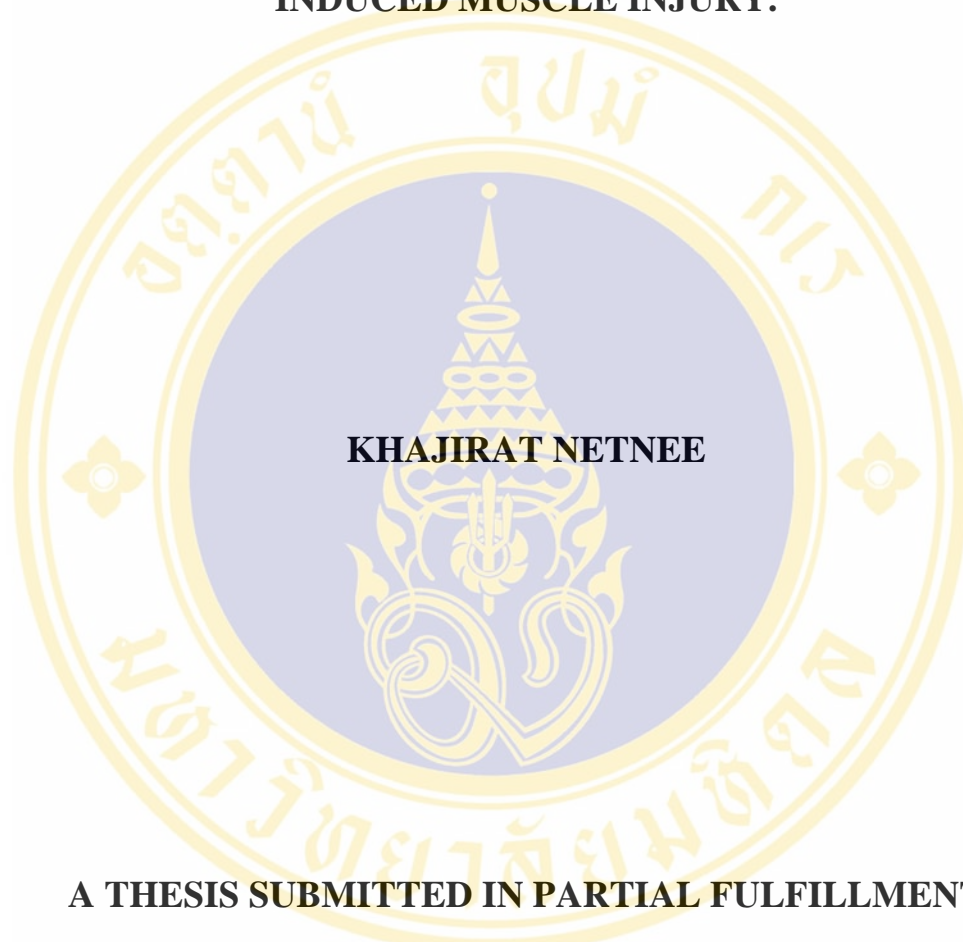


**EFFECT OF OVARECTOMY AND ESTROGEN
SUPPLEMENTATION ON THE RECOVERY FROM EXERCISE-
INDUCED MUSCLE INJURY.**



**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(EXERCISE PHYSIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY**

2008

COPYRIGHT OF MAHIDOL UNIVERSITY

Thesis
Entitled

**EFFECT OF OVARECTOMY AND ESTROGEN
SUPPLEMENTATION ON THE RECOVERY FROM EXERCISE-
INDUCED MUSCLE INJURY.**



Khajirat Netnee

Miss Khajirat Netnee
Candidate

T. Yimlami

Dr. Tossaporn Yimlami, Ph.D.
Major-Advisor

Pawinee Piyachaturawat

Prof. Dr. Pawinee Piyachaturawat, Ph.D.
Co-Advisor

Kanokpan Wongprasert

Asst. Prof. Dr. Kanokpan Wongprasert,
Ph.D.
Co-Advisor

G. Chaturapanich

Asso. Prof. Dr. Ganyapong Chaturapanich,
Ph.D.
Co-Advisor

B. Mahasavariya

Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies

Chumpol Pholpramool

Prof. Dr. Chumpol Pholpramool, Ph.D.
Chair
Master of Science Programme in Exercise
Physiology
Faculty of Science

Thesis
Entitled

**EFFECT OF OVARECTOMY AND ESTROGEN
SUPPLEMENTATION ON THE RECOVERY FROM EXERCISE-
INDUCED MUSCLE INJURY.**

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Physiology)

on
May 29, 2008

Khajirat Netnee

Miss Khajirat Netnee
Candidate

Jantarima Pandaranandaka

Dr. Jantarima Pandaranandaka, Ph.D.
Chair

Tossaporn Yimlami

Dr. Tossaporn Yimlami, Ph.D.
Member

G. Chaturapanich

Asso. Prof. Dr. Ganyapong Chaturapanich,
Ph.D.
Member

Kanokpan Wongprasert

Asst. Prof. Dr. Kanokpan Wongprasert,
Ph.D.
Member

B. Mahaisavariya

Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

Pawinee Piyachaturawat

Prof. Dr. Pawinee Piyachaturawat, Ph.D.
Member

Skorn Mongkolsuk

Prof. Skorn Mongkolsuk, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my advisor, Dr. Tossaporn Yimlamai, for his encouragement, valuable advice and support throughout the project. Above all, without his sympathy, this work would never have been accomplished. My deepest appreciation is also expressed to my co-advisor, Assistant Professor Kanokpan Wongprasert for her kindness and excellent guidance with histological techniques.

I would like to thank my examine committee, Professor Pawinee Piyachaturawat and Asso. Prof. Dr. Ganyapong Chaturapanich, who provide many valuable suggestions of this thesis.

I would also like to express my gratitude to the King Prajadhipok and Queen Rambhai Barni Memorial Foundation and Thesis Scholarships, Mahidol University for the research grant.

Finally, my deep thankfulness is extended to my family for their everlasting love and constant support without which I would never be able to achieve my goal and also my colleges for their sincere willing to help and encouragements.

Khajirat Netnee

EFFECT OF OVARIECTOMY AND ESTROGEN SUPPLEMENTATION ON THE RECOVERY FROM EXERCISE-INDUCED MUSCLE INJURY.

KHAJIRAT NETNEE 4736527 SCEP/M
M.Sc. (EXERCISE PHYSIOLOGY)

THESIS ADVISORS: TOSSAPORN YIMLAMAI, Ph.D., PAWINEE PIYACHATURAWAT, Ph.D., GANYAPONG CHATURAPANICH, Ph.D., KANOKPAN WONGPRASERT, Ph.D., JANTARIMA PANDARANANDAKA, Ph.D.

ABSTRACT

The aim of this study was to determine the effects of ovariectomy and estrogen supplement on indices of muscle injury and functional recovery following exercise. Female Sprague Dawley rats were divided into five groups; sham control (SHAMC), sham exercise (SHAME), ovariectomized control (OVXC), ovariectomized exercise (OVXE), and ovariectomized exercise with estrogen supplement (OVXES). In the exercise groups, injury was induced by a single bout of downhill running (90 min at 17 m/min, 16° inclination), whereas the unexercised groups served as control. Immediately after and at 3 days and 14 days after injury, soleus muscle was removed for histological, biochemical, and functional studies.

Results showed that downhill running increased both serum creatine kinase ($p < 0.05$) and myeloperoxidase activities ($p < 0.05$) compared to the control level immediately post-injury. Estrogen supplement tended to attenuate this effect especially during the 3 days post-injury. Muscle wet weights were unchanged among groups but the muscle wet-to-dry weight ratio increased significantly in the exercised groups immediately following exercise, and was restored to the baseline value by 14 days of recovery. Maximal twitch and tetanic force production were significantly reduced in exercised rats relative to that of uninjured rats immediately post-exercise. Myofiber cross-sectional areas (CSA) were similarly reduced in the exercised rats. Estrogen replacement restored the mean fiber CSA, improved the muscle function, and increased the numbers of activated satellite cells staining positive for MyoD in all exercised animals at day 3 of recovery. Cyclo-oxygenase-2 activity of injured soleus muscle increased significantly ($p < 0.05$) at 3 days following downhill running in OVXE, but remained the same in OVXES and SHAME groups.

These results suggest that estrogen has a protective effect against injury following downhill running, increases satellite cells activation, and improves skeletal muscle strength during recovery. However, this estrogenic effect seems to be independent on the COX-2 pathway.

KEYWORDS: EXERCISE/ MUSCLE INJURY/ MUSCLE REGENERATION/
ESTROGEN

ผลของการตัดรังไข่และฮอร์โมนเอสโตรเจนต่อการฟื้นฟูของกล้ามเนื้อภายหลังจากการบาดเจ็บจากการออกกำลังกาย (EFFECT OF OVARIECTOMY AND ESTROGEN SUPPLEMENTATION ON THE RECOVERY FROM EXERCISE-INDUCED MUSCLE INJURY)

ขจีรัตน์ เนตรณี 4736527 SCEP/M

วท.ม. (สรีรวิทยาของการออกกำลังกาย)

คณะกรรมการควบคุมวิทยานิพนธ์: ทศพร ยิ้มลมัย, Ph.D., ภาวิณี ปิยจตุรพานิช, Ph.D., กัลยพงษ์ จตุรพานิชย์, Ph.D., กนกพรธณ วงศ์ประเสริฐ, Ph.D., จันทริมา ปิณฑรนนทกะ, ป.ร.ด.

บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อระบุผลของการตัดรังไข่และฮอร์โมนเอสโตรเจนต่อการบาดเจ็บของกล้ามเนื้อและการฟื้นฟูภายหลังจากการบาดเจ็บจากการออกกำลังกาย

ในการศึกษาครั้งนี้แบ่งหนูทดลองออกเป็น 5 กลุ่มคือกลุ่มควบคุมของหนูปกติที่ไม่ตัดรังไข่, กลุ่มหนูปกติที่ออกกำลังกายไม่ตัดรังไข่, กลุ่มควบคุมของหนูที่ตัดรังไข่, กลุ่มหนูที่ตัดรังไข่และออกกำลังกาย และหนูที่ตัดรังไข่ ออกกำลังกายและให้ฮอร์โมนเอสโตรเจน ในหนูกลุ่มที่ออกกำลังกายได้มีการเหนี่ยวนำให้มีการบาดเจ็บของกล้ามเนื้อโดยการวิ่งลงทางชันที่ 16° เป็นระยะเวลา 90 นาทีที่ความเร็ว 17 เมตรต่อนาที ขณะที่หนูที่ไม่ออกกำลังกายจะเป็นหนูกลุ่มควบคุม ทันทีหลังจากออกกำลังกาย และในช่วงวันที่ 3 และ 14 หลังการบาดเจ็บ จึงนำกล้ามเนื้อโซเลียสมาศึกษาผลการเปลี่ยนแปลงด้านโครงสร้าง, ชีวเคมี และความแข็งแรงในการหดตัวของกล้ามเนื้อ

ผลการศึกษาพบว่า การออกกำลังกายทำให้ระดับเอ็นไซม์ครีเอติน ไคเนสและมัยโอโกลบินหรือคซีเดสซึ่งใช้เป็นตัวบ่งชี้ของการบาดเจ็บเพิ่มขึ้นอย่างมีนัยสำคัญทันทีหลังจากสิ้นสุดการออกกำลังกายเมื่อเทียบกับกลุ่มควบคุม การให้ฮอร์โมนเอสโตรเจนมีแนวโน้มที่จะช่วยลดการบาดเจ็บนี้ในช่วง 3 วันแรกของการบาดเจ็บ ส่วนน้ำหนักของกล้ามเนื้อไม่มีการเปลี่ยนแปลงระหว่างกลุ่มในขณะที่อัตราส่วนของน้ำหนักกล้ามเนื้อสัมพันธ์เพิ่มขึ้นอย่างชัดเจนภายหลังจากการบาดเจ็บและกลับสู่ภาวะปกติภายใน 14 วันหลังการบาดเจ็บ นอกจากนี้ความแข็งแรงและขนาดของกล้ามเนื้อของหนูกลุ่มที่ออกกำลังกายยังลดลงอย่างเห็นได้ชัดทันทีภายหลังจากการออกกำลังกาย และการออกกำลังกายยังทำให้มีการเพิ่มจำนวนของแซดเทิลไลท์เซลล์ในกล้ามเนื้อในหนูกลุ่มที่ออกกำลังกายในวันที่ 3 ของการบาดเจ็บ โดยเฉพาะอย่างยิ่งในหนูกลุ่มที่ให้ฮอร์โมนเอสโตรเจนทดแทน นอกจากนี้การเปลี่ยนแปลงของเอ็นไซม์ไซโคออกซิจีเนส 2 ในกล้ามเนื้อโซเลียสมีการเพิ่มอย่างชัดเจนในหนูกลุ่มที่ตัดรังไข่และออกกำลังกาย 3 วันหลังการบาดเจ็บ แต่ในหนูกลุ่มที่ตัดรังไข่และออกกำลังกายและให้ฮอร์โมนเอสโตรเจน และ กลุ่มที่ออกกำลังกายไม่ตัดรังไข่ไม่พบการเปลี่ยนแปลง

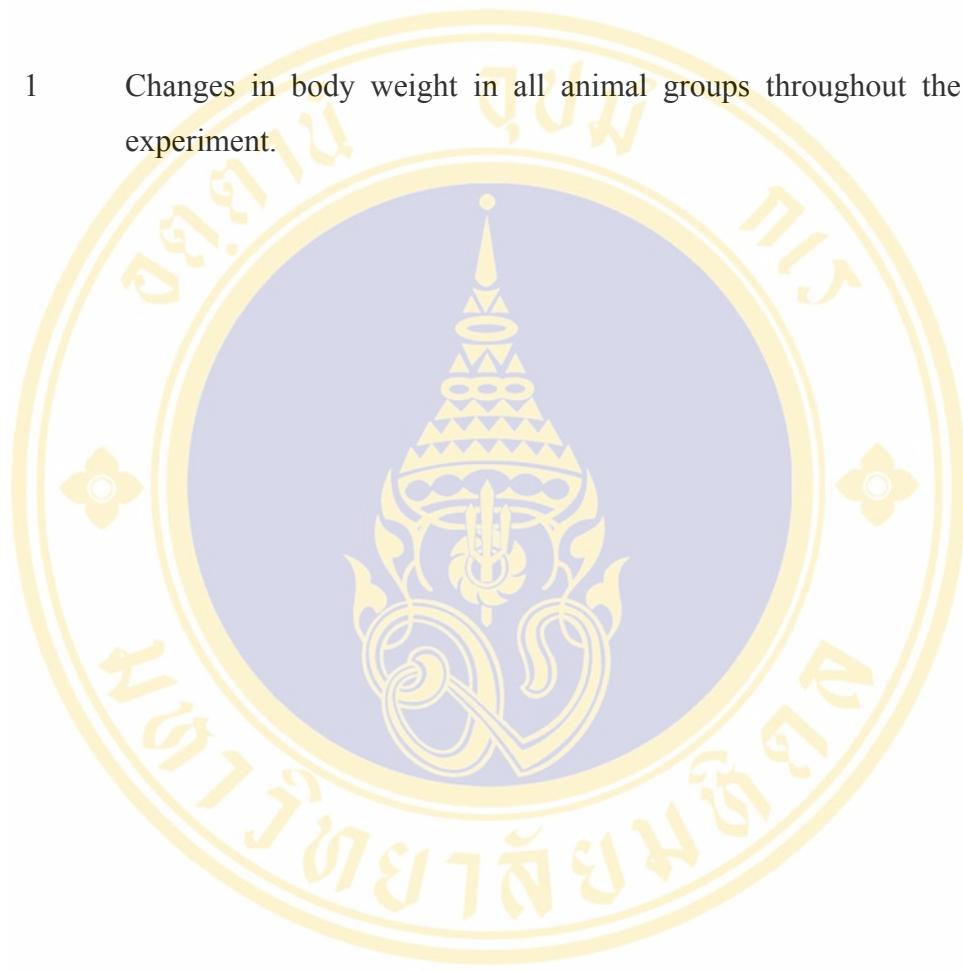
การศึกษานี้สรุปได้ว่าฮอร์โมนเอสโตรเจนสามารถช่วยลดการบาดเจ็บของเส้นใยกล้ามเนื้อโดยลดการอักเสบและเพิ่มจำนวนของแซดเทิลไลท์เซลล์ และความแข็งแรงของกล้ามเนื้อระหว่างการฟื้นตัวหลังการออกกำลังกายและผลของการเปลี่ยนแปลงนี้ไม่มีความสัมพันธ์กับการทำงานของเอ็นไซม์ไซโคออกซิจีเนส 2.

CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	x
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	5
A. MODEL OF SKELETAL MUSCLE DAMAGE	
B. THE PROCESS OF SKELETAL MUSCLE HEALING FOLLOWING INJURY	
C. SEX INFLUENCES ON MUSCLE DAMAGE	
III MATERIALS AND METHODS	23
IV RESULTS	32
V DISCUSSION	62
VI CONCLUSION	74
REFERENCES	75
APPENDIX	89
BIOGRAPHY	94

LIST OF TABLE

TABLE		PAGE
1	Changes in body weight in all animal groups throughout the experiment.	35



