039± 0.001	0.036± 0.002	0.150± 0.011	0.148± 0.010
OVX ex	23.85± 0.53	0.036± 0.001	0.036± 0.002	0.134± 0.016	0.151± 0.004
OVX+E ₂ sed	23.72± 0.43	0.037± 0.006	0.036± 0.001	0.158± 0.006	0.143± 0.003
OVX+E ₂ ex	24.08± 0.50	0.036± 0.002	0.034± 0.002	0.153± 0.015	0.156± 0.006

B) Organ weight

Group	Liver (g)	p value (v.SHAM sed)	Heart (g)	p value (v.SHAM sed)
SHAM sed	0.904±0.063	-	0.102±0.002	-
SHAM ex	1.075±0.075	0.060	0.109±0.006	0.165
OVX sed	0.940±0.068	0.356	0.105±0.007	0.382
OVX ex	0.939±0.059	0.494	0.106±0.002	0.470
OVX+E ₂ sed	1.025±0.049	0.084	0.104±0.004	0.390
OVX+E ₂ ex	1.088±0.041	0.020 *	0.115±0.004	0.011 *

The effects of OVX and 17β-estradiol supplementation on voluntary wheel-running

activity. The average distances run per day for the SHAM, OVX, and OVX+E₂ mice in the wheel-running groups are shown in Figure 3. Ovariectomy resulted in a significant 73% decrease in running distance compared to the SHAM animals (p=0.0005).

Supplementation of the OVX animals with 17β-estradiol resulted in a 110% increase in running distance compared to the OVX group (p=0.016), although the distances were still significantly less than the SHAM group (p=0.004).

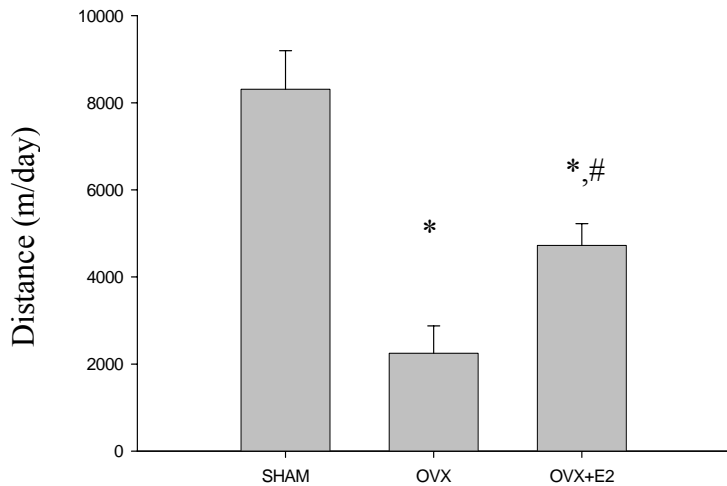


Figure 3. Average distance run per day for each group. N=5 for SHAM and OVX+E₂ groups, N=6 for OVX group. Data is presented as means ± SEM. *Indicates significantly different from SHAM group, #indicates significantly different from OVX group (p<0.05).

The effects of OVX and estradiol supplementation on blood glucose and NEFA levels.

Average fasted non-esterified free fatty acid (NEFA) and blood glucose levels are summarized in Table 4. SHAM sedentary animals were not significantly different compared to any other group. However, NEFA levels in OVX sedentary mice were significantly greater than NEFA levels in SHAM exercised mice (p=0.05). Further, when NEFA levels from sedentary and exercised groups were combined for each condition (SHAM, OVX, OVX+E₂), the OVX mice had significantly greater NEFA levels than SHAM mice (p=0.021), but were not significantly different from OVX+E₂ mice.

Blood glucose was higher in OVX sedentary mice as compared to SHAM sedentary (p=0.031), OVX+E₂ sedentary (p=0.031), and OVX exercised (p=0.031) mice (Table 4).

Table 4. Fasted NEFA and glucose levels in blood. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM. P-values are presented for values as compared to the SHAM sed group.

Group	Average NEFA (mmol/L)	p value	Average Blood Glucose (mg/dl)	p value
SHAM sed	0.1312±0.0187	-	188.5±14.48	-
SHAM ex	0.1129±0.0219	0.271	198.3±15.27	0.327
OVX sed	0.1616±0.0330	0.094	221.42±20.07	0.031*
OVX ex	0.2057±0.0517	0.121	176.42±8.68	0.321
OVX+E ₂ sed	0.1320±0.0230	0.490	177.1±9.09	0.329
OVX+E ₂ ex	0.1777±0.0311	0.110	195.9±19.4	0.336

Glycogen staining in the TA muscle of each group. An example of how individual fibers were categorized is shown in Figure 4A. Results of PAS staining are shown in Figure 4B as the percentage of total fibers which were glycogen positive (+/+), partially positive (+/-), and negative (-/-). The percentage of positive fibers and partially positive fibers was significantly greater in exercised animals versus sedentary animals for each intervention. The percentage of negative fibers was significantly lower in exercised animals versus sedentary animals for each intervention (p<0.001). OVX sedentary mice had a significantly lower percentage of positive fibers as compared to SHAM sedentary mice (p=0.017). The percentage of positive and partially positive fibers was increased, and the percent of negative fibers decreased in the OVX exercised group compared to the OVX sedentary group (p=0.003, p=0.002, p=0.0004, respectively). Further, no differences were detected between the OVX exercise group and the SHAM sedentary group. No differences were detected between the OVX+E₂ sedentary and the SHAM sedentary groups. OVX+E₂ exercised animals had a greater percentage of partially positive fibers and a decreased percentage of negative fibers as compared to OVX+E₂

sedentary animals ($p=0.03$, $p=0.05$, respectively). The percentage of positive and partially positive fibers were increased, and the percentage of negative fibers decreased in the OVX+E₂ exercised animals compared to SHAM sedentary animals ($p=0.05$, $p=0.02$, $p=0.02$, respectively) (Figure 4B).

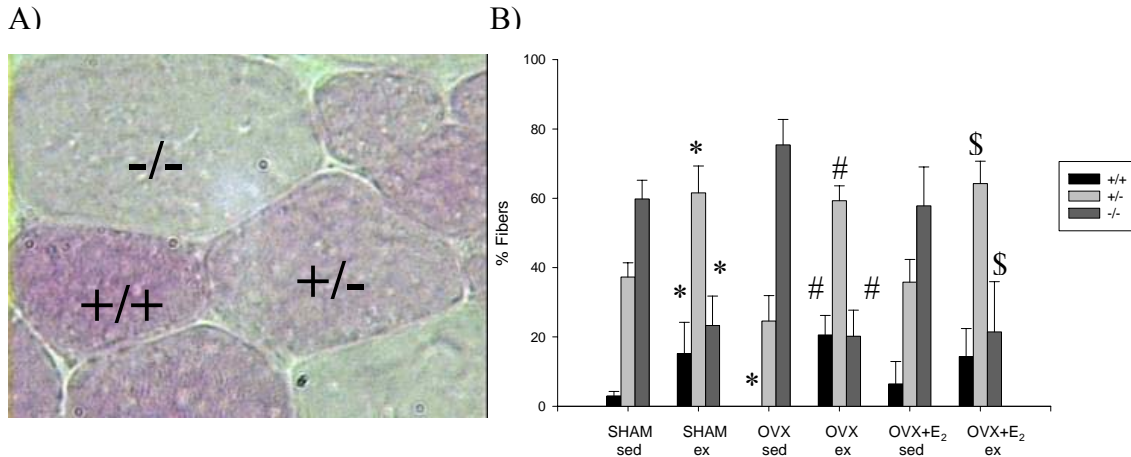


Figure 4.

A) Example of PAS stain quantification. +/+ indicates fiber counted as positive, +/- indicates fiber counted as partially positive, -/- indicates fiber counted as negative.

B) Results of PAS staining for each group. N=4 for SHAM sed/ex, OVX+E₂ sed. N=3 for OVX+E₂ ex group. N=5 for OVX sed/ex groups. Values indicate the % of fibers counted (+/+, +/-, -/-) with a minimum of 50 total fibers. Presented as average % \pm SEM. *Indicates significantly different from SHAM sed. #Indicates significantly different from OVX sed. \$Indicates significantly different from OVX+E₂ sed.

Triglyceride staining in the TA muscle of each group. TA cross-sections stained with Oil-Red-O were qualitatively observed under a light microscope. In the SHAM sedentary animals, there was minimal presence of triglyceride, indicated by the absence of red pigment, as shown in Figure 5. In the SHAM exercise group, there was frequent appearance of triglyceride, indicated by the dark red droplets (Figure 5). The OVX sedentary animals exhibited large volumes of red droplets. Exercised OVX animals also had large amounts of dark red droplets (Figure 5). The 17 β -estradiol-supplemented, sedentary mice had fibers that were low in droplet presence. In the OVX+E₂ exercise group there was a frequent appearance of red droplets in the muscle (Figure 5).

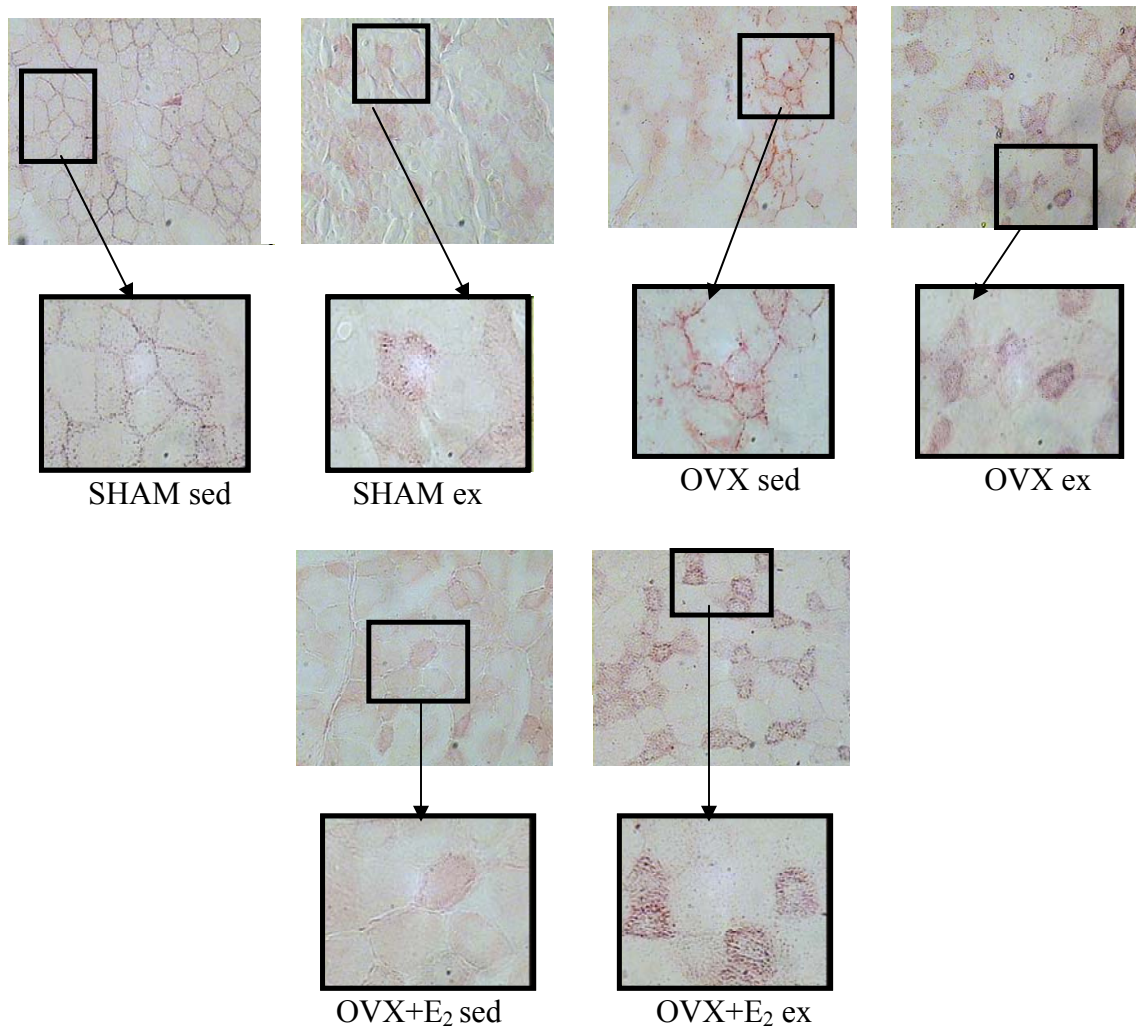


Figure 5. Example of Oil-red-O stain for SHAM sed/ex, OVX sed/ex, and OVX+E₂ sed/ex animals. Dark red color indicates presence of intramuscular triglyceride.

Metabolic gene expression in the quadriceps muscle. Expression levels of GP and CS were not different among any of the groups (Figures 6A-B). Further, the ratio of GP:CS did not yield any significant differences in gene expression (Figure 6C).