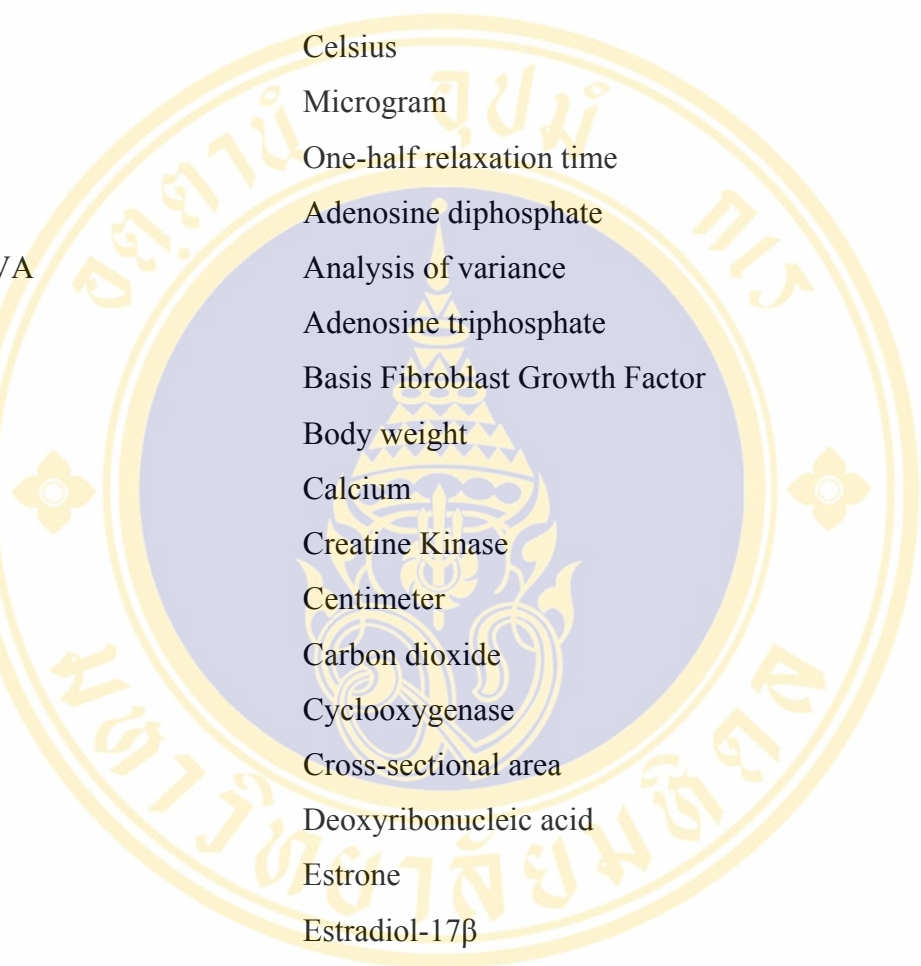
LIST OF FIGURES

FIGURE		PAGE
1	A schematic illustration of healing skeletal muscle	10
2	Satellite cell activation in response to an injury	14
3	Factors modulate satellite cell activity	17
4	An example of peak twitch tension (g)	26
5	Schematic of the experimental protocol	30
6	Changes in body weight in all experimental groups at immediately after, 3, and 14 days after injury	36
7	Soleus muscle wet weight per body weight ratio at immediately, at 3 days, and 14 days post-injury	37
8	EDL muscle wet weight per body weight ratio at immediately, at 3 days, and 14 days post-injury.	38
9	Uterine weight per body weight ratio at immediately, at 3 days, and 14 days post-injury.	39
10	Changes in peak twitch force of soleus muscles at immediately (0-1h) after, at 3 days, and 14 days after injury	40
11	Changes in specific peak twitch tension (sP_t) of soleus muscle at immediately (0-1h) after, at 3 days, and 14 days after injury.	41
12	Time to peak tension (TPT) of soleus muscle at immediately after, 3 days, and 14 days after injury.	42
13	Half relaxation time ($RT_{1/2}$) of soleus muscle at immediately after, 3 days, and 14 days after injury.	43
14	Peak tetanic force of SOL muscles (mN) at immediately, at 3 days, and 14 days post-injury.	44
15	Specific tetanic tension (sP_o) of soleus muscle at immediately after, 3 days, and 14 days after injury.	45

LIST OF FIGURES (CONT.)

FIGURE		PAGE
16	Representative section of soleus muscle (H&E stained) at immediately (0-1h) after, at 3 and 14 days after injury.	46
17	Soleus muscle fiber size in H&E stained sections at immediately, 3 days, and 14 days after injury.	47
18	Creatine Kinase activity at immediately after injury at 3 days, and 14 days post-injury.	51
19	Myeloperoxidase activity (MPO) at immediately, at 3 days and 14 days after injury.	52
20	Water content (%) of SOL muscle at immediately and at 14 days after injury.	53
21	Wet-to-dry weight ratio of SOL muscle at immediately and 14 days after injury.	54
22	The myofibers expressing activated (MyoD) satellite cells markers in transverse sections from soleus muscles at 3 days post-exercise.	56
23	Changes in relative numbers of myofibers expressing activated (MyoD) satellite cells markers in rat soleus muscle at immediately (0-1h) after, 3 days and 14 days after injury.	58
24	Changes in total numbers of myoneuclei per fiber in rat soleus muscle at immediately (0-1h) after, 3 days and 14 days after injury.	59
25	Changes in cyclo-oxygenase activity (COX-2) at immediately (0-1h), 3 days and 14 days after injury.	60
26	Changes in cyclo-oxygenase activity (COX-2) expressed as % of control value at immediately (0-1h) after, 3 days and 14 days after injury.	61
27	An example of peak twitch tension (g)	93
28	An example of peak tetanic tension (g)	93

LIST OF ABBREVIATIONS



°C	Celsius
μg	Microgram
$1/2RT$	One-half relaxation time
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	Basis Fibroblast Growth Factor
BW	Body weight
Ca ²⁺	Calcium
CK	Creatine Kinase
cm	Centimeter
CO ₂	Carbon dioxide
COX	Cyclooxygenase
CSA	Cross-sectional area
DNA	Deoxyribonucleic acid
E1	Estrone
E ₂ /E ₂	Estradiol-17β
E3	Estriol
EDL	Extensor digitorum longus
EGF	Epidermal Growth Factor
FGF	Fibroblast growth factor
g	Gram
G-6-P	Glucose-6-phosphate
h	Hour
H&E	Hematoxyline and Eosin
HGF	Hepatocyte Growth Factor
HRT	Hormone replacement therapy

LIST OF ABBREVIATIONS (CONT.)

Hz	Hertz
IGF-1	Insulin-like Growth Factor
IL-6	Interleukin-6
kg	Kilogram
L_0	Optimal muscle length for contraction
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mN	Millinewton
mo	Month
mpc	myogenic precursor cell
MPO	Myeloperoxidase
MRFs	Myogenic regulatory factors
N	Newton
Na^+	Sodium
n	Number of rats
NADPH	Nicotinamide adenosine dinucleotide phosphate
NF- κ B	Nuclear factor kappa B
nm	Nanometer
O_2	Oxygen
OVX	Ovariectomized rats
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PGE_2	Prostaglandin E2
PLA2	Phospholipase A2
P_0	Peak tetanic force
P_t	Peak twitch force

LIST OF ABBREVIATIONS (CONT.)

ROS	Reactive oxygen species
SEM	Standard error of mean
SHAM	Sham-operated rats
SOL	Soleus
sP _o	Specific peak tetanic tension
sP _t	Specific peak twitch tension
TGF- β 1	Transforming Growth Factor Beta-1
TNF α	Tumor necrosis factor- α
TNF β	Tumor necrosis factor- β
TPT	Contraction time
μ l	micro liter
μ m	micrometer
UV	Ultraviolet
wks	weeks

CHAPTURE I

INTRODUCTION

Background and Rationale

Skeletal muscle injuries are increasingly become the challenging problems in sports medicine. They contribute to the majority of sport-related injuries that occur in both professional athletes and recreational athletes (Garrett., 1990). Moderate to severe muscle injury may result in the athlete being unable to train at the required intensity or complete for several weeks (Orchard and Best., 2002). A variety of conditions can lead to muscle injuries including direct (e.g. trauma, laceration, and strain) and indirect (e.g. ischemia, and eccentric exercise) causes (Kasemkijwattana et al., 2000). Although skeletal muscle is known to exhibit a remarkable capacity to regenerate and restore function, the recovery is frequently incomplete until several weeks after injury. Therefore, the development of strategies or interventions to enhance muscle healing and hasten recovery has important implication for minimizing disability after injury.

Recently, it has been reported that the recovery of injured muscle varied considerably. It is dependent on the extent and the scope of injured tissues including degree of muscles damage, the following inflammatory response, ischemia (Garrett, 1990; Nikolaou et al., 1987), and the age and sex among the others (Pansarasa et al., 2000). Of these factors, there is increasing interest in sex-related difference in the recovery of skeletal muscle following injury. Evidence has been accumulated in the past decade from both animals and human studies. They revealed that female is less susceptible to muscle injury than male (Tiidus., 2001 and Clarkson and Hubal., 2001 for review). For instance, the plasma creatine kinase (CK) activity, an indirect marker of muscle damage (Amelink and Bar., 1986), ultrastructural disruptions (Komulainen et al., 1999), and inflammatory responses (Schneider et al., 1996) in female are reported lower than those in male animals. Further, more recent evidences indicatethat

the loss of ovarian hormones (i.e. ovariectomy surgeries) has detrimental effect on skeletal muscle force-generating capacity and prevents the muscle recovery from disuse atrophy (Brown et al., 2005; McClung et al., 2006; Stinick et al., 2006). These changes, however, are reversed by estrogen replacement. These data imply that female sex hormones may play an important role in muscle damage and regeneration after injury or disuse atrophy. However, the underlying mechanism responsible for this protective effect remains unclear. One potential mechanism seems to involve the increased myogenic satellite cells activity (Hawke and Garry., 2001).

Skeletal muscle is considered to be post-mitotic cell. Postnatal muscle growth is, therefore, dependent on an undifferentiated mononuclear myogenic stem cell called satellite cells (Hawke and Garry., 2001). Skeletal muscle satellite cells are also activated during recovery from injury and provide a critical myonuclei source for muscle mass recovery. In mature skeletal muscle, the satellite cells are normally quiescent and reside beneath the basal lamina. Once become activated, the satellite cells re-enter the cell cycle, proliferated and differentiated to myotubes which in turn fuse with the existing muscle fiber or replace damaged fibers. Currently, the precise mechanism that regulates the satellite cell activity in response to muscle injury is not well understood.

A number of factors can modulate satellite cells and have been implicated in regulating the degeneration and regeneration of skeletal muscle following injury. These include various growth factors, cytokines, nitric oxide and prostaglandins that released during inflammatory phase after muscle injury (Hawke and Garry., 2001; Chambers and McDermott, 1996). Among these factors, prostaglandin is one of the most attractive mediators (Prisk and Huard., 2003). Prostaglandins have been reported to play a major role in tissue inflammation (Dubois et al., 1998; Prisk and Huard., 2003) and in various stages of myogenesis (Prisk and Huard., 2003 and Bondesen et al., 2004). Prostaglandins are signaling molecules synthesized from arachidonic acid that are released from membrane phospholipids by phospholipase. The arachidonic acid is then converted to prostaglandin by the cyclooxygenase (COX) enzymes. Two isoforms of COX have been identified. COX-1 is constitutively expressed and is involved in cellular homeostasis with the maintenance of tissue physiology, whereas COX-2 is a rapidly inducible isoform upregulated by reactive oxygen species,

cytokines, and mitogens (Dubois et al., 1998). Recently, it has been reported that COX-2 is upregulated in various type of injury including local freeze injury, exercise, and hindlimb suspension, and appears to be involved in regeneration of injured tissues (Bondesen et al., 2004; Mendius et al., 2005).

There are several lines of evidences suggest the role of prostaglandin in mediating the regeneration of skeletal muscle following injury. Mendias et al (2004), by using specific COX inhibitors, showed that inhibition of COX-2 alone resulted in decreased satellite cells proliferation, whereas the inhibition of both COX-1 and COX-2 resulted in decreased satellite cell differentiation and fusion. In addition, Bondesen et al (2004) demonstrated that an inhibition of COX-2, but not COX-1, could reduce the inflammatory response to injury, the number of myoblast, and myofiber growth.