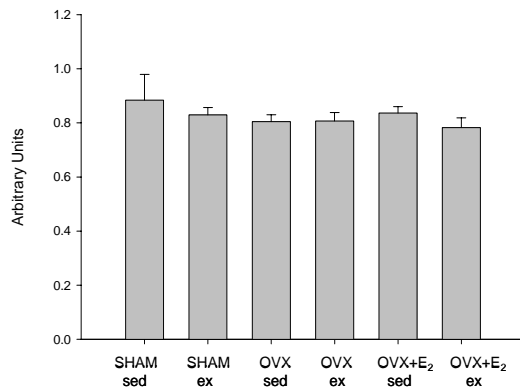


Figure 6A. Relative expression of normalized GP. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

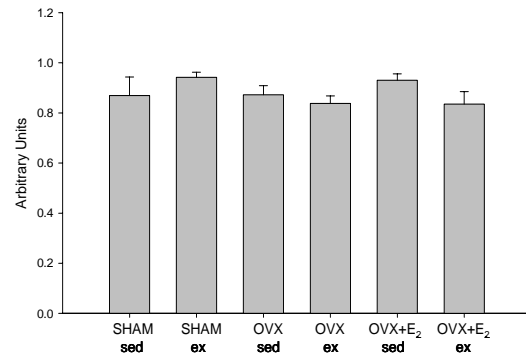


Figure 6B. Relative expression of normalized CS. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

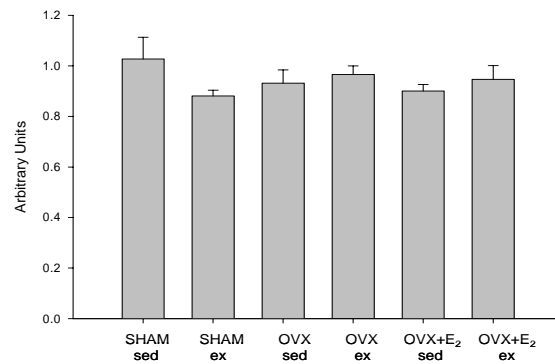


Figure 6C. Ratio of GP:CS. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

No differences in expression levels of FAT/CD36 or CPT-1 were found between any groups (Figure 7A-B). The ratio of FAT/CD36:CPT-1 did not exhibit any significant differences between groups (Figure 7C).

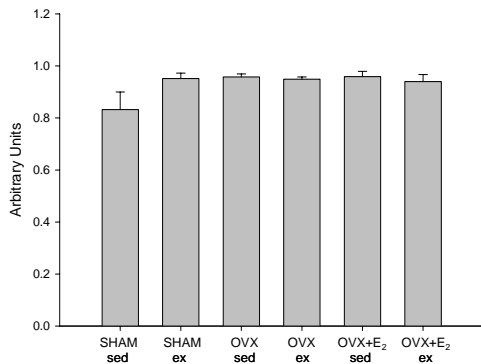


Figure 7A. Relative expression of normalized FAT/CD 36. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

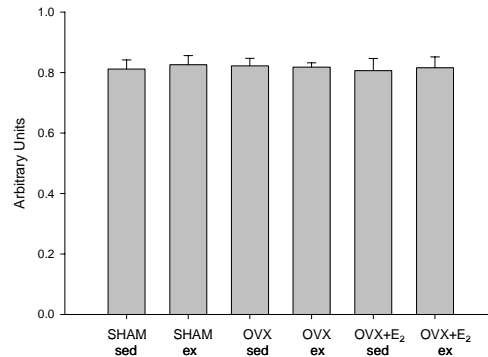


Figure 7B. Relative expression of normalized CPT-1. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

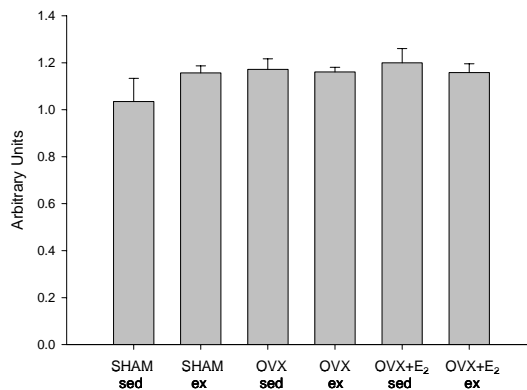


Figure 7C. Ratio of FAT/CD36:CPT-1. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. Values are presented as means ± SEM.

MAPK phosphorylation in adipose tissue from each group. Representative blots for phosphorylated and total ERK1/2 are shown in Figure 8. Phosphorylation of ERK1 (p-ERK1) was significantly greater in the sedentary OVX mice as compared to all other groups ($p < 0.05$) (Figure 9). When normalized to total ERK1, differences were detected between the sedentary SHAM and OVX mice ($p = 0.016$) and between the OVX and OVX+ E₂ sedentary mice ($p = 0.037$) (Figure 10).

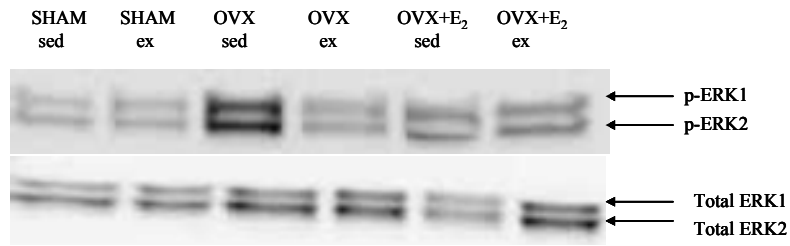


Figure 8. Representative blot of phosphorylated and total ERK1/2

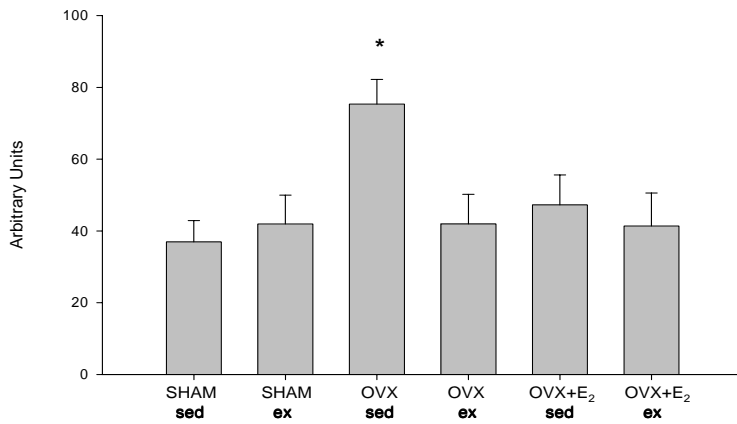


Figure 9. Absolute phosphorylation of ERK1 was higher in the OVX sed group. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. *Indicates significantly different from all groups (p<0.05)

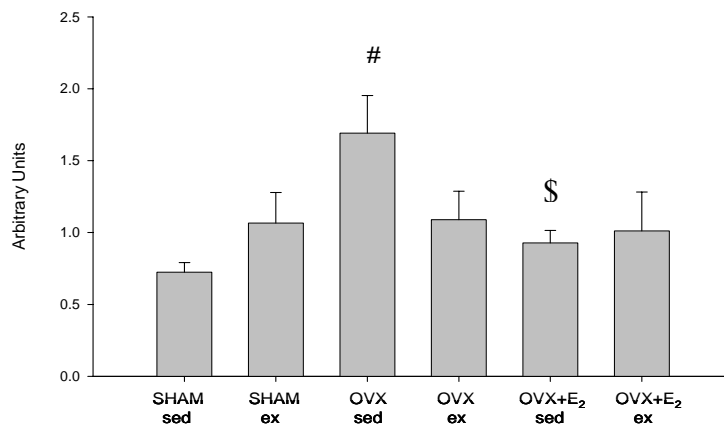


Figure 10. Phosphorylation of ERK1 normalized to total ERK1. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. #Indicates significantly different from SHAM sed (p=0.016) \$Indicates significantly different from OVX sed (p=0.037)

Phosphorylation of ERK2 (p-ERK2) was also significantly greater in the sedentary OVX mice as compared to all other groups ($p < 0.05$), (Figure 11). When normalized to total ERK1, differences were detected between the sedentary SHAM and OVX mice ($p = 0.016$) and between the SHAM and OVX+ E₂ sedentary mice ($p = 0.004$) (Figure 12).

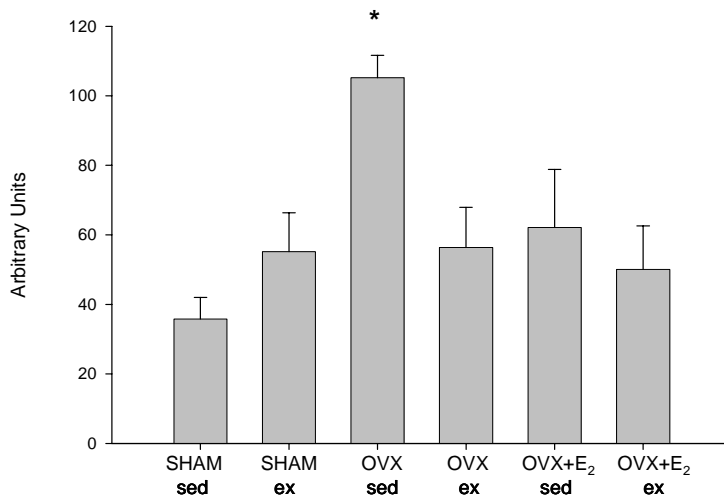


Figure 11. Absolute phosphorylation of ERK2 was higher in the OVX sed group. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. *Indicates significantly different from all groups ($p < 0.05$)

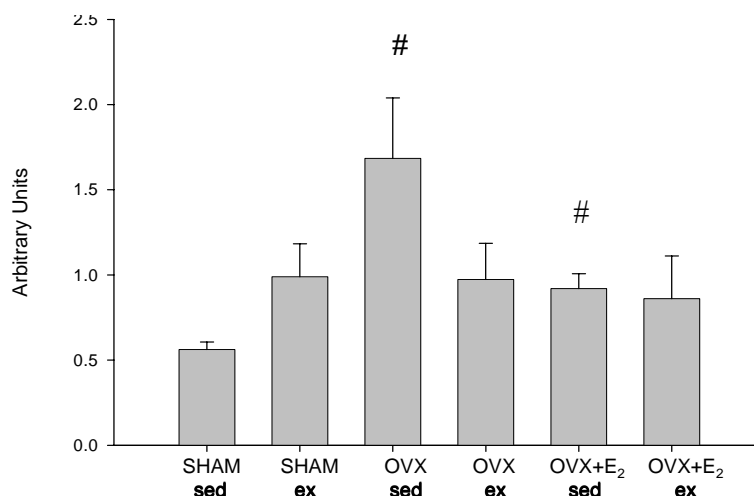


Figure 12. Phosphorylation of ERK2 normalized to total ERK2. N=5 for SHAM sed/ex, OVX+E₂ sed/ex groups. N=6 for OVX sed/ex groups. #Indicates significantly different from SHAM sed ($p < 0.05$)

Discussion

This study demonstrates that 10 weeks after undergoing ovariectomy surgery (OVX) mice present with significantly elevated levels of blood glucose, NEFA, and increased visceral fat mass compared to SHAM animals. Providing the OVX mice with a voluntary running wheel significantly attenuated the increase in blood glucose and visceral fat mass when compared to the sedentary SHAM mice even though the OVX mice ran significantly less than active SHAM mice. Supplementation with 17 β -estradiol did not completely recover running distances in the OVX mice, but did prevent changes in blood glucose, NEFA, and visceral fat mass. ERK1/2, a regulator of HSL-mediated lipolysis, was increased in adipose tissue of sedentary OVX mice as compared to sedentary SHAM mice. Both exercise and 17 β -estradiol supplementation in the OVX mice returned ERK1/2 phosphorylation levels to that of the SHAM mice. Exercise in combination with 17 β -estradiol supplementation did not result in further reductions in ERK1/2 phosphorylation. PAS staining of individual fibers in the TA muscle indicated a decrease in glycogen storage in sedentary OVX mice as compared to sedentary SHAM mice. Voluntary wheel running increased the percentage of fibers positive for glycogen in all groups, however, minimal differences were detected between active groups. The appearance of red droplets, indicative of IMTG, was frequently detected in all active groups and sedentary OVX mice. In contrast, sedentary SHAM and OVX+E₂ mice had minimal appearance of red droplets. In spite of changes in PAS and Oil-Red-O staining, no changes in mRNA expression of CPT-1, FAT/CD36, CS, or GP in skeletal muscle were detected between any of the groups.