Another evidence to support the important role of COX-2 in regulating skeletal muscle regeneration comes from transgenic animals. Knockout mice lacking COX-2 protein (COX-2^{-/-}) showed the decreased in skeletal muscle regeneration. This effect was associated with the presence of fewer myoblast in regenerating muscle and the attenuated myofiber size (Bondesen et al., 2004). Moreover, COX-2^{-/-} satellite cells exhibited impaired activation and proliferation according to Bondesen et al (2006).

Taken together, to our knowledge, there has been limited information available regarding the role of female sex hormones on skeletal muscle regeneration, therefore, this study was undertaken to examine the role of estrogen (if any) on satellite cells function and the recovery of skeletal muscle following exercise-induced injury.

Hypothesis

We hypothesized that estrogen treatment could attenuate muscle damage and stimulate satellite cell activation and/or proliferation, and thus promoting muscle recovery after injury. Furthermore, we determined whether this estrogen's effect was mediated by the COX-2 pathway.

Objectives

1. To examine the effect of ovariectomy and estrogen supplementation on muscle damage and contractile function after injury.

2. To compare the effect of ovariectomy and estrogen supplementation on satellite cells function following exercise- induced injury.
3. To determine whether this estrogenic-dependent effect on satellite cells activation was mediated via the COX-2 pathway.



CHAPTER II

LITERATURE REVIEWS

Models of Skeletal Muscle Damage/Injury

Muscle damage can occur in a variety of situations including after demanding or unaccustomed exercise. Such injury could arise from various types of muscle contraction; shortening, where the muscle is activated and shortened (also called concentric or dynamic), lengthening, where the muscle is activated and lengthened (also called eccentric or plyometric), and isometric, where the muscle is activated and maintained at the same length (also called static) contractions.

Of these three types of contraction, lengthening contractions cause severely damage to muscle (Close et al., 2005). Eccentric contractions can produce damage to muscle fibers as documented by morphologic analysis of muscle biopsy samples, although some questions have been raised over the accuracy of this technique to assess muscle damage (Friden et al., 1984; Stauber et al., 1990). When the myofibrils of a muscle fiber are stretched while contracting, some sarcomeres may resist stretch more than the others. This is probably due to the overlapping of the myofilament is closer to their optimum value or the cross-sectional area of the myofibril is slightly greater at that point. As a consequence, weaker sarcomeres take up most of the stretch. If this occurs on the descending limb of the length–tension curve, these sarcomeres get progressively weaker until there is no overlap between the myofilaments. The rising passive tension in elastic elements balances the tension in the remaining unstretched sarcomeres. During a series of eccentric contractions, more and more sarcomeres will become overstretched, beginning with the weakest and including progressively stronger sarcomeres. Each time the muscle relaxes, myofilaments in some overstretched sarcomeres may not reinterdigitate, and the sarcomere becomes disrupted. Overstretched, disrupted sarcomeres lie scattered at random along the length of the myofibril. Once one or more sarcomeres have become disrupted, the damage

may spread longitudinally to adjacent sarcomeres in the myofibril and transversely to adjacent myofibrils. A point will be reached at which the structural distortions produced by the presence of overstretched sarcomeres lead to membrane damage, including membranes of the sarcoplasmic reticulum, transverse tubules, or the sarcolemma. This is accompanied by the uncontrolled movement of Ca^{2+} into the sarcoplasm, triggering the next stage in the damage process. However, these proposal mechanisms have not been universally accepted. In the alternative view, the damage is largely the result of excitation–contraction (E-C) uncoupling. The supporting evidence for this hypothesis is based on intracellular Ca^{2+} measurements; one could argue that an increase in Ca^{2+} is secondary to mechanical changes in the fiber. In addition, an E-C coupling mechanism does not readily explain a shift in the length–tension relation of the muscle, a characteristic feature associated with damage from eccentric contractions. An event supplementary to sarcomere disruption is the opening of stretch-activated cation channels as a result of membrane stresses produced by the mechanical changes. This leads to inward movement of Na^+ and Ca^{2+} into the sarcoplasm. To summarize, the sequence of events would begin with disruption of sarcomeres. Structural distortions triggered by the disruptions lead to membrane damage and interference with E-C coupling. At the same time, the accompanying stresses applied to membranous structures lead to opening of cation channels. All of this produces an increase in sarcoplasmic Ca^{2+} levels and the Ca^{2+} triggers proteolysis associated with fiber breakdown and repair (Proske U and Allen., 2005). Moreover, subsequent damage is linked to inflammatory processes and may also function in regeneration process (Clarkson and Hubal., 2002).

Physical trauma includes crush, contusion, or cut injuries can be used to induce skeletal muscle injury, the extent of damage and duration of regeneration needs is dependent upon the type of injury. Furthermore, physical trauma can induce a local injury that does not necessarily have an effect on the entire muscle (See Chambers and McDermott., 1996 for review).

Myotoxins have been also used extensively in animals to induce muscle injury and regeneration. Substances or toxins frequently used to induce injury are local anesthetic, snake venome and barium chloride. Injection of local anesthetics may produce damage that is specific to only the site of injection. While the snake venome

notoxin produces a severe degeneration of the muscle and is lethal, the barium chloride appears to produce injury without being as lethal as notoxin.

Another mode of inducing injury is muscle ischemia. This model allowed for injury to the muscle without damage to circulation or basement membrane. However, there are several drawbacks to this model. In addition to requiring surgery, the protocol preferentially damages fast-glycogenolytic fibres, which is a problem in muscles of mixed fiber population.

The Biological Process of Skeletal Muscle Healing Following Injury

Muscle injuries can occur via a variety of mechanisms including direct (e.g. laceration, contusion, and strain) and indirect (e.g. ischemia and neurological dysfunction) causes. Although the functional recovery of the injured muscle varies from one type of muscle injury to another, the healing processes are similar among the various types of muscle injuries. Following injury muscle undergoes a three distinct set of healing phases, consisting of degeneration, inflammation, and regeneration phases (Tero AH Järvinen et al., 2005).

1. Degeneration phase

This destruction phase is characterized by the rupture and ensuing necrosis of the myofibers, the formation of a hematoma between the ruptured muscle stumps, and the inflammatory cell reaction (Tero AH Järvinen et al., 2005).

Injury to muscle disrupts the integrity of the sarcomere, sarcolemma, and basal lamina, leading to the ingress of extracellular calcium as well as the activation of complement cascade. Intrinsic proteases then autodigest disrupted and subsequently necrotic myofibers. The tendon-myofiber-tendon units are disrupted and the ruptured myofibers retract forming a gap. Since skeletal muscle is richly vascularized, and capillary injury can result in hematoma that fills this gap. The upregulation of adhesion molecules and cytokines at the site of injury can also influence local vascular permeability and blood flow, thus accelerating the ensuing inflammatory response and resultant edema. Toxic free radical species is developed during this phase and can interfere excitation-contraction coupling, induction of proteolysis, and subsequent

necrosis of myofibers both in the traumatized tissue and in healthy tissue located nearby.

At present, little attention has been paid to finding ways to limit the degeneration that occurs with muscle injury. In order to affect this phase, one must employ preventive medicines techniques. Some have suggested the use of antioxidant substances prior to sporting activities. However, studies on antioxidant or free radical inactivating substances like vitamin E, vitamin C, and N- acetyl cysteine have failed to show promising results (Prisk V and Huard., 2003).

2. Inflammation phase

Inflammation is an early response to muscle tissue injury. It involves the coordination between the immune system and injury tissue. This phase is, perhaps, the least distinct phase as its process overlap with all of the other phases of muscle injury and repair. During this phase of damage, necrosis of muscle fibres is triggered by disruption of the sarcolemma. This causes an increase in the permeability of the muscle fiber and increased calcium influx. This calcium influx can increase calcium-activated proteases such as calpains, which can cleave myofibrillar proteins and are thought to drive degeneration (Close et al., 2005).

This initial period of degeneration is then followed by invasion and activation of inflammatory cells triggered by factors released from the damaged muscle. First to invade the damaged sites are neutrophils that promote inflammation and attract macrophages by releasing chemoattractants. In addition to phagocytosis, neutrophils invasion can cause further damage through the generation of superoxide and other ROS via a respiratory burst, which is catalysed by the enzyme NADPH oxidase, located in plasma membrane. Additionally, myeloperoxidase can generate hyperchlorous acid, a highly reactive oxidizing agent. Macrophages then invade the damaged site and phagocytose cellular debris from the site of injury. Like neutrophils, macrophages are capable of producing oxygen free radicals. Activated macrophases also release proinflammatory cytokines, which in turn may exacerbate damage by potentiating cytotoxic mechanisms of other inflammatory cells to enhance free radical production and enzyme release (Prisk V and Huard., 2003). The exact time course of the inflammation response after exercise is variable, dependent on several factors such

as exercise mode, intensity or duration, and the muscle groups utilized, however, recent studies suggest that cellular infiltration of the damaged muscle peaks within 24 h post-injury and can persist for days to weeks (Tidball., 1995).

More recently, the invasion of inflammatory cells have also been suggested to play a role in muscle growth and repair following injury. For example, Teixeira et al (2003) reported that neutrophils could facilitate muscle regeneration by activation of satellite cells. However, the mechanism by which this occur remains poorly understood and need a further investigation.

1. Regeneration phase

This is a period during which the maturation of the regenerated myofibers, the contraction and reorganization of the scar tissue and the recovery of the functional capacity of the muscle occur (Tero AH Järvinen et al., 2005).

Muscle regeneration begins during the first week post-injury. Because the nuclei within myofibers are postmitotic, myogenesis primarily depends on satellite cells, which are quiescent muscle precursor cells that lie beneath the basal lamina surrounding each myofiber. Satellite cells are activated as early as 24 hours post-injury. In response to injury, satellite cells reenter the cell cycle and proliferate. These activated precursor cells, or myoblasts, then undergo differentiation and join with each other to form multinucleated myotubes. The newly formed multinucleated myotubes then fuse with the part of the injured myofiber that has survived the initial trauma. Eventually, the regenerating parts of the myofibers acquire their mature form with normal cross-striations and peripherally located myonuclei. During the phagocytic phase of muscle damage there is an associated division of surviving satellite cells, which mature into myoblasts and use to form new myotubes. However, it does appear that invasion by macrophages seems to be an essential prerequisite for regeneration, possibly by somehow stimulating satellite cell division. Indeed, it is strongly suggested that macrophage infiltration is an important part of the regeneration phase particularly in terms of satellite cell proliferation (Tero AH Järvinen et al., 2005).