Ovariectomy induces the metabolic syndrome

Based on the findings of this study, it appears that after 10 weeks of ovariectomy, mice developed symptoms indicative of the metabolic syndrome. In particular, OVX mice had increased central obesity, increased blood glucose levels, and signs of dyslipidemia compared to the SHAM animals. Other studies have observed similar changes in fat mass (64), blood glucose (17), and blood lipid profile (131) in OVX mice. Based on these findings, it appears that the ovary plays a role in fatty acid dynamics (See Figure 13. below)

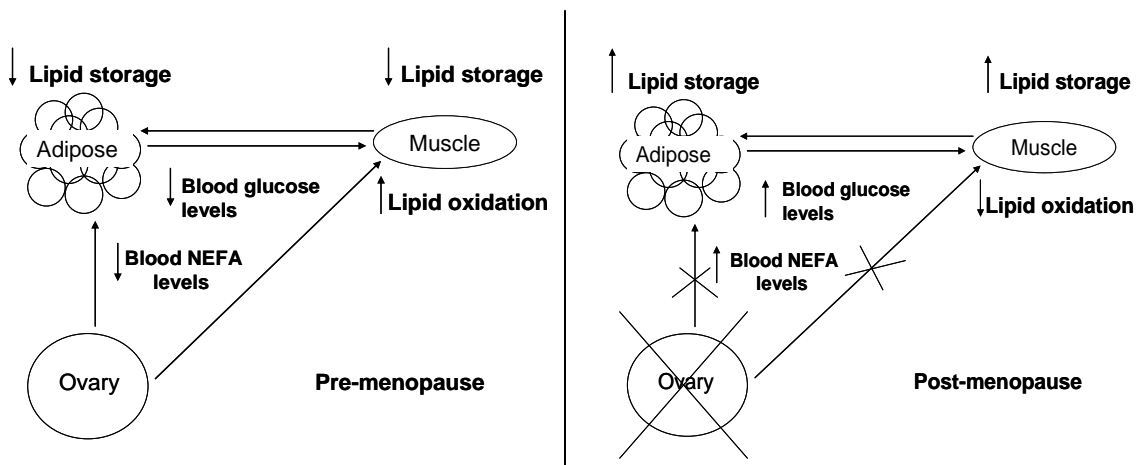


Figure 13. Role of the ovary and ovarian hormones in fatty acid dynamics.

However, in line with previous studies (60; 61; 100), 17β -estradiol supplementation prevented the increases in visceral fat mass, blood glucose, and NEFA levels in OVX mice. D'Eon et al. found increased levels of serum free fatty acids (FFA) following fasting in OVX mice, which was successfully attenuated with 17β -estradiol supplementation (44). Blood glucose levels in mice 13 weeks following ovariectomy were significantly increased compared to both SHAM and 17β -estradiol-supplemented mice (122). Many studies have demonstrated an increase in fat mass in mice following

ovariectomy (33; 41; 43; 96; 188), as well as an attenuation of fat accumulation when OVX mice are supplemented with 17 β -estradiol (68; 134; 153; 188). Thus, these data suggest that natural decreases in 17 β -estradiol during the onset of menopause may mediate some of the documented changes in metabolic parameters that occur in aging women.

Voluntary wheel running attenuated increases in visceral fat mass and blood glucose levels in OVX mice. This finding is particularly interesting considering the OVX animals ran 73% less than the SHAM mice and 53% less than the OVX+E₂ mice. So, even reduced levels of physical activity were effective at preventing metabolic changes in these mice. Shinoda et al. demonstrated that forced treadmill running improves body composition in OVX rats over a similar duration of time. However, the running protocol employed by Shinoda et al. was extremely vigorous compared to voluntary wheel running, with rats running up to 60 minutes at 26m/min and 10% slope 5 times per week over the 8-week study (188). Both voluntary wheel running and 17 β -estradiol supplementation attenuated onset of metabolic syndrome in OVX mice, however, there was no additive effect of the two treatments. The results suggest that low volumes of physical activity are just as effective as HRT in preventing the metabolic syndrome after the loss of ovarian hormone function.

Elevated NEFA levels suggest active lipolysis in OVX animals

When sedentary and exercised animals were pooled, the OVX animals exhibited increased levels of circulating NEFA. Previous studies suggest that ERK1/2 enhances HSL activity and subsequently enhances lipolysis (69; 93). In sedentary OVX animals ERK1/2 phosphorylation was greater than all other groups. Phosphorylation of ERK1/2

at Thr 202/Tyr 204 is necessary for complete catalytic activation of ERK1/2. Phosphorylated ERK1/2 can phosphorylate HSL at Ser600, which increases HSL activity and subsequently enhances lipolysis (193). In the sedentary OVX animals, NEFA levels were elevated, thus, an increase in factors contributing to lipolysis, such as ERK1/2 phosphorylation would be expected. Voluntary wheel running, as well as 17 β -estradiol supplementation reduced ERK1/2 phosphorylation to levels equivalent to SHAM animals. Thus, this finding does not explain why the active OVX animals had elevated NEFA in that voluntary wheel running was not successful at decreasing ERK1/2 phosphorylation. At this time, there is no commercially available antibody to determine the phosphorylation status of HSL600 in our samples. Thus it is unclear how HSL is affected by this change in ERK1/2 phosphorylation. It is possible that another factor, such as AMPK, contributed to changes in lipolysis and thus increased NEFA levels in exercised OVX animals. For example, other studies have shown AMPK to be activated and to exert a significant effect on lipolysis in adipose tissue taken from physically active animals (107; 110; 150). While many attempts were made to determine AMPK phosphorylation, the presence of AMPK was not detectable in adipose tissue in this study. This may be in part due to the fact that our measures were made in tissue from animals at rest, while other studies have measured AMPK in adipose tissue immediately after exercise (107; 110; 150). Further, regulation of HSL occurs by phosphorylation at multiple sites. The Ser563, Ser659, and Ser 660 sites are phosphorylated by Protein Kinase A (PKA) (29). Phosphorylation at these sites is additive in nature to HSL activation (47), and these remaining sites will need to be measured in these samples. In

addition, adipose tissue glycerol lipase (ATGL) was recently discovered and it is possible that ATGL contributes to changes in these animals (220).

While the role of ERK1/2 in adipose tissue lipolysis in OVX mice may be uncertain, it is clear that changes in ovarian hormone status affected the phosphorylation status of ERK1/2, and the phosphorylation status was reversed by voluntary wheel-running and 17 β -estradiol supplementation.

No differences in metabolic gene expression in the skeletal muscle were detected

Expression levels of CPT-1, FAT/CD36, GP and CS were not significantly different among any of the groups. CPT-1 promotes the oxidation of fat and decreases esterification of FFA (26). Previous studies have shown that CPT-1 expression increases with obesity (154), thus, it was somewhat surprising that no differences were detected in the OVX sedentary animals. However, because no gender differences have been detected in CPT-1 expression (20), perhaps ovarian hormones do not contribute to regulation of CPT-1 expression. Further, it is possible that the mice in this study were not obese for a long enough period of time to bring about changes in CPT-1 expression at the mRNA level in skeletal muscle.

FAT/CD36 plays an important role in FFA uptake at the level of the plasma membrane (108; 205). Even though the OVX mice appear to have increased fat storage, these mice did not exhibit differences in mRNA expression of FAT/CD36 compared to the SHAM group. In humans, it has been shown that short-term exercise training results in changes in FAT/CD36 expression (84). However, in this study, long-term exercise training failed to induce any changes in FAT/CD36 expression in muscle (183). This may indicate that mRNA changes in FAT/CD36 occur quickly, allowing for any

adaptations in protein expression to occur. Recently, it has been suggested that contraction-induced translocation of FAT/CD36 to the mitochondrial membrane is a significant contributor to FFA oxidation (73). This would suggest that the critical factor affecting FAT/CD36 is localization and not expression, thus, it is necessary to perform follow-up experiments at the protein level.

Citrate Synthase (CS) is an indicator of oxidative capacity which also promotes the oxidation of fat and decreases esterification of FFA (26). In spite of increased fat storage, no differences in CS expression in OVX mice compared to SHAM mice were found. Roepstorff et al. found gender differences to exist in CS protein activity, but no differences in CS mRNA expression (171). This may indicate that CS expression is regulated at the level of protein translation. Therefore, follow-up experiments at the protein level should be performed.

GP contributes to carbohydrate utilization by catalyzing the breakdown of glycogen for glycolysis (89). Even though the sedentary OVX mice appear to have decreased glycogen storage, these mice did not exhibit differences in GP mRNA expression compared to the SHAM group. Interestingly, none of the active groups demonstrated changes in GP mRNA. Other studies utilizing endurance exercise training have also failed to demonstrate changes in GP expression at the mRNA level (118). It was predicted that OVX mice would show differences in GP mRNA expression since other studies have found gender- and obesity-related differences in GP activity (4; 88). As with CS, this may suggest that follow-up experiments at the protein level should be performed to detect changes in GP activity.

It was somewhat surprising not to detect differences in CPT-1, FAT/CD36, CS, or GP mRNA expression based on the PAS and Oil-Red-O staining results in the TA muscle from the different groups. Ovariectomy resulted in a significantly lower percentage of PAS positive fibers as compared to SHAM sedentary mice, indicating a potential change in glycogen dynamics in the muscle. Exercise resulted in an increased percentage of PAS positive and partially positive fibers, and a decrease in the percentage of negative fibers as compared to the sedentary animals in all groups. Therefore, the ability of exercise to increase glycogen storage was not defective in any group. Based on these results, it appears that OVX results in a net loss in glycogen positive fibers, suggesting that glycogen is not stored effectively or it is utilized at a higher rate in OVX mice than in the SHAM animals. Beckett et al. found a similar decrease in muscle glycogen content following ovariectomy (14). Interestingly, exercise is effective at restoring the number of glycogen positive fibers in OVX animals to a level equal to that of SHAM animals. Thus, exercise fixed the impaired glycogen storage found in sedentary OVX animals. Both exercise and 17 β -estradiol supplementation was effective at increasing the number of glycogen positive fibers in the OVX animals.

In addition to differences in glycogen levels, OVX animals exhibited a high frequency of Oil-Red-O staining in the muscle, which indicates that the muscle is storing lipid. It is unclear whether this excess storage is due to a failure to oxidize the lipid. CS mRNA expression would suggest that oxidative capacity may not be reduced in these animals, as no differences in expression were detected. However, it is possible that measurement of CS at the protein level would be more effective at detecting differences. Oil-Red-O staining resulted in high amounts of red droplets in the SHAM, OVX+E₂,

OVX exercised groups, as well as the OVX sedentary group, and small amounts of red droplets in the SHAM and OVX+E₂ sedentary groups. This would suggest that exercise and ovariectomy may result in increased uptake of FFA into the muscle. IMTG content in response to exercise training has been widely studied, with endurance exercise training resulting in increased levels of IMTG in the muscle (65), as well as increased ability to oxidize IMTG (65; 163). The high amounts of red droplets in the SHAM and OVX+E₂ exercised groups as compared to their sedentary counterparts are thus in line with previous findings. Obesity results in increased uptake of FFA into the muscle as well (66; 130). However, the ability to oxidize IMTG may be impaired (144). The OVX mice in this study displayed centripetal obesity, thus, the high amount of red droplets in the sedentary OVX animals would be expected and may be indicative of a reduced oxidative capacity of the muscle.

While the Oil-Red-O findings are in line with previous studies, there is a disconnect with the mRNA expression results within this study. Measurement of both CPT-1 and FAT/CD36 mRNA expression indicated no differences between groups. An increase in FAT/CD36 would be expected to accompany increased IMTG, as FAT/CD36 contributes to FFA uptake into muscle cells. Further, in animals capable of increased oxidation of IMTG, such as the SHAM exercise trained group, an increase in CPT-1 expression would be expected, as CPT-1 regulates transport of FFA into the mitochondria for oxidation. However, as previously discussed, these expected differences may not have been observed since FAT/CD36 may be regulated through post-translational modification (73; 171). Therefore, protein expression or protein activity, rather than mRNA expression, should be measured.

Future Directions

There are a few areas in which additional experiments should be performed in order to expand upon the findings of this study. While the lack of differences in mRNA expression is understandable given the results of other studies, targets could be measured at the protein level, or translocation could be measured to test for differences in activity. To test the functional effects of changes in ERK1/2 phosphorylation, downstream activation of targets (i.e., HSL, ATGL) should be measured. Recent pilot data indicates that ATGL expression, which promotes lipolysis and contains 2 amino acid residues capable of phosphorylation by ERK1/2, is elevated in exercised SHAM and OVX mice, as well as OVX sedentary mice. These data suggest that active lipolysis is increased in OVX mice. The increase in ATGL expression in the SHAM mice indicates lipolysis is occurring during exercise, however, there is most likely an increase in fat oxidation as well which is absent in the OVX mice. Clearly, more data will need to be collected to further clarify these findings.

Overall Conclusions

It appears that both voluntary wheel running and 17 β -estradiol supplementation are effective in preventing symptoms of the metabolic syndrome and changes in cellular signaling of adipose tissue in OVX mice. However, in light of the Women's Health Initiative (WHI), the question of hormone replacement safety arises. Two of the 17 β -estradiol-supplemented mice died of unknown causes during this study. Further, upon sacrifice, two mice in the OVX+E₂ group had enlarged bladders, a side effect which has been found in other labs as well (personal communication with Dr. Dawn Lowe). The results of studies from WHI suggest that HRT increases a woman's risk of stroke, blood

clots, heart disease, and certain cancers. With the efficacy of even low volumes of voluntary wheel running in preventing obesity and metabolic syndrome following ovarian hormone loss, exercise should be suggested as an alternative to HRT.

When comparing the results to the original hypotheses, some findings confirmed the hypotheses, and some findings refuted the hypotheses. Comparisons between the hypotheses and results are shown below.

H₁: Ovariectomized mice undergoing voluntary exercise training will have similar fat masses to female control mice, but higher masses than ovariectomized mice supplemented with 17 β -estradiol.

The findings of this study supported the hypothesis that OVX exercised mice would have similar fat masses to female control mice. However, OVX exercised mice did not have higher fat masses than OVX+E₂ mice.

H₂: Ovariectomized mice undergoing voluntary exercise training will have similar levels of circulating free fatty acids to control mice, but higher levels than ovariectomized mice supplemented with 17 β -estradiol.

The findings refute this hypothesis, in that exercise training did not decrease NEFA levels in OVX mice.

H₃: Ovariectomized mice undergoing voluntary exercise training will have similar fasting blood glucose levels to control mice, but impaired fasting blood glucose levels compared to ovariectomized mice supplemented with 17 β -estradiol.

The findings support the hypothesis that OVX exercised mice would have similar fasting blood glucose to control mice. However, fasting blood glucose levels in the OVX exercised mice were not impaired compared to OVX+E₂ mice.

H₄: The sedentary ovariectomized mice will have greater amounts of intramuscular triglycerides than the exercise-trained ovariectomized mice or 17β-estradiol-supplemented mice.

Due to qualitative, rather than quantitative analysis of Oil-Red-O staining, this hypothesis cannot be analyzed. While quantitative comparisons of groups cannot be made, visually it appeared that muscle tissue from the SHAM sed, OVX sed, OVX ex, and OVX+E₂ ex groups all contained large amounts of IMTG based on the frequent appearance of red droplets.

H₅: The sedentary ovariectomized mice will have decreased expression levels of lipid oxidative genes as compared to the exercise-trained ovariectomized mice or 17β-estradiol-supplemented mice.

The findings refuted this hypothesis, as no differences in expression of lipid oxidative genes were found between any groups.

H₆: The sedentary ovariectomized mice will have increased expression levels of genes promoting lipid storage as compared to the exercise-trained ovariectomized mice or 17β-estradiol-supplemented mice.

The findings refuted this hypothesis, as no differences in expression of genes promoting lipid storage were found between any groups.

H₇: The sedentary ovariectomized mice will have increased phosphorylation of ERK1/2 (Thr 202/Tyr 204) in adipose tissue at rest as compared to the sedentary

SHAM mice and ovariectomized mice supplemented with 17 β -estradiol. The exercise-trained SHAM mice and 17 β -estradiol-supplemented mice will have increased phosphorylation ERK1/2 (Thr 202/Tyr 204) compared to their sedentary counterparts. The exercise-trained OVX mice will have phosphorylation levels of ERK1/2 (Thr 202/Tyr 204) similar to sedentary OVX mice.

As predicted, ERK1/2 phosphorylation was increased in the sedentary OVX mice compared to the sedentary SHAM and OVX+E₂ mice. Also, OVX exercised and sedentary groups did demonstrate ERK1/2 phosphorylation levels that were not significantly different. However, the exercised SHAM and OVX+E₂ mice did not increase ERK1/2 phosphorylation compared to their sedentary counterparts.

REVIEW OF LITERATURE

The purpose of this review of literature is to examine the role of exercise and estrogens in preventing obesity and the metabolic syndrome in post-menopausal females.

Obesity:

The increased incidence of obesity and obesity-related conditions has placed significant strain on individual health, the medical community, and the economy alike. The most recent figures from the Center of Disease Control estimate 65% of adults, and 15% of children in the United States are overweight, with 30% falling in the category of obese (1). Along with the increased incidence of obesity, the relative risk for Type II diabetes and cardiovascular disease (CVD) has risen dramatically in obese individuals (55-57; 102; 196). Android, or centripetal obesity is of particular concern, since it is highly correlated with the development of obesity-related conditions. Finally, the treatment of obesity-related conditions has placed a significant burden on the economy, with an estimated \$122.9 billion spent annually (CDC), thus, the physiological and economic costs of obesity are having a significant impact on American society.

Obesity-related conditions:

Studies have linked visceral fat accumulation to an increase in occurrence of Type II diabetes, cardiovascular disease, and risk of myocardial infarction in both men and women (219). Taken together, these conditions which have been attributed to obesity have been termed the metabolic syndrome. On the surface, obesity has been dubbed the major cause of the metabolic syndrome, however, the underlying mechanisms responsible for the associated metabolic changes are an increase in visceral adiposity and insulin

resistance (5; 71; 167). Many different groups, including the International Diabetes Federation (IDF), World Health Organization (WHO), and Adult Treatment Panel III (ATP III), have developed lists of factors used to diagnose the presence of the metabolic syndrome. The criteria set forth by the IDF and WHO are most commonly used for diagnosis. For diagnosis of the metabolic syndrome under the IDF criteria, waist circumference must be greater than or equal to 80 cm, indicating central obesity, along with 2 of the 4 following criteria:

- 1) Elevated triglycerides (greater than or equal to 1.7 mmol/L)
- 2) Low HDL cholesterol (less than 1.29 mmol/L)
- 3) Elevated blood pressure (systolic greater than or equal to 130 mmHg or diastolic greater than or equal to 85 mmHg)
- 4) Elevated fasting plasma glucose (greater than or equal to 5.6 mmol/L)

In order to be diagnosed with the metabolic syndrome with the WHO criteria, a patient must exhibit diabetes mellitus, decreased glucose tolerance, increased fasted blood glucose or insulin resistance, plus two of the following criteria:

- 1) Blood pressure greater than or equal to 140/90 mmHg
- 2) Dislipidemia (triglycerides greater than or equal to 1.695 mmol/L and/or high density lipoprotein cholesterol (HDL-C) less than or equal to 1.0 mmol/L),
- 3) Central obesity (waist:hip ratio greater than 0.85, and/or body mass index (BMI) greater than 30 kg/m^2 , and
- 4) Microalbuminuria (urinary albumin excretion ratio greater than or equal to 20 mg/min or albumin:creatinine ratio greater than or equal to 30 mg/g)

Age, genetic predisposition, physical inactivity, diet, and possibly hormonal status (168) in females all contribute to an increased risk for the development of the metabolic syndrome (170).

Path from obesity to metabolic syndrome:

On a cellular level, various theories have been developed to explain the progression from obesity to the onset of the metabolic syndrome. Obesity results in increased amounts of white adipose tissue (WAT) and greater amounts of visceral fat than subcutaneous fat (132). WAT has a large capacity to store lipids, and this increase in lipid storage has a pathophysiological effect as well (211). Obese patients demonstrate low-grade inflammation that originates from WAT, and is characterized by an increase in inflammatory markers such as interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF- α) (13; 210). These markers have local effects on the WAT, as well as systemic effects that may contribute to insulin resistance (202). TNF- α contributes to insulin resistance by inducing phosphorylation of serine residues on the insulin receptor substrate-1 (IRS-1) protein, thus preventing interaction with the insulin receptor β subunit, and disrupting the insulin signaling pathway (13). IL-6 interferes with the signal transduction mechanisms involved in insulin signaling, although the mechanism of action is unknown (12). The secretion of IL-6 is greater from visceral adipose tissue than subcutaneous adipose tissue (12) and serves as a major marker for cardiovascular problems such as atherosclerosis (182) and coronary heart disease (12), which are part of the metabolic syndrome. However, the role of IL-6 is controversial because it also increases with exercise and has been suggested to increase glucose uptake by activating AMPK (128; 152; 179). By increasing levels of inflammatory mediators which

contribute to insulin resistance and cardiovascular disease, obesity can result in development of the metabolic syndrome (101; 109; 146).

The decline in circulating estrogens accompanying menopause plays a strong role in metabolic syndrome onset in post-menopausal females. While many studies have addressed the metabolic syndrome from a mechanistic standpoint in males and pre-menopausal females, much work remains in order to understand the metabolic syndrome in post-menopausal females.

Formation of the estrogens:

Estrogens are steroid hormones which operate as the primary sex hormones in females. Three major types exist, estradiol, estriol, and estrone, with 17β -estradiol the predominant form present in pre-menopausal females (21). All estrogens begin as androgens and are converted to estrogens via enzymatic action of aromatase. During steroidogenesis, cholesterol is converted to pregnenolone by cholesterol side chain cleavage enzyme, followed by the conversion of pregnenolone to androstenedione through a series of enzymatic reactions. Androstenedione is then either converted to estrone by aromatase, or to testosterone by 17β hydroxysteroid dehydrogenase (17β HSD). Aromatase then converts the testosterone to estradiol (166) (See Figure 13 below).

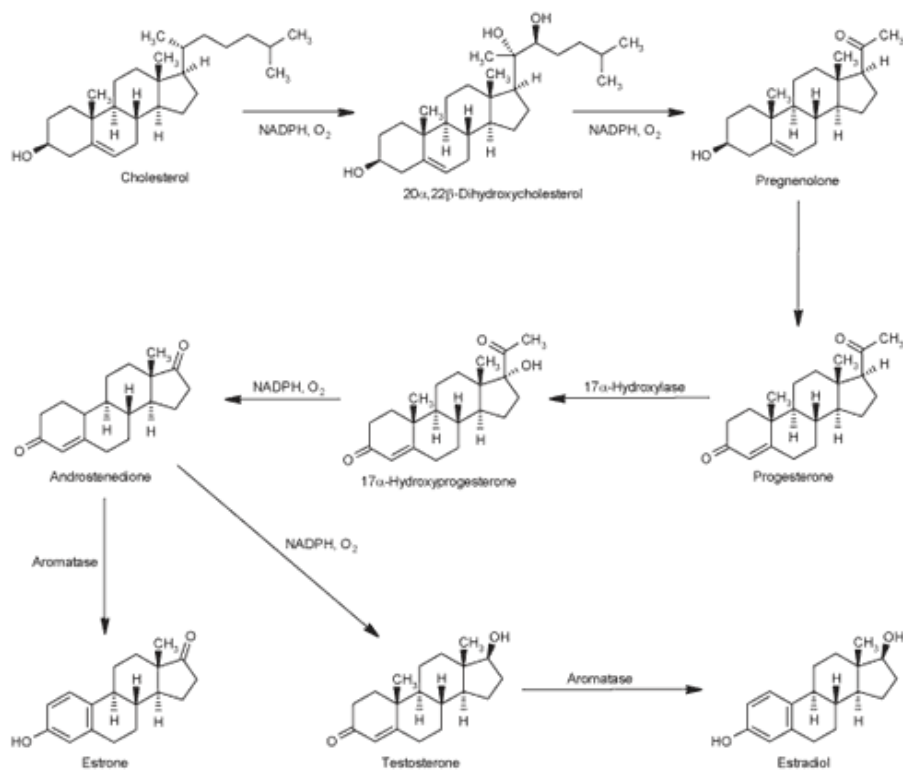


Figure 14. Formation of estrogen, estrone, and progesterone (166).

Function of the estrogens:

Estrogens play an important role in regulation of numerous physiological processes, including the menstrual cycle, the development of female secondary sex characteristics, bone growth, etc (129).

The menstrual cycle is regulated by estrogens and is necessary for reproduction. The menstrual cycle consists of three phases: menstruation, the follicular phase, and the luteal phase. The typical menstrual cycle is 28 days long, with day one defined as the onset of menses. During the menstrual cycle, levels of estradiol, progesterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) fluctuate in order for ovulation and menstruation to occur (124). The menstrual cycle continues for females

during their reproductive years until the onset of menopause, which is on average at age 51 (203).

In addition to the regulation of the menstrual cycle, estrogens are also important in the development of female secondary sex characteristics and bone growth. Secondary sex characteristics are those which distinguish males from females but do not play a role in reproduction. In females, these characteristics include the development of breasts, widening of the hips, and increased fat deposit in the thigh and hip region, also known as gynoid fat distribution (124). This fat distribution is typical of females, whereas males more often have increased fat in the visceral region, known as android distribution (120). Thus, females are commonly referred to as having a “pear” shape, while males have an “apple” shape (140). Many studies have demonstrated that visceral fat accumulation places individuals at greater risk for cardiovascular disease (CVD) and the metabolic syndrome than fat accumulation in the lower half of the body (28; 42). Thus, as estrogens contribute to gynoid fat distribution, females are typically at decreased risk for CVD and the metabolic syndrome based on body shape (28; 67).

Furthermore, estrogens play a strong role in bone growth during development in both males and females. Due to larger amounts of estrogens, females reach the end of their growth spurts, signaled by closing of the epiphyseal plates, more quickly than their male counterparts (42). In addition, estrogens have various effects on the brain, cognitive function (for review see Luine 2008) (126), and susceptibility to cancer (155; 186; 187) throughout the female lifespan. It is clear that estrogens are important to function and maintenance of many tissues, however, mechanistically, much remains to be discovered. Elucidating the mechanisms behind actions of the estrogens would be an important step

in understanding the changes which occur during menopause and could potentially lead to the development of alternatives to Hormone Replacement Therapy (HRT).

Menopause:

According to the World Health Organization (WHO), menopause is defined as the complete cessation of menstruation resulting from loss of ovarian follicular activity. The age at which menopause is reached varies greatly, however, the average woman reaches menopause at age 51 (203). In addition to natural menopause, numerous women undergoing hysterectomy to remove the uterus also may have their ovaries removed, resulting in a form of surgically-induced menopause (23; 91; 215). These women experience many of the same physiological changes as post-menopausal women (23; 91; 215).