Muscle regeneration also depends on other cellular processes involving nonmuscle cells. One of the earliest of these is the inflammatory response, which facilitates myogenesis via phagocytosis of cellular debris and the release of

chemoattractants and growth factors. The cellular events that occur during muscle regeneration are orchestrated by a number of growth factors and cytokines. Growth factors released at the injury site, including Insulin-like Growth Factor-1 (IGF-1), basis Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), and Transforming Growth Factor Beta-1 (TGF- β 1), have been shown to influence the proliferation and differentiation of myoblasts and muscle stem cells in vitro (Prisk V and Huard., 2003). As the local environment during muscle regeneration overlaps with the inflammatory process, prostagladins released in the injured muscle may also contribute or be essential to the action of growth factors in myofiber regeneration (Tero AH Järvinen et al., 2005). As shown in figure 1:

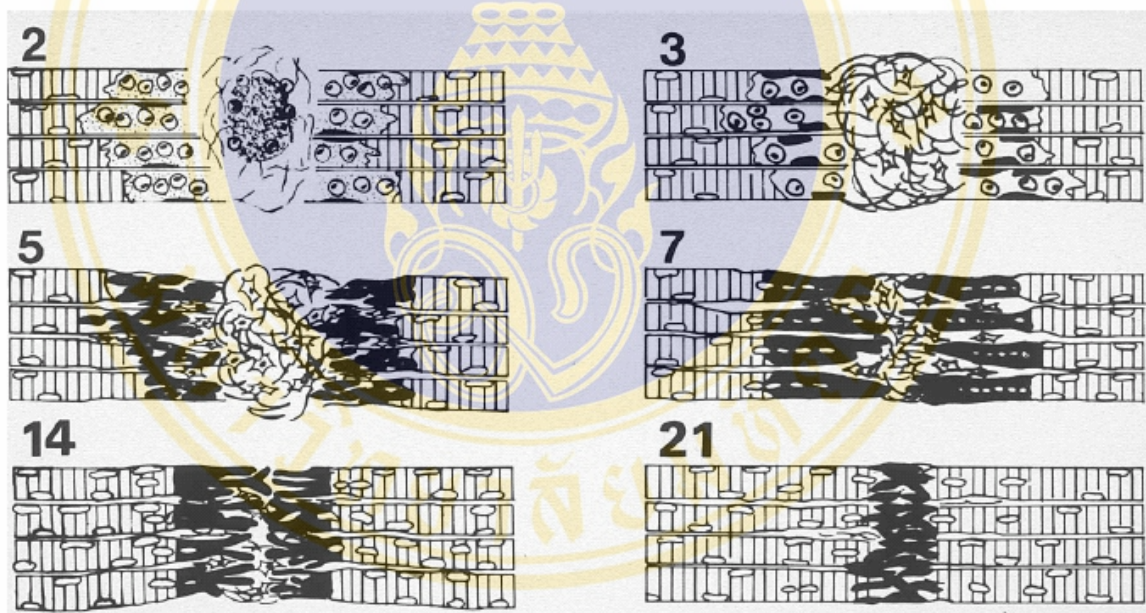


Figure 1 A schematic illustration of healing skeletal muscle. Day 2: the necrotized parts of the transected myofibers are being removed by macrophages while, concomitantly, the formation of the connective tissue scar by fibroblasts has begun in the central zone. Day 3: satellite cells have become activated within the basal laminar cylinders in the regeneration zone. Day 5: myoblasts have fused into myotubes in the regeneration zone, and the connective tissue in the central zone has become denser. Day 7: the regenerating muscle cells extend out of the old basal lamina cylinders into

the central zone and begin to pierce through the scar. Day 14: the scar of the central zone has further condensed and reduced in size, and the regenerating myofibers close to central zone gap. Day 21: the interlacing myofibers are virtually fused with little intervening connective tissue (scar) in between (Tero AH Järvinen et al., 2005).

Skeletal Muscle Regeneration

Skeletal muscle exhibits a high capacity to repair and regenerate in response to injury. This regenerative capacity is primarily attributed to satellite cells, which are located between the basal lamina and the sarcolemma of muscle fibers. In general, muscle regeneration can be subdivided into four important stages: (1) satellite-cell activation, (2) myoblast or precursor proliferation, (3) differentiation, and (4) return to quiescence (Ashley et al., 2005).

Satellite-Cell Activation.

Satellite-cell activation is the process by which satellite cells exit G_0 and enter into the cell cycle. This activation can be measured using a wide variety of methods. Observations of changes in morphology and contents have been extensively used to develop structural criteria for the activation state of satellite cells. A quiescent satellite cell is a spindle-shaped cell with little cytoplasm and few organelles, whereas an activated satellite cell has hypertrophied organelles and an expanded cytoplasm.

Satellite-Cell Proliferation.

The second important stage in skeletal muscle regeneration is the proliferation of satellite cells and myogenic precursor cells. Daughter cells (myoblasts) then commit to enter the myogenic lineage by the expression of Myf5 and MyoD approximately 6 h after activation *in vivo*. These committed myoblasts continue to proliferate and express muscle regulatory genes, including myogenin, until the balance of protein expression pushes the cells toward differentiation.

Precursor Differentiation.

Differentiation is the process whereby proliferating myoblasts derived from activated satellite cells and other myogenic precursors will withdraw from the cell cycle and either fuses to existing fibers in repair of damaged segments or to each other to form new fibers. Fusion events occur after cells exit mitosis and enter into G_1 . The onset of differentiation in cell or fiber cultures can be marked by the expression of the myogenic regulatory genes myogenin and MRF4. Early differentiation can also be measured by an increase in creatine kinase activity in muscle tissue, particularly the isoform creatinekinase BB, and later by the shift in expression of contractile protein isoforms from immature or developmental isoforms to adult isoforms. Because satellite cells are self-renewing and satellite-derived myoblasts do not uniformly fuse into fibers, at least some myoblasts likely maintain, resume, or take up the satellite-cell position, for example, as evidenced by retention of markers of earlier DNA synthesis, and may eventually return to quiescence.

Return To Quiescence.

Under normal conditions the proportion of satellite cells that actually reside in G_0 rather than in a long lag phase in G_1 is not known, as are many important aspects of satellite-cell quiescence. For example, it is unknown whether the same satellite cells that originally respond to an activating stimulus will return to quiescence, or whether the satellite-cell compartment is only repopulated by daughter cells. It is likely, however, that the expression of particular genes and proteins must be up-regulated during late differentiation in order to return satellite cells to quiescence.

Satellite cell number is dependent on the species, age, and muscle fiber type (Schultz et al., 1994). It has been reported that satellite cells constitute up to 30% of the muscle nuclei in the neonate and decrease with age to 4% in the adult and 2% in the senile (29–30 mo) mouse (Snow., 1977). This decrease in the percentage of satellite cells with aging could result from an increase in myonuclei (Type I fibers) and a decrease in total number of satellite cells (Type II fibers). The satellite cell distribution between muscle groups is a result of the heterogeneity in satellite cell content (Hawke and Garry., 2001).

Myogenic regulatory factors of satellite cell activation and differentiation

Quiescent satellite cells adjacent to mature fibers express c-Met and M-cadherin proteins. The primary MRFs, Myf5 and MyoD, are required for the determination of myoblasts, whereas the secondary MRFs, myogenin and MRF4, function to regulate terminal differentiation. The MRF expression program during satellite cell activation, proliferation, and differentiation is analogous to the program manifested during the embryonic development of skeletal muscle. Quiescent satellite cells express no detectable levels of MRFs. MyoD is rapidly up-regulated within 12 h of experimentally induced muscle injury prior to expression of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation. Myogenin is expressed last during the time associated with fusion and differentiation. Quiescent satellite cells express no detectable MRFs but do express the c-Met receptor tyrosine kinase, the receptor for hepatocyte growth factor (HGF/SF). Activated satellite cells (satellite cells entering the cell cycle) first express either Myf5 or MyoD followed soon after by coexpression of Myf5 and MyoD. Following proliferation, myogenin and MRF4 are expressed in cells beginning their differentiation program. Skeletal muscle from MyoD^{-/-} mice displays a strikingly reduced capacity for regeneration following injury. Electron microscopic examination of MyoD-deficient muscle reveals morphologically normal satellite cells whose numbers are increased 1.8-fold in MyoD^{-/-} muscle and 13-fold in mdx:MyoD^{-/-} muscle. These data suggest a model in which up-regulation of MyoD is required for satellite cells to enter the mpc proliferative phase that precedes terminal differentiation. In the absence of MyoD, myogenic progenitors undergo an apparent increase in numbers as a consequence of an increased propensity for self-renewal rather than progression through their developmental program. Taken together, these data suggest that MyoD^{-/-} myogenic cells represent an intermediate stage between a satellite cell and a mpc (Seale and Rudnicki., 2000).

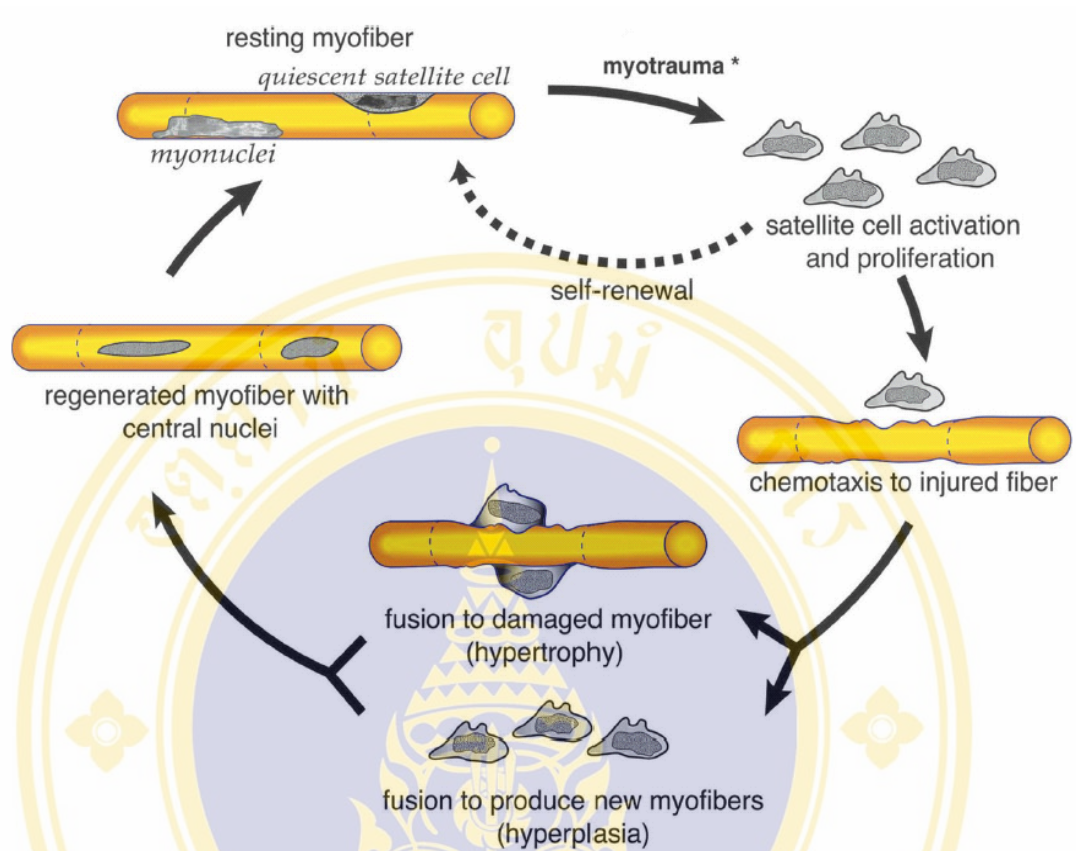


Figure 2 Satellite cell activation in response to an injury (Hawke and Garry, 2001).

Factors that modulate satellite cell activation

Skeletal muscle regeneration is a complex process and can be influenced by various factors including several growth factors, cytokines, prostaglandins and free radicals. All of these factors are capable of modulating satellite cell activity.

Role of Growth factors and Cytokines

Cytokines such as interleukins and tumor necrosis factor- α (TNF- α) are small polypeptides produced by circulating and tissue resident leucocytes as well as other cells (Dietrich et al., 1999). Previous studies suggested that cytokines play variety roles during muscle regeneration. Of these, tumor necrosis factor- α (TNF- α) is particular interest since there is direct evidence that TNF- α has a physiological role in the regeneration of the injured skeletal muscle, as the inhibition of its activity during the healing results in a slight deficit in the strength of the recovering skeletal muscle

(Schmalbruch and Hellhammer., 1977). A large number of growth factors such as the members of fibroblast growth factor (FGF), insulin-like growth factor (IGF), and transforming growth factor- β (TGF- β) families; hepatocyte growth factor (HGF) and the platelet-derived growth factor are known to be expressed in skeletal muscle (LeRoith et al., 1992; Carlson and Faulkner., 1989) Their expression can be induced by micro-traumas, external stretching or mechanical loading. Considering that these growth factors are potent mitogenic activators for numerous different cells, they are also likely to be involved in the activation of the regeneration of the injured muscle cells. These growth factors are potential activators of myogenic precursor cell (mpc; satellite cell) proliferation (Clarke et al., 1993). Some of them are also powerful stimulators for mpc differentiation and the fusion of myotubes into multinucleated mature myofibers later during the regeneration process (Borycki et al., 1999; Carlson and Faulkner, 1989; Clarke et al., 1993).

Role of Prostaglandins

Prostaglandins have been identified and implicated as major factors in tissue inflammation for many years after discovery that aspirin and other NSAIDs, which inhibit prostaglandin synthesis, also attenuate acute inflammation. Furthermore, injection of prostaglandins into various tissues can potentate the signs of inflammation induced by bradykinin and histamine. Many prostaglandins are synthesized by the cyclooxygenase enzyme. Cyclooxygenase exists in several isoforms and catalyzes multiple steps in conversion of arachidonic acid to various prostaglandins. Proinflammatory mediators induce the synthesis of prostaglandins through phospholipase A2 (PLA2) mediated release of membrane-associated arachidonic acid and induction of cyclooxygenase enzyme activity. Three isoforms of cyclooxygenase have been described to date. Cyclooxygenase-1 (COX-1) is produced constitutively, synthesizes prostaglandins important for homeostasis, and appears to play a small role in early inflammation. Cyclooxygenase-2 (COX-2) is an inducible isoform that plays a major role in mediation of pain and inflammation after injury. Increased COX-2 products, such as PGE₂, appear to sensitize local nociceptor terminals, thereby increasing peripheral hypersensitivity to pain. There is also evidence that interleukins and other inflammatory mediators lead to prostaglandin-mediated sensitization to pain

in spinal cord and other areas of central nervous system. Likewise, multiple studies have proven the effectiveness of selective COX-2 inhibitors in the reduction of postoperative and arthritic pain. Cyclooxygenase-3 (COX-3) is a recently described isoform of cyclooxygenase that appears to be involved in process such as fever and is inhibited by acetaminophen. Unlike COX-1 and COX-2, COX-3 does not appear to have significant involvement in tissue inflammation.

Immediately after muscle injury, satellite cells and stem cells within the basal lamina of myofibers are released and activated from quiescent state to the activated state to divide and eventually participate in the regeneration of myofibers. Mitogenic stimulation of multiple quiescent cell types by the addition of serum to medium typically results in increased expression of COX-2 *in vitro*. This finding correlates with the complex role of COX-2 in the cell cycle dynamics of neoplasia, and one could postulate that COX-2 plays a related role in initiating proliferation of satellite cells or muscle stem cells (Prisk V and Huard., 2003). Moreover, Steiner et al. (1995) noted that the rise in COX-2 protein levels after changing from serum depletion to serum stimulation of myoblasts is only transient, suggesting that COX-2 expression might only be of significance in stimulating re-entry of satellite cells into the cell cycle rather than being essential for continued exponential proliferation of those cells.

Role of Reactive Oxygen Species (ROS)

Free radicals are molecules or molecule fragments containing an unpaired electron in their outer valence shell. This unpaired electron is usually extremely exchangeable, which is the chemical and physical reason for the reactivity of radical species. Free radicals have a potent oxidizing effect, which is the basis for their destructive effect against lipids, proteins, nucleic acid and the extracellular matrix.

It has been demonstrated that intense muscular activity increases the production of free radicals and reactive oxygen species (ROS) and this is associated with skeletal muscle damage and a subsequent decrease in physical performance (Chevion et al., 2003; Close et al., 2004; Radak et al., 1999). Prolonged exercise leads to the production of ROS by the mitochondrial electron transport chain in muscle cells through an increase in oxygen consumption, and also xanthine oxidase is activated via ischemia-reperfusion process during exercise (Aoi et al., 2004). Furthermore,

prolonged exercise is partly related to information via phagocyte infiltration caused by ROS (Aoi et al., 2004). In other words, there are other secondary sources of free radical such as the release of radicals by macrophages recruited to repair damage tissue (Close et al., 2005; Urso et al., 2003; Powers et al., 2005). The balance between free radical generation and antioxidant activity in animal cells is critical to pathogenesis of oxidative stress-related disorders. Free radicals can cause damage by lipid peroxidation of unsaturated fatty acids in the muscle membrane. They can also cause oxidative damage to DNA and proteins.

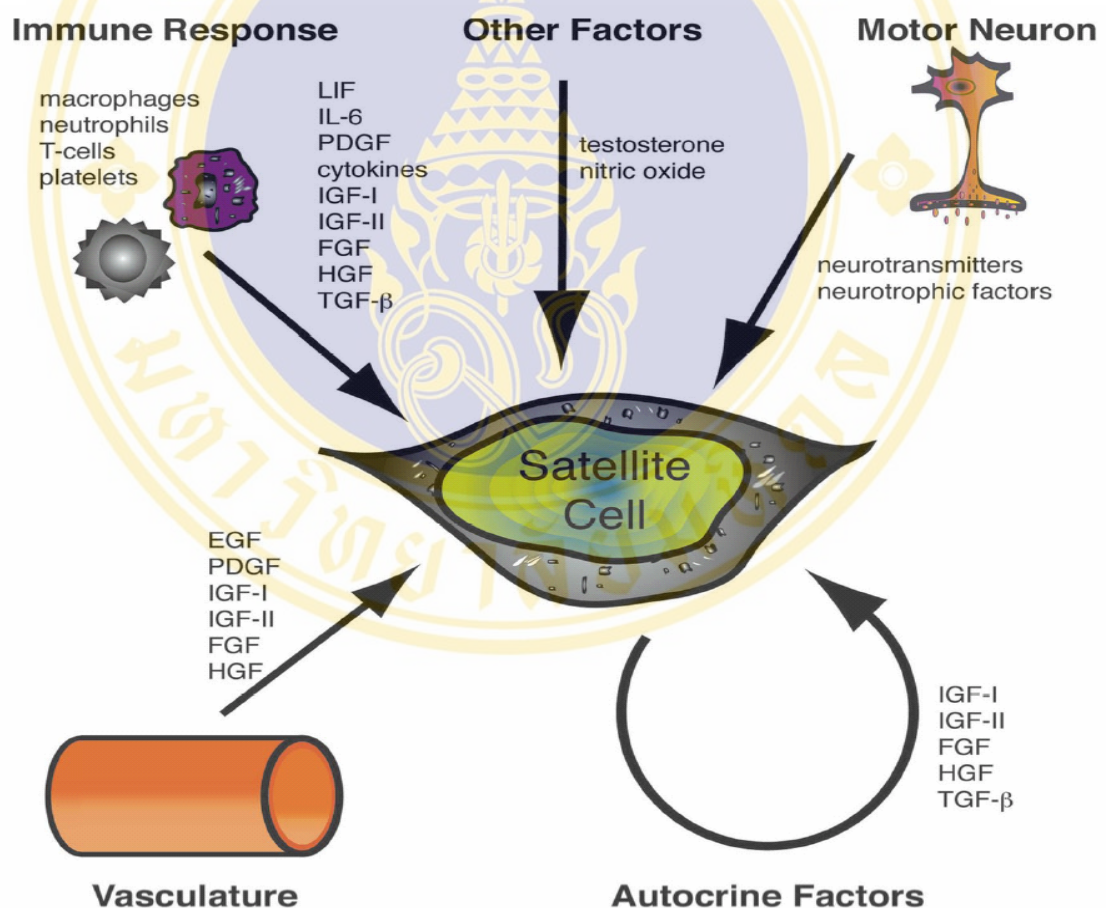


Figure 3 Factors modulate satellite cell activity (Hawke and Garry, 2001).