All females have a finite number of follicles/oocytes, which are gradually exhausted with every menstrual cycle during the reproductive years. At birth, 1-2 million follicles are present, but by the onset of menopause, only about 1,000 remain. Some of the follicles are lost to ovulation, however, most are lost due to degeneration and resorption, a process termed atresia. In young females, follicles respond to increased levels of LH and FSH during the menstrual cycle, resulting in ovulation. As a woman nears menopause, the few follicles remaining fail to properly respond to LH and FSH and the menstrual cycle becomes irregular. The stage where the menstrual cycle is irregular, but still occurring, is termed perimenopause and covers a time period of 3-10 years prior to menopause. As follicles are the major source of estrogen production, the levels of estrogens decline during perimenopause and production almost stops completely once the menopausal transition is complete (49; 185; 206). The transition from perimenopause to

post-menopause is defined by a 12-month absence of a menstrual period, at which point estrogen levels are very low and FSH levels are high due to decreased negative feedback of estrogen (8).

Menopause and disease risk:

This drop in production of estrogens results in the loss of menses, and is associated with changes in metabolism, fat distribution, blood lipid profile, and blood glucose levels (129; 184). These conditions are observed in women who go through natural menopause, as well as those who undergo hysterectomy (23; 91; 215). Thus, in both natural and surgical menopause, conditions such as the metabolic syndrome, cardiovascular disease, and osteoporosis may result as the level of estrogens declines (46; 216).

Cardiovascular Disease:

After menopause, females greatly increase their risk of developing cardiovascular disease (CVD) (119; 176). Pre-menopausal women have an occurrence of coronary artery disease (CAD) that is much lower than males (119). However, after the onset of menopause, occurrence of CAD increases significantly (62), accounting for 59% of the mortality rate in post-menopausal women (156), and resulting in women equaling the mortality rate of age-matched males. The development and progress of CAD is impacted by the sex hormones, in that after menopause, females exhibit changes in blood lipid profile, clotting factors, endothelial factors, LDL and HDL cholesterol levels, and substrate utilization, all of which may impact the progression of CAD (127; 168; 197). While initial studies proved inconclusive (194), the latest updates from the American

Heart Association indicate that hypertension is linked to the decrease in sex hormones occurring with menopause (123; 164).

Metabolic Syndrome:

Not only does hormone status impact female risk for CVD, it also places women at an increased risk for development of the metabolic syndrome (34). With the decrease in physical activity and subsequent increase in obesity in recent years, the metabolic syndrome has increased in prevalence across many populations. However, it appears that changes in hormone status may contribute to onset of the metabolic syndrome in post-menopausal women (34).

In addition to weight gain, post-menopausal women are also at an increased risk for developing insulin resistance, another component of the metabolic syndrome (34). While some studies conclude that the main cause of insulin resistance in post-menopausal women is increased body mass, rather than hormone status, others argue that menopausal status plays an important role. It has been suggested that only a portion of the gradual decline in insulin sensitivity following menopause is attributable to increases in visceral fat mass (24). In addition to changes in insulin sensitivity, sex hormones have been demonstrated to alter glucose and lipid utilization (168). These changes in fuel utilization may contribute to the weight gain and increased risk for development of diabetes mellitus observed in post-menopausal women.

While physiological changes which place post-menopausal women at an increased risk for developing the metabolic syndrome have been well documented, there is no currently accepted way to treat post-menopausal women. Following the Women's Health Initiative (WHI), Hormone Replacement Therapy (HRT) has been ruled out as a

viable treatment for most women after estrogen production declines, due to increased occurrence of stroke, blood clots, and heart disease with HRT use. Thus alternative treatments for post-menopausal women should be sought out.

Exercise results in many positive physiological changes that improve overall health. In most populations, exercise has been successfully utilized as both a preventative measure and a treatment for obesity-related conditions. However, the effectiveness of exercise in attenuating the onset of metabolic syndrome in post-menopausal females is currently unknown.

Fuel utilization during exercise:

In order to grasp how exercise can prevent and treat the metabolic syndrome, it is important to have an understanding of how exercise impacts fuel utilization. During exercise, fat and carbohydrate are the main substrates oxidized for production of ATP (207). Carbohydrates are stored in the form of glycogen in muscle and liver, with plasma glucose also significantly contributing to energy during exercise. Oxidation of muscle glycogen is the major source of carbohydrate-produced energy during moderate to high intensity exercise (89). As the duration of exercise increases, glycogen stores become depleted and reliance on blood glucose increases (89). Fat is predominantly stored as triacylglycerol in adipose tissue, with some free fatty acids (FFAs) present in the bloodstream and a small amount of fat is stored as intramuscular triglycerides (IMTG) in muscle (207). Oxidation of FFAs derived from adipose tissue or IMTG is a major source of energy for contracting muscle. The relative contribution of FFAs and IMTG during

submaximal exercise remains controversial, with results of some studies favoring increased IMTG oxidation (94; 159; 160) whereas others suggest increased oxidation of FFAs (51; 106), regardless, both appear to be used.

An increased utilization of fat allows for an attenuated rate of glycogen depletion (136; 158) and slowed plasma glucose appearance and oxidation (58; 137; 157). This glycogen sparing from increased fat oxidation can result in an increased endurance capacity because glycogen stores are a limiting factor in endurance activity (89). The respiratory exchange ratio (RER) is an indicator of fuel utilization; a value of 0.7 indicates fat oxidation whereas a value of 1.0 suggests increased carbohydrate oxidation. At rest, RER is on average 0.8, increasing to 1 or greater with increasing exercise intensity. According to Brooks and Mercier, the relative exercise intensity where the predominant fuel being oxidized switches from fat to carbohydrate is known as the “cross-over” point (25). In untrained individuals, this point is at approximately 50% of $VO_2\text{max}$ (25), although results of some studies suggest that the cross-over point may be as high as 65% of $VO_2\text{max}$ (87). The point in exercise where oxidation of carbohydrate begins to predominate is dependent upon a number of factors, including training status, diet, and glycogen availability. Adaptation to intense endurance exercise may raise the crossover point, as suggested by the finding that when subjects underwent 12 weeks of high intensity training which raised their $VO_2\text{max}$ by 26%, there was ~5% reduction in RER at the same relative intensity (94). These results suggest that training decreased the utilization of carbohydrate sources. Diet also plays an important role in fuel utilization during exercise. Diets high in fat have been found to increase oxidation of fat during exercise (19; 59; 76; 77; 77; 115). For example, subjects cycling at 65% of their $VO_2\text{max}$

while on a high carbohydrate diet had an RER of 0.92, while 7 days of a high fat diet resulted in an RER of 0.81 in the same subjects (99). It is clear that a number of factors, including training and diet may impact the cross-over point during exercise.

Factors Affecting Relative Carbohydrate and Fat Oxidation During Acute Exercise:

Fiber type, size, and capillary density have all been demonstrated to impact fat oxidation during exercise. Slow twitch oxidative Type I fibers, as well as Type IIa, have a greater ability to oxidize fat both at rest and during exercise compared to fast twitch glycolytic Type IIx/b fibers which are much more reliant on glycolysis at rest and during exercise. Slow twitch fibers exhibit lower absolute maximal force output compared to fast twitch fibers, however, slow twitch fibers have increased time to fatigue (133). Typically, endurance athletes, such as marathon runners, have a larger percentage of Type I and Type IIa fibers, while power athletes, such as sprinters, tend to have more Type IIx/b fibers (72). The ability of Type I and Type IIa fibers to store IMTG and oxidize a greater amount of fat during exercise is at least partly attributable to increased mitochondrial density as compared to Type IIx/b fibers (72). As mitochondria are the machinery for aerobic respiration, increased mitochondrial number corresponds to a greater aerobic capacity and oxidation of fat during exercise. Furthermore, fiber size is related to oxidative capacity. Type I fibers typically have the smallest cross-sectional area (CSA), while Type IIx/b fibers have the largest CSA. In addition to fiber type and CSA, capillary density impacts the oxidative capacity of skeletal muscle during exercise (2; 89). Increased capillary density contributes to greater levels of fat oxidation during exercise, thus Type I fibers in particular are capillary dense (112). Having a smaller CSA

and increased capillary density is advantageous for oxygen diffusion into the fiber, thus increasing the capacity for aerobic respiration (2; 89).

Analysis of the impact of exercise intensity on fuel oxidation gives further insight into the effects of muscle fiber type on carbohydrate and fat oxidation. During low intensity exercise, Type I fibers are preferentially recruited and RER values are relatively low, indicating a greater reliance on fat oxidation. As exercise intensity increases, RER increases, indicating greater carbohydrate oxidation, and simultaneously more Type IIx/b fibers are recruited (63). The increase in RER can be attributed, at least in part, to the tendency of Type IIx/b fibers to oxidize carbohydrate rather than fat, shifting total oxidation from FFAs and IMTG toward glycogen and plasma glucose (63). The increase in glycolysis and glycogenolysis associated with carbohydrate oxidation with increasing exercise intensity has been associated with an inhibitory effect on fat oxidation via increased malonyl CoA, which inhibits the transfer of long chain FFAs into the mitochondria by inhibiting carnitine palmitoyl transferase (217).

Mitochondrial Enzymes and Exercise Training:

Endurance training results in increased numbers of mitochondria within the muscle and increased oxidative enzyme expression (82; 83; 139), which includes enzymes involved in β -oxidation and lipolysis (31; 160; 204), and mitochondrial enzymes, such as enzymes of the Krebs cycle and enzymes associated with oxidative phosphorylation (90).

Oxidation of fat during exercise is dependent upon a number of enzymes. Carnitine palmitoyl transferase (CPT-1) plays a key role in the regulation of fat oxidation. CPT-1 is a mitochondrial enzyme which mediates the transport of long chain fatty acids

into the mitochondria for oxidation. In the skeletal muscle of rats where overexpression of CPT-1 is induced, fat oxidation is increased and the amount of triacylglycerol present in the muscle is decreased. Thus CPT-1 promotes the oxidation of fat and decreases esterification of FFA (26). Conversely, decreased levels of CPT-1 are seen in skeletal muscle from obese subjects compared to lean subjects (154). Further, no gender-specific differences in CPT-1 activity have been observed (20), however, very few studies have been performed.

Whereas CPT-1 is critical in mitochondrial FFA uptake, Fatty Acid Translocase/Cluster of Differentiation (FAT/CD36), plays an important role in FFA uptake at the level of the plasma membrane. Stimulation of muscle via contraction results in increased translocation of FAT/CD36 to the plasma membrane, which results in an increase in FFA transport into the cell (108; 205). Exercise training has been shown to increase expression of FAT/CD36 in skeletal muscle (84). Recent studies have suggested that FAT/CD36 is located in mitochondrial and plasma membranes, and co-immunoprecipitates with CPT-1, with increasing levels of co-immunoprecipitation following exercise training (183). In obesity, there is an increase in plasma FFA, as well as in amount of FAT/CD36 located at the plasma membrane, which promotes increased uptake of FFA into the muscle (40; 73; 125). Gender differences in FAT/CD36 expression in skeletal muscle of obese subjects have been observed, with decreased expression of FAT/CD36 in adipose tissue of obese males and increased expression of FAT/CD36 in obese females compared to their lean counterparts (53). While plasma FFA and cellular FFA transport are increased, the oxidation of FFA is compromised in obesity. One possible reason for impaired FFA oxidation in spite of increased FFA

uptake is that malonyl CoA, an inhibitor of CPT-1, is increased in obesity, thus preventing FFA transport into the mitochondria for oxidation (10). Another explanation that has been proposed is that the decreased mitochondrial density in obese subjects contributes to decreased oxidative capacity of FFA (105). Recently it has been suggested that decreased contraction-induced translocation of FAT/CD36 to the mitochondrial membrane is responsible for the impaired FFA oxidation in obesity (73). Regardless of the explanation, obesity is associated with increased FFA transport across the plasma membrane via FAT/CD36 without a subsequent increase in FA oxidation.

In order to get a clear picture of fuel metabolism, it is necessary to look at storage and oxidation of carbohydrate in addition to fat utilization. Glycogen phosphorylase (GP) is an enzyme which breaks glycogen, the storage form of carbohydrate, releasing glucose-1-phosphate. Once free, glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase in order to be utilized for glycolysis (89). Thus, the GP contributes to carbohydrate utilization by catalyzing the breakdown of glycogen for glycolysis. Numerous variables can impact GP activity. Gender differences have been observed in GP expression in response to physical activity in that voluntary wheel running decreased GP activity in female, but not male mice (88). In addition, skeletal muscle from obese subjects exhibits increased GP activity (4), thus suggesting that obese individuals are more likely to use carbohydrate as a substrate.

Citrate Synthase (CS) is a critical enzyme in the Krebs' Cycle, which catalyzes the formation of citrate from Acetyl-CoA and oxaloacetate. While GP is indicative of carbohydrate utilization via glycolysis, CS is an indicator of mitochondrial function. CS expression and activity is increased with exercise training (86). Gender differences in CS

mRNA expression in skeletal muscle have been observed, with increased levels in females as compared to males, however, no differences in CS activity were observed at rest (171). Further, in the same study, 90 minutes of exercise resulted in increased activity of CS in both trained and untrained females, but no change was observed in males in either group (171). Whereas acute exercise increased CS activity in females only, exercise training increased CS activity in males and females to an equal degree (171). As one would predict, activity of CS is decreased in obese subjects, and to an even greater degree in obese subjects with non-insulin-dependent diabetes mellitus (105; 189). Thus, obese subjects demonstrate an impaired capacity for oxidation via the Krebs's cycle.