Several investigators suggested that ROS might be part of the indispensable physiological mediators associated with the NF- κ B activation cascade (Chung et al., 2002). NF- κ B has been shown to play a key role in the expression of many genes that

are central to inflammatory process. Once activated, NF- κ B increases transcription of pro-inflammatory molecules such as tumor necrosis factor (TNF $_{\alpha}$ and TNF $_{\beta}$) and enzymes like iNOS and COX-2. Activated NF- κ B has been detected in a variety of inflammatory setting in situ, including in atherosclerotic and restenotic lesions, in septicemia in humans, in rheumatoid synovium, and in UV-damaged skin. Some of the NF- κ B-induced proteins are known to act as potent NF- κ B activators that can generate an auto-activating loop. As a consequence, more inflammatory mediators are synthesized and the chronic inflammatory conditions would prevail (Chung et al., 2002).

Sex influences on muscle damage and membrane disruption

There are sex-related differences in muscles during prolonged intense activity leading to fatigue, where female muscles have been found to be more fatigue resistant and to recover faster than male muscles (Tiidus., 2001). Interestingly, female rat muscles show fewer histopathological changes after repeated eccentric contractions than male muscles. Moreover, compared with normal females, male and ovariectomized female rats exhibit higher indexes of exercise-induced muscle membrane damage and increased stress protein expression, and this difference disappears after estradiol treatment. The mechanisms behind these sex-related differences in skeletal muscle are not known, but it appears that they are a consequence of different sex hormonal status.

Recent human studies also reported that women have attenuated serum creatine kinase activities after eccentric exercise-induced muscle damage compared with men. Serum creatine kinase activity, although weakly related to actual cytoskeletal muscle disruption, is a relatively good indicator of muscle membrane stability and exercise-induced muscle membrane disruption. Therefore, such studies strongly suggest that females have greater protection than males against muscle membrane disruption consequent to intense exercise as a result primarily of circulating oestrogen levels. This factor may also have implications for sex differences in the post-muscle damage inflammatory response.

Relatively fewer studies have reported on sex differences in post-exercise muscle cytoskeletal and sarcomere disruption. Amelink et al. (1991) found lower post-exercise muscle damage in vitamin E-deprived female rats than in vitamin E-deprived males. A more recent study by Komulainen et al. (1999) also reported that in male rats' disruption of the muscular micro-architecture and histopathological changes reflecting losses of dystrophin and desmin, the appearance of disorganized actin and fibre swelling occurred to a greater extent and at an earlier time course than in females. This histological evidence was further supported by similarly higher postexercise muscle activities of the lysosomal enzyme β -glucuronidase in male versus female animals. Muscle β -glucuronidase activity has previously been correlated with the extent of exercise-induced muscle damage. Roth et al. (2000) recently found greater evidence of ultrastructural muscle damage in older than younger women after a 9 week strength training programme, whereas no such differences existed between men of comparable ages. They suggested that these differences in training-induced muscle damage between younger and older women may be partly due to the lower levels of oestrogen found in older post-menopausal women.

Estrogen and Muscle Damage

Estrogens are a group of 18-carbon steroids secreted primarily by the ovary and, to a lesser extent, the adrenals in females, and in smaller quantities from the testes and adrenals in males (Bunt J, 1990). The term estrogen refers to three structurally similar steroid hormones, estradiol-17 β (E2), estrone (E1) and estriol (E3). Of these, E2 is the primary estrogen in humans and the one with the greatest estrogenic properties, and as such is studied in the majority of investigations (Kendall and Eston., 2002).

Estrogen and Muscle Function

The effects of the female sex hormone estrogen on skeletal muscle are not completely understood. Muscle force-generating capacity has been shown to increase (Suzuki and Yamamuro., 1985), decrease (Warren et al., 1996), and not change (McCormick et al., 2004) after the removal of ovarian hormones from growing, immature rodents. Furthermore, estradiol replacement reversed the changes in force generation in those studies, suggesting that estrogen was the ovarian hormone

responsible for the initial changes after ovariectomy. As the ages of rodents used in those studies are equivalent to adolescent or younger in humans, this might illustrate the complex and critical roles of ovarian hormones during muscle development. More recently, Moran et al (2005) investigated the effects of ovarian hormones on intact skeletal muscle contractile function using mature rodents. They found that the loss of ovarian hormones has detrimental effects on skeletal muscle force-generating capacities. This is partly due to the altered actin-myosin interaction. In humans, there are conflicting reports which indicate that estrogen may or may not influence the maintenance of muscle mass and quality in terms of strength in women who are undergoing hormonal decline (Bassy et al., 1996; Bemben and Langdon., 2002; Seeley et al., 1995; Widrick et al., 2003). In one study, Kurina et al (2004) followed 563 middle-aged women for 3 years and observed that those who became post-menopausal lost an average of grip strength compared with woman remained pre-menopausal. Thus, estrogen seems influence the recovery of skeletal mass and function.

Estrogen as an Antioxidant

It has been demonstrated *in vitro* and *in vivo* in both rat and human investigations, that estrogen (at physiological and supraphysiological concentrations) possesses a potent antioxidant characteristic, although the mechanisms by which estrogen acts as an antioxidant have not been fully determined. Estrogens possess a hydroxyl group on their A (phenolic) ring, in the same configuration and position as tocopherol (vitamin E) [known to possess a strong antioxidant capacity] and similar to thyroxine, which also possesses potent antioxidant activity. Estrogen may donate hydrogen atoms from the phenolic hydroxyl group, thus terminating peroxidation chain reactions, in similar-manner as tocopherol (Kendall and Eston., 2002).

Estrogen and Membrane Stabilization

Estrogen is believed to have membrane stabilizing characteristics, due to its figuration and antioxidant capacity. It has been suggested that estrogen may protect membranes from peroxidative damage by decreasing membrane fluidity and increasing membrane stability in ways similar to cholesterol. Estrogen is a fat-soluble hormone and this type of stabilization involves an interaction between membrane

phospholipids and estrogen in ways similar to the stabilizing mechanisms of tocopherol and cholesterol. As steroid hormones are lipophilic, they intercalate into the bilayer of the cell plasma membrane, potentially altering the fluidity and function of the membrane (Kendall and Eston., 2002).

Estrogen and Gene Regulation

Pro-inflammatory cytokines, such as IL-6 and TNF α have been shown to increase during the muscle damage and repair cycle. Nuclear factor kappa B is known to regulate gene expression involving various cytokines and cell adhesion molecules and it has been shown that tocopherol inhibits the activation of this factor (Kendall and Eston., 2002).

Estrogen and Inflammatory Response

Tiidus and Bombardier (1999) measured post-exercise tissue myeloperoxidase activity in male and female rats which were treated (40 μ g/kg bodyweight) and untreated with estrogen. Their results suggested that estrogen might significantly affect post-exercise leucocyte infiltration into skeletal muscle. However, the mechanism by which this may have occurred is difficult to determine, as the control of infiltration by neutrophils and macrophages is complex. Several factors can influence these processes including calcium homeostasis and calpain production, cytokines, oxygen free radical activity and prostaglandin E₂.

Nevertheless, Stupka et al (2000) reported that the possible difference in the extent of muscle damage between males and females is due to differences in the inflammatory response but not the differences in sarcomere damage. They demonstrated that following an eccentric protocol, women showed less muscle inflammation compared with men despite the same amount of z-line streaming. This may suggest that female muscle may be compromised in terms of regeneration, despite experiencing a similar amount of initial damage.

St Pierre Schneider et al (1996) investigated the time course and concentration of leucocyte invasion in injured soleus muscles of male and female mice, to determine if any gender differences existed. They found that estrogen prevented elevated macrophage concentrations in blood vessels by limiting the availability of endothelial

cell adhesion molecules. This suggests that estrogen could reduce macrophage or other leukocyte emigration into inflamed tissue. As a result, the removal of damaged myofibre is slower in female mice.



CHAPTER III

MATERIALS AND METHODS

A. Chemicals

All chemicals used for myeloperoxidase (MPO) activity assay, 17- β estradiol, protease cocktails inhibitor, and Krebs-Heseleit buffer were purchased from Sigma Chemical Co. (St. Louis., Mo., U.S.A.). The Bicinchoninic Acid (BCA) protein assay which was used to quantify protein content was obtained from Pierce (Rockford., U.S.A.). Creatine kinase (CK) activity and cyclo-oxygenase activity assay kits were obtained from Abbott Laboratories and Cayman, U.S.A., respectively.

For satellite cell identification study, MyoD primary antibody was purchased from Santa Cruz Biotechnology., (California., U.S.A.) whereas Alexa-Fluor 568 and Tropo3 secondary antibodies were obtained from Jackson Laboratory (Bar Harbor., U.S.A.).

B. Animals

Adult female Sprague Dawley rats (8 wks old) were obtained from the National Laboratory Animal Centre of Thailand, Salaya, Nakhon Pratom. The animals were housed individually in a stainless cage shoebox in a temperature-controlled environment (23-25°C) with a 12 h light/dark cycle at Laboratory Animal Husbandry Unit, Faculty of Science, Mahidol University. The animals were given free access to food and water *ad libitum*. All animal experiments were performed in accordance with the animal use and care guidelines established by the Faculty of Science Ethics Committee on the Use of Experimental Animals.