Acute Cellular Regulation of Fat Oxidation:

AMP-activated protein kinase (AMPK) has been shown to regulate energy balance and homeostasis. Increased AMPK activity is thought to contribute to increases in fat oxidation, glucose uptake, glycolysis, and mitochondrial biogenesis when activated by muscle contraction (75). Malonyl CoA is an intermediate in the formation of FFA's, as well as an inhibitor of CPT-1 which controls FA entry into the mitochondria for oxidation (135). Acetyl CoA carboxylase (ACC) regulates synthesis of malonyl CoA, thus enhanced activation of ACC results in decreased fat oxidation. When phosphorylated, AMPK can phosphorylate ACC, inhibiting ACC activity and reducing the formation of malonyl CoA (165). This inhibition of malonyl CoA synthesis inhibits lipogenesis and promotes lipid oxidation, thus AMPK activity plays a role in lipid utilization. This suggests AMPK is a major contributor to the regulation of fat oxidation during exercise.

Cellular Regulation of Lipolysis:

Hormone Sensitive Lipase (HSL) is responsible for catalyzing the hydrolysis of triacylglycerol and functions as the rate limiting step in lipid utilization (85). HSL is present in many tissues, but is expressed at high levels in adipose tissue (45). In order for lipid to be utilized as an energy source, it must be broken down from the storage form of triacylglycerol to diacylglycerol and then ultimately into individual FFA's. Once triacylglycerol is hydrolyzed into individual FFA's, which are transported in the blood and taken up by the muscle, these FFA's may be oxidized in the mitochondria. Regulation of HSL activity occurs primarily via phosphorylation, however, translocation and composition of the lipid droplet to be hydrolyzed also impact HSL activity (85). In humans, HSL can be activated via phosphorylation at Ser552, Ser649, and Ser650, and Ser554. In rats and mice, Ser554 corresponds to Ser 565, and Ser563 is orthologous to human site Ser 552 (45). In the presence of β -adrenergic stimulation, the Ser563, Ser659, and Ser 660 sites are phosphorylated by Protein Kinase A (PKA) via the cyclic AMP pathway. The Ser565 site is phosphorylated by AMPK and calcium calmodulin-dependent kinase II (CaMKII), while the Ser600 site is a target of the mitogen-activated protein kinase via extracellular signal-related kinase (ERK). Studies have suggested that phosphorylation of HSL at Ser 565 inhibits lipolysis, while phosphorylation at Ser 563 results in increased HSL activity (45). When the Ser565 site is phosphorylated, it may prevent phosphorylation of the regulatory Ser565 site, inhibiting HSL activity (45; 85). However, studies on the potential inhibitory effects of AMPK on HSL have produced mixed results. In a study by Watt et al., subjects completed 60 minutes of cycling at 70% of VO_2 max under both normal and low glycogen conditions. At rest, HSL activity and

α 2-AMPK phosphorylation were the same under normal and low glycogen conditions. When glycogen content was low, α 2-AMPK increased to a greater extent during exercise than under normal conditions. Further, HSL activity was lower following exercise under low glycogen conditions. In spite of decreased HSL activity, IMTG was decreased by exercise in the low glycogen group. In L6 myotubes, addition of aminoimidazole-4-carboxamide (AICAR), an activator of AMPK, resulted in attenuation of HSL activity in the presence of epinephrine. Based on these results, Watt et al. concluded that AMPK phosphorylation of HSL inhibits HSL activity and that a mechanism other than HSL may contribute to the utilization of IMTG during exercise (213). In contrast, a similar study conducted by Roepstorff et al. concluded that the increased AMPK phosphorylation following exercise in a glycogen-reduced state did not impact HSL activity (174). However, Roepstorff et al. suggest that increases in other factors, such as ERK phosphorylation of HSL, may have negated inhibitory effects of AMPK phosphorylation of HSL.

In addition to regulation by PKA and cAMP mechanisms, HSL activity may be altered by ERK1/2. When ERK1/2 is activated via phosphorylation at the Thr 202/Tyr 204 residues, ERK1/2 phosphorylates HSL at the Ser600 residue. Phosphorylation of HSL at the Ser600 residue by ERK1/2 enhances HSL activity and subsequently enhances lipolysis. By inhibiting activation of ERK1/2 in 3T3-L1 adipose cells, Greenberg et al. found a 30% decrease in catecholamine-stimulated lipolysis. Further, by activating ERK1/2, a 2-fold increase in glycerol release was observed in 3T3-L1 cells (69). Figure 14 below illustrates the regulation of HSL activity via phosphorylation by various substrates.

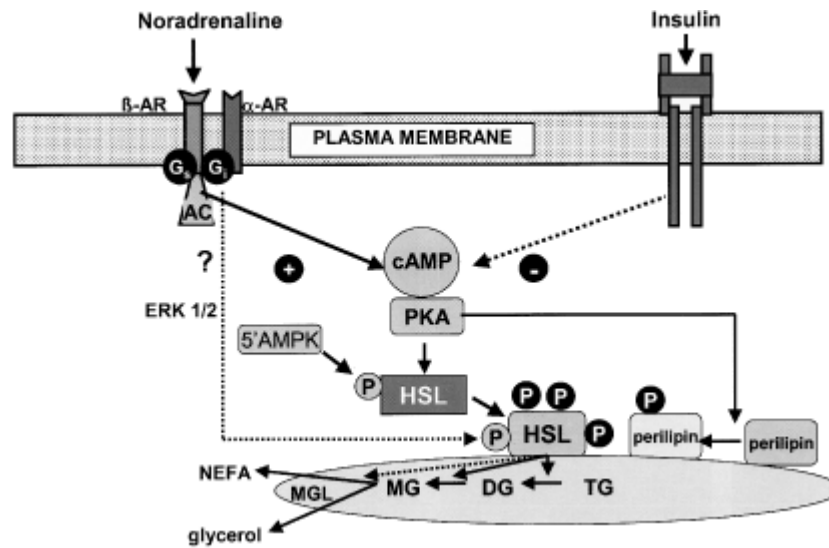


Figure 15. Cellular regulation of HSL in adipose tissue (85).

Thus, exercise uses a dynamic interaction between lipolysis and fat oxidation in the muscle to effectively use fat as a substrate. The regular use of fat by the muscle is critical in maintaining a favorable body composition. Thus, it is critical to understand the mechanisms that regulate both lipolysis and fat oxidation.

Obesity vs. Exercise Training:

Alteration in oxidative gene expression, such as CPT-1, FAT/CD36, GP, and CS, may contribute to metabolic changes observed in obese and endurance-trained subjects. Both obesity and endurance training result in increases in uptake of FFAs and IMTG accumulation. This is known as the “athlete’s paradox,” as endurance trained athletes exhibit an increase in IMTG accumulation similar to the IMTG accumulation observed in obese individuals (65). In the obese, this increase in IMTG contributes to insulin resistance (104). However, endurance-trained individuals with increased IMTG show increased insulin sensitivity (65). This difference in insulin utilization accompanying IMTG accumulation in trained and obese individuals is believed to be due to differences in lipid oxidation rates. Trained individuals are able to oxidize more of the accumulated

IMTG, thus increasing insulin sensitivity (65). Various theories have been proposed to explain the link between IMTG accumulation and insulin resistance in sedentary and obese populations. One explanation is that metabolites of IMTG, such as diacylglycerol (DAG) or ceramide, activate a serine kinase cascade, which in turn decreases activity of the insulin receptor substrate-1 (IRS-1) associated phosphatidylinositol 3-kinase (70; 97; 161). Decreased activity of IRS-1 results in impaired glucose transport, thus it contributes to insulin resistance (111). Another theory suggests that the activation of inflammatory pathways (NF κ B and JNK) during lipid uptake/fat accumulation promote phosphorylation of IRS-1 and thus impair insulin signaling (97; 212).

Peripheral insulin sensitivity is positively affected by exercise. Improvements in blood glucose regulation occur after an acute exercise bout, as well as long-term with regular exercise training (38; 39). Acute exercise promotes increased translocation of GLUT4 to the cell membrane for glucose uptake by activating AMPK (121), while exercise training can improve the muscles' ability to respond to insulin by increasing the sensitivity of the insulin receptor thus enhancing glucose uptake (181). The increased insulin sensitivity and blood glucose handling result in a decreased risk for development of Type II diabetes. With weight loss, there are additive changes in insulin sensitivity due to exercise (66). However, changes in insulin sensitivity still occur with exercise in the absence of changes in body composition (15). Through alteration of insulin sensitivity and blood glucose regulation, exercise decreases the risk of developing Type II diabetes and being diagnosed with the metabolic syndrome.

Due to the physiologic adaptations invoked with endurance training, trained individuals benefit from enhanced fat oxidation which results in the sparing of

carbohydrate. During an exercise bout of low-moderate relative intensity, trained individuals exhibit a decreased uptake in plasma glucose and a sparing of glycogen through slowed glycogenolysis (9; 37; 54; 78; 79; 95). On the other hand, obese individuals do not have enhanced fat oxidation in spite of increased fat uptake into muscle cells (209). Thus, obese individuals will store more fat due to an impaired ability to oxidize the fat.

Estrogens and Fuel Metabolism:

In addition to obesity, conditions where ovarian hormone levels are altered, such as menopause, contribute to alteration in fuel utilization. Whereas it is well established that glycogen content, diet, training status, and intensity level all influence fuel utilization during exercise, the role of gender and thus gender-specific hormones is still under investigation. Results of several studies have shown that females possess greater levels of fat oxidation during exercise at the same relative intensity as males (178; 198-200).

In females, the cross-over point occurs at a higher relative exercise intensity compared to males, thus females exhibit a greater amount of fat oxidation at all relative intensity levels (208). These findings would suggest a potential role of the sex hormones, the estrogens in particular, in fat oxidation. Changes following ovarian hormone loss provide further support for a role of sex hormones in fuel metabolism. Many animal studies have been conducted using ovariectomy as the model of menopause. Hansen et al. found that in ovariectomized female rats estradiol supplementation resulted in decreased synthesis of FFA's, whereas progesterone supplementation elevated FFA synthesis compared to estradiol supplementation. This would suggest that estradiol may promote FFA oxidation over FFA storage (74).

Ovarian Hormones May Promote Fat Oxidation:

It would be logical to hypothesize that greater fat oxidation by females during exercise might be indicative of gender differences in AMPK activation as well. Most recently, Roepstorff et al. demonstrated that women oxidize significantly more fat than men during submaximal exercise independent of AMPK activation (173). Specifically, phosphorylation of α AMPK (Thr172), as well as activity of α_2 AMPK, were equal in males and females at rest, but elevated only in males following a bout of exercise. These findings suggest that AMPK is not the mechanism responsible for gender differences in fat oxidation, leaving the area open to further exploration (173). From an anatomical standpoint, Roepstorff et al. suggest that gender differences in fat oxidation are due to sex-specific muscle morphology based on a positive correlation between fat oxidation and type I fibers, as well as capillary density (173). However, D'Eon et al. recently demonstrated that estradiol and 2-hydroxyestradiol (2-HE2), an estrogen metabolite, can increase AMPK phosphorylation in C₂C₁₂ myotubes (43). With low doses of estradiol, AMPK phosphorylation was observed to increase ~1.6 fold, while in high doses, AMPK phosphorylation increased ~3.0 fold. At all concentrations tested, 2-HE2 resulted in increased AMPK phosphorylation in C₂C₁₂ myotubes (43). These results suggest that estradiol and estradiol metabolites may contribute to gender differences in AMPK activation.

Using OVX mice, I have found decreased AMPK phosphorylation in the tibialis anterior (TA) muscle compared to their SHAM counterparts at rest. However, when fatigue was induced using *in situ* contractions, AMPK phosphorylation increased from baseline in the OVX group, whereas the SHAM group showed no further increases in

phosphorylation. In contrast, when the muscle was placed in an *in vitro* bath, phosphorylation of AMPK was almost undetectable in both the SHAM and OVX groups at rest and increased to the same degree in both groups following contraction. These data suggest that a circulating factor present in the blood of female animals with ovaries intact contributes to AMPK phosphorylation at rest. However, removal of the ovaries results in decreased AMPK phosphorylation at rest and increased AMPK phosphorylation in response to contraction stimulus (Wohlers et al. 2008-in review). These results demonstrate that ovarian function impacts AMPK phosphorylation at rest and following contraction in skeletal muscle, thus potentially altering fuel utilization. This is in line with the results of Roepstorff et al. in which females had elevated AMPK phosphorylation at rest and did not increase AMPK phosphorylation with exercise, while males exhibited significantly increased AMPK phosphorylation with exercise (173). Taken together, these studies demonstrate that the ovarian hormones may induce changes in AMPK phosphorylation in human and mouse skeletal muscle.

Phosphorylation of ERK1/2 is also altered by ovarian hormone status. Specifically, I found ERK2 phosphorylation to increase in both SHAM and OVX mice in response to *in situ* contraction of the TA muscle. However in the OVX group, the increase in phosphorylation is significantly greater than in the SHAM group (Wohlers et al. 2008-in review). Thus, in skeletal muscle, ovarian hormones appear to inhibit the ability of muscle to increase ERK1/2 phosphorylation with contraction.

Ovarian Hormones May Affect Lipolysis:

Based on the observed differences in fat mass between pre-and post-menopausal females, it may be hypothesized that ovarian hormone status may result in differences in

lipolysis and/or lipogenesis. Roepstorff et al. addressed this hypothesis in a study of males and females and found that HSL mRNA expression and protein content was higher in females than males (195). However, total HSL activity was not different between the sexes. Males exhibited increased phosphorylation of Ser659, and HSL activity normalized to HSL protein content, as compared to females. Roepstorff et al. suggested that increased oxidation of fat in females versus males is due to the greater IMTG content in the muscle, rather than enhanced lipolysis in the adipose tissue of females versus males (195). There are few studies examining regulation of lipolysis specifically in females versus males.

In cats, at 24 weeks following ovariectomy, fat mass increased to a greater degree than lean mass, resulting in a significant shift in body composition. Cats were less physically active following surgery, with a 52% decrease in activity by week 24. Serum glucose and triglyceride concentrations, as well as circulating leptin levels, were significantly increased at week 24 as compared to all other time points. LPL, HSL, and adiponectin mRNA expression in adipose tissue was decreased, and IL-6 expression was increased at 24 weeks as compared to baseline. The decreases in HSL and LPL would suggest alterations in lipolysis and uptake occur following loss of ovarian hormones, while the decrease in adiponectin would suggest ovarian hormone loss might reduce insulin sensitivity. Thus, ovariectomy resulted in anthropomorphic changes, as well as alteration in expression of genes which impact fuel utilization (16).

While the benefits of exercise have been widely demonstrated in males and pre-menopausal females, little is known about the benefits of exercise as a preventative measure for the health problems that many women face when they reach menopause. Until recently, HRT was the most common treatment to smooth the transition into menopause and prevent the onset of post-menopausal conditions such as osteoporosis and metabolic syndrome.

Women entering menopause are susceptible to changes in fat metabolism that place them at increased risk for the metabolic syndrome:

Following the onset of menopause, physiological changes occur which increase the risk of developing the metabolic syndrome. Unfortunately, at this point preventative treatment for women remains unclear. However, one would predict that increased physical activity would be effective. Metabolic syndrome prevalence is lower in adolescent males and females who are moderately physically active than in those with low levels of activity (148). Exercise has been utilized as an effective treatment of the metabolic syndrome in pre-menopausal women, mostly through exercise effects on insulin sensitivity and weight loss. Multiple studies have suggested that physical activity in women decreases risk of CVD and is an effective treatment in those who already have CVD, however, these studies focus on pre-menopausal women (See review Oguma 2004) (147).

HRT:

Definition:

Hormone replacement therapy (HRT) is a means to increase or replace female hormones during and following menopause. HRT typically consists of estrogen

supplementation alone, or estrogen in conjunction with progesterone. Prior to the Women's Health Initiative, HRT was widely prescribed in order to prevent the negative side effects associated with menopause, including the metabolic syndrome, cardiovascular disease, and osteoporosis.

Health benefits of HRT:

As described above, menopause results in increased risk of development of the metabolic syndrome, CVD, and osteoporosis. Use of HRT has been shown to decrease the risk for and/or improve symptoms of, these conditions. HRT has positive effects on bone health and remodeling. HRT can attenuate the onset of the metabolic syndrome by decreasing abdominal fat by approximately 6.8% (180). Also, some studies suggest HRT use leads to improvements in insulin sensitivity (143; 190). However, results on the effects of HRT on insulin sensitivity are mixed, as the type of HRT, method of use, and study population produced different results (6). HRT leads to improvements in blood lipid profile, resulting in increased HDL cholesterol and decreased LDL cholesterol (141). By impacting body composition, insulin sensitivity, and blood lipid profile, HRT decreases the risk of developing the metabolic syndrome in post-menopausal women.

Negative side effects of HRT:

Based on the positive effects of HRT and osteoporosis, it would appear that use of HRT in post-menopausal women is favorable. However, negative side-effects reportedly linked to HRT use have led many to question its use. These side effects include increased risk for stroke, blood clots, and heart disease (145). Further, some studies show increased occurrence of various cancers among women taking HRT as compared to post-menopausal women not on HRT (145). These findings have been summarized in the

Women's Health Initiative, which began in 1991, and have resulted in a significant decline in prescription of HRT to post-menopausal women.

Is Exercise Training a Useful Alternative to HRT in Post-menopausal Women?:

It is clear that menopause results in many changes that negatively impact the health of females. With the recommendation to reduce HRT use following the release of the Women's Health Initiative, another mechanism of treatment should be sought out. Exercise has been utilized as an effective treatment of the metabolic syndrome in males and pre-menopausal females, mostly through exercise effects on weight loss and insulin sensitivity. Multiple studies have suggested that physical activity in women decreases risk of CVD and is an effective treatment in those who already have CVD. However, these studies focus on pre-menopausal women (See review Oguma 2004) (147). Based on the knowledge of the beneficial effects of exercise, and its successful use in other populations as preventative medicine, study of exercise effects in prevention of the metabolic syndrome in post-menopausal women is warranted.

In summary, exercise is beneficial for cardiovascular health, as well as for preventing the onset of the metabolic syndrome. Further, exercise training results in positive metabolic changes, such as an increased ability to oxidize fat, thus sparing glycogen stores. Post-menopausal women experience alterations in metabolism that increase risk of developing the metabolic syndrome and CVD. In light of the WHI, and based on knowledge of therapeutic and preventative uses of exercise, the possibility of exercise prescription in place of HRT in prevention of the metabolic syndrome in post-menopausal women should be pursued.

APPENDIX A: Limitations

This study involves the analysis of mice ovariectomized at 8 weeks of age in attempting to understand mechanisms involved with menopause, which occurs much later in the lifespan, at approximately 17-25 months. Therefore, it is possible that factors not present in young mice may be involved in the onset of metabolic syndrome in post-menopausal females. However, since the occurrence of metabolic syndrome increases with age independent of estrogen status, it is important to understand the impact of aging and estrogen status as individual phenomenon. Thus, we believe that the model selected is most appropriate, as it is the only way to look at the effects of estrogen independent from changes that occur with age. Furthermore, as this study was performed in mice, findings would need to be confirmed in humans before they could be applied to treatment of post-menopausal women.

APPENDIX B: Animal Protocol Approval



UNIVERSITY OF MARYLAND

GRADUATE STUDIES AND RESEARCH

Institutional Animal Care & Use Committee

James M Dietz

Jmdietz@umd.edu
phone: (301) 405-6949

March 30, 2007

Dr. E. Spangenburg
Kinesiology Department
CAMPUS

Dear Dr. Spangenburg:

This letter is to inform you that the members of the Institutional Animal Care & Use Committee (IACUC), at their **November 16, 2006** meeting, reviewed your protocol:

**Use of Exercise as a Preventive Treatment for
Menopause-induced Obesity**

R-06-77

At that time, the IACUC requested additional information before your protocol could receive approval. You have now provided this office with that information. The approval date is **March 30, 2007**.

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **March 30, 2010**. Moreover, federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (**March 2008 & March 2009**). All subsequent work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

A handwritten signature in black ink, appearing to read 'J. Dietz', written over a white background.

James M. Dietz
Chair, IACUC
Professor, Biology

Animal Use Protocol Form - Research

University of Maryland, College Park

(NOTE: Form must be submitted by the 1st of any month to be considered by the IACUC that month)

PROTOCOLS FORMS ARE GENERALLY NOT REVIEWED IN AUGUST

Principal Investigator: Espen E Spangenburg		Date of submission:
Department: Kinesiology	Phone: 5-2483	
E-mail: espen@umd.edu	FAX: 5-5578	
Co-investigators (faculty):		
Collaborating scientists (including postdocs, visitors, etc.) include institution or address & phone number		
Graduate students:		
Lindsay Wohlers		
Technicians:		
Undergraduates:		
Facility in which animals will be housed:		
Anticipated start date: Oct. 1, 2006	Anticipated completion date: August 31, 2008	
Project title: Use of exercise as a preventive treatment for menopause-induced obesity		
Is this protocol included in a grant proposal?	Yes:	No: X
If yes, who is sponsor?	NIH:	NSF: USDA: Other (specify):
ORAA Proposal ID number (if known):		

I acknowledge responsibility for the conduct of these procedures with animals. I attest to the accuracy and completeness of the information provided. I promise to conduct this work with animals in accordance with the protocol as approved by the IACUC and the Campus Animal Care and Use Guidelines. I will not make any changes in the above protocol without first obtaining the approval of the IACUC and I will not use any procedures which are not included in this form.

	Printed Name	Signature	Date
Investigator:	Espen E Spangenburg, Ph. D.		
Supporting Signatures			
Facility Supervisor	Kathy Nepote, DVM		
Department Chair	Jane Clark, Ph. D.		

Before completing this and the subsequent sections of the protocol form you should familiarize yourself with the material described in Section IV below.

SECTION II

If the animal procedures planned involve simple field observations with *NO IMPACT ON EITHER THE ANIMALS OR THEIR ENVIRONMENT* then continue here. Otherwise, skip to section III.

- a. **Lay summary:** On a separate sheet, provide a 1-2 paragraph overview of the proposed research in terms understandable by a non-scientist. Include an explanation of research goals, rationale for animal research, and how this work will benefit society and knowledge. Do not include undefined scientific terms. Since this summary may be made available to the public if requested, it is imperative that you carefully consider its content.
- b. In more scientific terms, describe the study activities. Include all precautions taken to ensure no adverse impact on the study animals or their environment.
- c. Include copies of all required permits and/or letters of permission, or provide documentation that such permits are not required.
- d. You and your department chair (and not the facility supervisor) should sign and submit this form, You do *not* need to complete Sections III and IV..

SECTION III

ANSWER ALL QUESTIONS AS INDICATED (circle the appropriate answer)

1. Does this research duplicate previous experiments? (If YES, justify on separate page)	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
2a. Will the animals be euthanized at the end of the experiment?	<input type="checkbox"/> NO	<input checked="" type="checkbox"/> YES
2b. Method of euthanasia The animal will be anesthetized through inhaled isoflurane. Upon reaching an appropriate surgical plane the desired muscle tissues will be removed. These tissues include the diaphragm, such the animal will euthanized through exsanguination.		
3a. Is surgery to be performed? (If yes, complete items 3b to 3d and add surgical SOP=s as appropriate.)	<input type="checkbox"/> NO	<input checked="" type="checkbox"/> YES
3b. Surgery room (building and room number): Central Animal Resource facility		
3c. Is the surgical procedure terminal?	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
3d. Is the surgical procedure a survival procedure?	<input type="checkbox"/> NO	<input checked="" type="checkbox"/> YES
4a. Have the husbandry SOP=s been approved by the IACUC?	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
4b. If you answered ANo@ in item 4a, on a separate page please explain why not and describe the procedures to be used.		
5a. Are infectious agents to be used:(SEE 6E)	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
The agent is (are)?		
5b. Are chemical hazards to be used?	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
The chemical hazards are?		
5c. Are radioisotopes to be used?	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
The radioisotopes are?		
5d. Are other biohazards (including recombinants) being used?	<input checked="" type="checkbox"/> NO	<input type="checkbox"/> YES
The biohazards are?		

5e. If answers to any part of question 5 is YES, IACUC approval is contingent upon IACUC receipt of written approval of your procedures by the Office of Environmental Safety.

SECTION IV. ANSWER EACH OF THE FOLLOWING QUESTIONS ON A SEPARATE SHEET(S) OF PAPER. PLEASE BE SURE AND USE THE APPROPRIATE NUMBER FOR EACH QUESTION.

6. **Lay summary:** Provide a 1-2 paragraph overview of the proposed research in terms understandable by a non-scientist. Include an explanation of research goals, rationale for animal research, and how this work will benefit society and knowledge. Do not include undefined scientific terms. Since this summary may be made available to the public if requested, it is imperative that you carefully consider its content.
7. **Research Goals:**
 - A. What are the scientific issues addressed by the research?
 - B. What are the specific goals of the animal studies described in this protocol?
8. **Alternatives:** Explain why animal studies are preferred to non-animal alternatives in achieving these research goals.
9. **Species:** Why is this the most appropriate species/strain to use in these studies?
10. **Design:** Summarize the experimental groups and the numbers of animals in each group. What is the basis for these numbers (statistical test, consultant, etc.)? These numbers should be easily tallied to reflect the numbers used in question 24. Clearly delineate those animals which are used more than once (in multiple experimental groups).
11. **Experimental Procedures:** Describe the animal procedures that are to be performed, detailing specifics of those procedures which impact the health and comfort of the study animals (e.g., frequency of performance of procedures, methods of restraint, blood sample volumes, needle sizes restrictions, etc.)
12. **Surgery:** If surgery is involved, described the surgical procedures to be performed. Include the procedures planned to ensure asepsis (e.g., surgical site preparation, instrument sterilization, etc.). If aseptic procedures are not being used, explain why not and describe the procedure(s) of choice. Finally, describe the immediate and post-operative (next day and thereafter) care.
13. **Anesthetic:** If any anesthetic is used, provide information on: (a) Anesthetic/analgesic to be used (types/names), (b) method(s) of administration, and (c) dosage(s) to be used.
14. **Investigator training:** Describe the training and qualifications of each person named in this protocol. Be specific about the hands-on training of those performing procedures that may cause animal discomfort (e.g., restraint, injections, blood collection, surgery, euthanasia, etc.). Include the status of all personnel with regard to required campus animal care training. Please be reminded that no one may work with animals until after they have completed the necessary campus training.

15. Animal Number and Welfare Status: (Note, the numbers on this chart must equal those presented in the research description (for category definitions see No 18).

Species	Category I (Little or no pain, anesthetic may be used for sedation)	Category II (anesthetic used for relief of potential pain or distress)	Category III (Pain not relieved)
Mouse	XXX		

For all animals in categories II and III, please fill in the following information on your **Alternative Search**. Please review Nos. 21 & 22 for the definition of ALTERNATIVES.

Category	Databases searched	Inclusive dates of literature searched	Date Search Conducted	Key words used in search
II				
III				

Describe the results from the literature searches (if alternatives were found but you decided not to use them, explain why)

If animals were listed in Category III, explain why pain or discomfort cannot be relieved and what procedures will be used to minimize pain/discomfort.

16. Do any approved SOP=s apply to this protocol? If so, please fill in the following table as appropriate and attach copies of the SOPs.

SOP Title	Approval date

SECTION V: PLEASE BE SURE AND REVIEW THE FOLLOWING INFORMATION

17. The Campus accepts only those euthanasia practices which are consistent with the recommendations in A1993 Report of the American Veterinary Medical Association (AMVA) Panel on Euthanasia.@ Copies of this document are available through CARF.
18. Definitions of animal welfare categories (See Question 15).
 - I. Little or momentary pain or discomfort (peripheral blood collection, injections, vaccinations, sedation, etc.).
 - II. Potential discomfort or pain which is relieved by the appropriate anesthetic or analgesic (surgery under anesthesia, cardiac puncture under anesthesia).
 - III. Discomfort or pain which is not relieved (toxicity studies, disease studies).
19. U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing states AThe animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results@
20. The Animal Welfare Act states that: AProcedures that may cause more than momentary or slight pain or distress to the animals will: a) Be performed with appropriate sedatives, analgesics or anesthetics unless withholding such agents is justified for scientific reasons, in writing, by the principal investigator and will continue for only the necessary period of time; b) Involve in their planning, consultation with the attending veterinarian..., c) Not include the use of paralytics without anesthesia...@
21. The Animal Welfare Act states: AThe principal investigatory has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals, and have provided a written narrative description of the methods and sources, e.g., the Animal Welfare Information Center, used to determine that alternatives were not available,@
22. Please remember that in discussing alternatives, we are considering the A3Rs.@
 - (A). Replacement: those methodologies (computer programs, tissue culture techniques, epidemiological data, etc.) which replace the use of animals.
 - (B). Reduction: Those methodologies which reduce the numbers of animals used in the protocol.
 - (C). Refinement: Those methodologies which refine the procedure to minimize the amount of discomfort that the animal may experience. (anesthetics, analgesics, training, etc)
23. The U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training includes: AProcedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge or the good of society.@
24. The Animal Welfare Act States: AActivities that involve surgery include appropriate provision for pre-operative and post-operative care of the animals in accordance with established veterinary medical and nursing practices. All survival surgery will be performed using aseptic procedures, including gloves, masks, sterile instruments, and aseptic techniques.@
25. The Animal Welfare Acts states: APersonnel conducting procedures to the species being maintained or studied will be appropriately qualified and trained in those procedures.@

6. Lay summary:

The **overall intent** of the study is to determine if regularly performed exercise can be used as substitute for hormone replacement therapy (HRT). Currently, it is fairly well established that woman who undergo menopause and do not use HRT become susceptible to the development of obesity. This has been shown to occur in rodent models of menopause as well. However, it is unclear if exercise can be used as a substitute for HRT. This is an important consideration since a recent NIH study found that HRT can increase a women's risk of stroke. **The hypothesis** is that exercise will alter gene expression in both muscle and adipose tissue independent of estrogen status that will encourage the maintenance of normal body mass. **The objectives** of the study are to determine if exercise can prevent the increase in adipose tissue mass in ovariectomized mice as effectively as HRT. In addition, the expression and activity of various fatty acid metabolic enzymes will be measured from muscle and adipose tissue to determine if they are affected by exercise or changes in estrogen status. **The significance of this study** is that it will allow us to gain a better understanding of the role reduced levels of estrogens plays in the development of obesity. In addition, it will allow us to determine if physical activity can attenuate this effect without the use of HRT. Finally, the experiments will provide us with potential gene targets that estrogen may use to prevent the accumulation of adipose tissue.

7. **Research Goals:**

A. What are the scientific issues addressed by the research?

To determine if exercise can prevent the development of obesity in a rodent model of menopause. In addition, we will seek to understand how estrogens are able to prevent the accumulation of adipose tissue by measuring the expression of key enzymes involved in fatty acid metabolism in skeletal and adipose tissue.

B. What are the specific goals of the animal studies described in this protocol?

The specific goal is to determine if regularly performed exercise prevents the accumulation of adipose tissue after a surgically-induced menopause procedure. In addition, one key test will be to exam if exercise is as effective as HRT. Finally, I will attempt to identify how exercise or HRT prevents the accumulation of adipose tissue after the ovariectomy surgery.

8. **Alternatives:** Explain why animal studies are preferred to non-animal alternatives in achieving these research goals.

Due to the invasive nature of the protocol, human studies are not possible and the amount of necessary tissue prevents any modifications to the protocol to allow for human use. Cell culture is also not a feasible option since it is not possible to mimic exercise using cell culture models.

9. **Species:** Why is this the most appropriate species/strain to use in these studies?

Mice are appropriate since they are strong runners. In fact, they will exercise for fairly long durations (5-10 km) with no intervention. Also, the use of ovariectomy in rodents is an extremely well described model of menopause.

10. **Design:** Summarize the experimental groups and the numbers of animals in each group. What is the basis for these numbers (statistical test, consultant, etc.)? These numbers should be easily tallied to reflect the numbers used in question 24. Clearly delineate those animals which are used more than once (in multiple experimental groups).

The specific groups to be studied are listed below. Each group will have an n of 12 for a total n of 72 animals. This number of animals is the minimal number per group needed to maintain statistical power for the experiment. The animals will be euthanized at the appropriate time point and the skeletal muscle and adipose tissue will be removed and frozen in liquid nitrogen for further processing.

Group 1 – Control Female and Sham surgery.

Group 2 – Control Female and Ovariectomy Surgery.

Group 3- Control Female and Ovariectomy Surgery and Estradiol Supplementation

Group 4 – Exercised Female and Sham surgery.

Group 5 – Exercised Female and Ovariectomy Surgery.

Group 6 - Exercised Female and Ovariectomy Surgery and Estradiol Supplementation

11. Experimental Procedures: Describe the animal procedures that are to be performed, detailing specifics of those procedures which impact the health and comfort of the study animals (e.g., frequency of performance of procedures, methods of restraint, blood sample volumes, needle sizes restrictions, etc.)

Female Swiss Webster (5 weeks old) mice will be acquired and allowed to acclimate to the new surroundings for 7 days. The animals will be divided into 6 separate groups. Three groups will undergo the Sham surgery and the other 4 groups will undergo the ovariectomy surgery (OVX) described in the next section. Two of the groups will be implanted with subcutaneous capsules that release estradiol (see next section). The animals will be allowed to recover for 1 week. After the one week is complete, three of the groups will be given access to a Fast-Trac running wheel in their cage (see figure 1) and the other set of animals will not have access to a running wheel in their cage. The Fast-Trac running wheel is a device sold as a form of enrichment specifically for laboratory animals. It is specifically designed to be incorporated into mouse cages and will fit the cages at the CARF. The running wheel does not interfere with normal husbandry practices of the animals. We will actively monitor the revolutions the animals run on the running wheels using a modified bicycle computer set up. This device will be externally mounted, so it will not bother the animal. Therefore, the animals will be housed 1 per cage to allow us to quantify how many revolutions each individual animal runs per day. All mice will be maintained with normal Standard Operating Procedures at the CARF, which includes food, bedding, nestlets and water. The animals will be checked daily to ensure the health of the animals is adequate. The PIs will monitor the animals' appearance and activity patterns. If the animal appears unhealthy in anyway, the experiment will be terminated and the university veterinarian (Dr. Nepote) will be contacted. The Fast-Trac will be replaced with a clean Fast-Trac on a weekly basis. If the Fast-Trac in the cage becomes excessively dirty, the device will be cleaned with alcohol wipes.

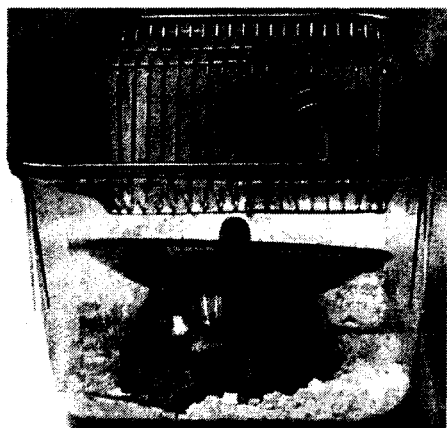


Figure 1. Pictures of the Fast-Trac running wheel that will be placed in the cages for the exercising mice.



The animals will be exposed to the running wheel for three months total. After the three months is complete, the animals will be anesthetized with isoflurane gas (2-3%). When the animals have reached an appropriate plane of anesthesia (as determined by lack of toe pinch reflex), we will remove various organs tissues (skeletal and adipose tissue) from the mouse. It is important that we use anesthesia since we need the tissue to be as fresh as possible (blood flow intact) when it is removed

12. Surgery: If surgery is involved, described the surgical procedures to be performed. Include the procedures planned to ensure asepsis (e.g., surgical site preparation, instrument sterilization, etc.). If aseptic procedures are not being used, explain why not and describe the procedure(s) of choice. Finally, describe the immediate and post-operative (next day and thereafter) care.

Female Swiss Webster mice (5 weeks old) will be acquired and allowed to acclimate to the new surroundings for 7 days. All procedures will be performed under aseptic conditions -- with the surgeons wearing sterile gloves. After acclimatization, the animals will be anesthetized using 2-3% (or to effect) isoflurane gas. After proper surgical depth is obtained (to be determined by toe pinch reflex), a small area on the dorsal flank will be shaved with clippers, cleaned using a small volume of Betadine, and gently scrubbed with 70% alcohol. This process will be repeated three times. All surgical tools will be autoclaved and will be disinfected between animals using a glass bead sterilizer. All surgeries will be performed on a heating pad to prevent hypothermia in the animals. For each ovary a 3/4 cm dorsal flank incision penetrating the abdominal cavity will be made. The parovarian fatty tissue will be identified and retracted. The exposed ovary and associated oviduct will be severed and removed. Hemostasis will be achieved by hemostat pressure for 1-2 minutes. Rarely, is a ligature (5-0 absorbable suture) around the severed ovarian vasculature required to maintain hemostasis. The incision will be closed (5-0 nonabsorbable suture in an interrupted pattern). In animals, that are to receive estrogen supplementation the animals will be implanted with a sterile SILASTIC capsule (Konigsberg Instruments, Pasadena, CA) containing 17 β -estradiol (180 μ g/ml) subcutaneously on the lower region of the back. Prior to the incision, the back will be shaved, repeatedly cleaned using a small volume of Betadine, and finally gently scrubbed with 70% alcohol. This paradigm of chronic low-dose estradiol treatment produces ~20 pg/ml in rats. These levels are equivalent to basal circulating levels found during the estrous cycle of rats. The incision will be closed (5-0 nonabsorbable suture in an interrupted pattern). To avoid dehydration of the animal, ~0.3 milliliters of warm sterile saline will be subcutaneously injected into the animal. After completion of the surgery, the animals will be returned to housing quarters when they have recovered from the anesthesia. The animal's eating and drinking behavior will be monitored. This can be determined by watching for decrements in the quantity of feces in the cage or measurable decreases in drinking water consumption. If the animal is not eating, not drinking, or is rapidly losing weight, it will be immediately terminated. After 10 days, animals will be anesthetized using 3-4% (or to effect) isoflurane gas until proper surgical depth is obtained (to be determined by toe pinch reflex). The sutures from the ovariectomy surgery will be removed.

13. Anesthetic: If any anesthetic is used, provide information on: (a) Anesthetic/analgesic to be used (types/names), (b) method(s) of administration, and (c) dosage(s) to be used.

- a. Isoflurane
- b. Inhalation
- c. 2-3%

14. Investigator training: Describe the training and qualifications of each person named in this protocol. Be specific about the hands-on training of those performing procedures that may cause animal discomfort (e.g., restraint, injections, blood collection, surgery, euthanasia, etc.). Include the status of all personnel with regard to required campus animal care training. Please be reminded that no one may work with animals until after they have completed the necessary campus training.

Dr. Spangenburg has been handling rodents since 1995 with no complications. He has published over 30 peer-reviewed articles, which document his use of animal models. Dr. Spangenburg has successfully completed animal training courses at Virginia Tech, the University of Missouri, and the University of California-Davis.

Dr. Spangenburg has completed the animal care training class necessary for the University of Maryland.

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