C. Exercise Protocol for inducing of muscle injuries

After 2 weeks of ovariectomy, rats were exposed to the rodent treadmill for 5-10 min on two separate occasions to acclimate them to downhill running, while the control (unexercised) animals remained the cages. After 2 weeks of estrogen treatment, all animals including the treated animals were subjected to acutely exercised (four animals at a time) on a motorized rodent treadmill for 90 min using an intermittent protocol (5 min running interspersed with 2 min rest) of downhill running (16° incline) at a speed of 17 m min⁻¹. This protocol has been proven to injure soleus and superficial (white) vastus muscles in rodents and was considered to be non-exhaustive (Amstrong et al., 1983). Immediately, day 3 and day 14 post-exercise, animals were anesthetized with thiopental (30 mg/kg BW, i.p.) and hindlimb muscles were removed. Animals were then euthanized using a lethal dose of thiopental and cardiac incision was done.

D. Blood sampling and biochemical analysis

Blood samples were collected from cardiac puncture into a 2-ml container containing the anticoagulant ethylenediamine tetraacetic acid (EDTA) (Becton-Dickinson Ltd., U.K.). The blood sample was left at room temperature for 15 min and centrifuged at 3,000 × g for 15 min to obtain serum. The serum was then transferred to appropriate containers and stored at -80°C until assay.

D.1 Serum Creatine Kinase (CK) levels

The activity of CK was used as an indicator of skeletal muscle injury. Serum CK activity was determined using creatine kinase assay kits (Cat. # 7D63-20 and 7D63-30., Abbott Laboratories). Briefly, Creatine Kinase (CK), present in samples, catalyzed the transfer of a high energy phosphate group from creatine phosphate to ADP. The ATP produced in this reaction was subsequently used to phosphorylate glucose to produce glucose-6-phosphate (G-6-P) in the presence of hexokinase. G-6-P was then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with the concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduce (NADPH). The rate of NADPH formation was monitored at 340 nm and was propotional to the activity of CK in the

sample. These reactions occurred in the presence of N-acetyl-L-cysteine (NAC), which was present as an enzyme reactivator.

D.2 Assay for myeloperoxidase (MPO) activity

Assay for MPO was performed by spectrophotometrically technique as described by Smith et al (1989). Briefly, Muscle samples were homogenized in 20 mM phosphate buffer (pH 7.4) containing protease inhibitor cocktail (Cat. # P8340 Sigma., USA). Muscle homogenates were then centrifuged at 15,000 g for 15 min at 4°C to pellet the insoluble cellular debris. The supernatant, which contained ~5% of the total MPO activity and >95% of water-soluble hemeproteins (myoglobin and hemoglobin), was discarded. The pellet was then rehomogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. After which, the samples were sonicated (3x10s) at 4°C, frozen in liquid nitrogen and thawed in 40° C water for two times, and the supernatant was kept at -80° C until assay. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of N,N,N',N'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0 per min at 490 nm and 37°C.

E. Determination of contractile properties

E.1 Force measurement

Isometric contractile properties of soleus (SOL) were determined as previously described by Felice et al (2004) with some modifications. All animals, regardless of group assignment, were anesthetized with an intraperitoneal injection of thiopental (30 mg/kg BW) with supplemental dose given as required. Once a surgical plane of anesthesia is reached, as determined by the lack of withdraw from the pain reflex test, SOL muscle from one leg were quickly removed and placed into ice-cold Krebs-Henseleit Buffer bubbled with 5% CO₂ and 95% O₂ for contractile experiment. The proximal and distal tendon of each muscle was securely tied with a surgical silk thread (3-0, Pearsalls Sutures, Somerset, UK). One end was securely anchored to a fixed clamp, while the other was tied to the force transducer (FT-03, Grass, Rhode Island, USA). Each muscle was placed into an organ bath containing Krebs Henseleit Buffer equilibrated with 5% CO₂ and 95% O₂ and thermostatically maintained at 32-33°C.

After 15-min equilibration, the muscle was stimulated by two platinum-plate electrodes flanked the length of the muscle preparation. Tension was recorded using the PowerLab 400 (Castle Hill., NSW., Australia). The optimal muscle length for contraction (L_o) was determined by stimulating the muscle to produce an isometric twitch response and adjusting muscle length to produce a maximum isometric twitch force (P_i). All stimulations were delivered by using a Grass-S48 stimulator with isolation unit (Grass Instruments, Quincy, MA).

E.2 Frequency-Force Relationship

The frequency-force relationship of the muscle was determined by stimulating an isolated soleus muscle at various frequencies of 20, 40, 60, 80, 100, and 120 Hz, with a 2-min rest period between each stimulus to prevent fatigue. The maximal isometric tetanic force (P_o) of the SOL muscle was determined from the plateau of the frequency-force relationship. Muscle cross-sectional area (CSA) was calculated as muscle mass (g)/ muscle length (cm) / muscle density 1.06 g/m^3 . Specific tension (sP_o) was expressed as the maximum force per CSA of the muscle (N/cm^2). Immediately after the functional measurements, the muscles were carefully blotted on filter paper, weighed and stored for immunohistological studies.

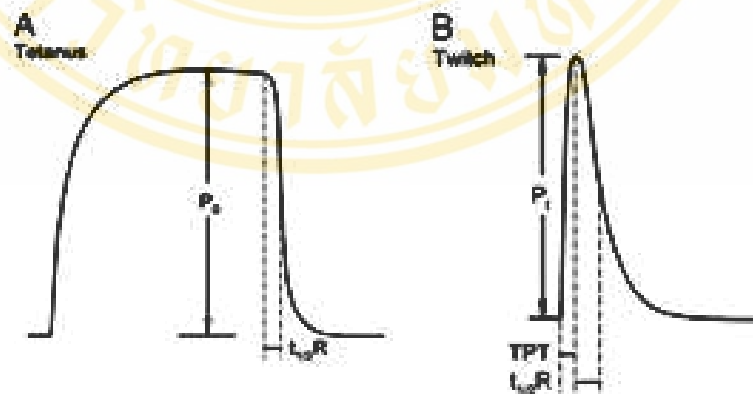


Figure 4 A representative tracing of peak tetanic tension and single twitch response.

F. Studies of histomorphology and histochemistry

F.1 Muscle histology

SOL muscles from the contralateral leg were fixed in neutral buffered formaldehyde (4% paraformaldehyde) for 12-18 h and then transferred to 70% ethanol before paraffin embedding. Transverse sections (6 μm thick) were cut at the midbelly of soleus muscles and mounted serially onto adherent-coated slides. The sections were then deparaffinized in xylene and ethanol, and stained with hematoxylin and eosin (H&E) for morphological analysis, cover slipped in aqueous mounting medium, and examined using a standard light microscope.

F.2 Satellite cells identification using histochemistry

Myogenic satellite cells were identified by immunofluorescence staining using a primary antibody to MyoD, an *in vivo* marker of activated and/or proliferated satellite cells. In brief, 6- μm paraffin-embedded sections were deparaffinized in xylene (x3) and graded ethanol (100% ETOH x2, 95% ETOH x2, 80% ETOH x1, and 70% ETOH x1). Antigen retrievals were accomplished by subjecting deparaffinized sections to water bathing at a gentle boil in sodium citrate buffer, pH 6.0, for 10-12 minutes, followed by equilibration to room temperature for a minimum of 20 minutes. The sections were then incubated in 1 M glycine for 3 h, blocked with 4% Bovine Serum Albumin + 1% Normal Goated Serum for 1 h, and incubated overnight at 4 °C in humidifying chamber with rabbit anti-MyoD antibody (1:100) in 0.1M PBS containing 10% normal goat serum and 1% Triton X-100. After which, the sections were incubated with Alexa-Fluor 568 secondary antibody (1:200) in 5% normal goat serum, 0.1% Triton X-100 in 0.1M PBS for 1 h at room temperature. Sections were then be counterstained with Tropo-3 (1:500) for the identification of myonuclei for 1 h. Sufficient washes were performed in phosphate-buffered saline (PBS) containing 1% Tween-20, pH 7.4. Immunofluorescence slides were stored at -20 °C in dark chamber until use. Imaging was taken using a digital camera Axiocam HRc with Carl Zeiss Axioskop 40 or the Nikon Eclipse E800 microscopes. Axiovision 3.1 software program was used to record the images, including the co-staining overlay images.

The number of muscle cells expressing a particular protein during the course of regeneration was examined on sections by counting the number of positive nuclei staining for MyoD. The systematic random fields for counting were selected with the magnification of x400 (x10 ocular and x40 objective lens). Four to five fields with positive staining (positive nuclei) were accepted as lesion area and were included in the analysis. The number of nuclei in cells with positive staining were compared with the total number of nuclei in the counted fields to give an estimation of the number of cells expressing the proteins at the time points investigated. The relative number of myofiber positive for the marker was calculated as number of positive fibers/total myoneuclei x 100.

F.3 Assay for cyclooxygenase activity

Muscle was homogenized in an ice-cold lysis buffer (0.1 M Tris-HCl, pH 7.8, containing 1 mM EDTA and a protease inhibitor cocktail and centrifuged at 15,000xg for 15 min at 4°C. Then, the supernatant was removed for the assay. The peroxidase activity of COX-2 was measured by using a commercially available kit. The samples were incubated with and without COX-2 inhibitor (DuP-697). The COX-2 activity was measured colorimetrically by monitoring the oxidation of N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) at the wavelength of 590 nm. COX-2 activity was calculated from the following formula;

$$\text{Total COX activity} = \frac{\Delta A_{590} / 5 \text{ min.} \times 0.21 \text{ ml (Total volume)}}{0.00826 \mu\text{M}^{-1} \times 0.01 \text{ ml (Sample volume)}} \div 2^*$$

$$= \text{nmol/min/ml (U/ml)}$$

* It takes two molecules of TMPD to reduce PGG₂ to PGH₂

ΔA_{590} : Average background OD value-Average sample OD value

The reaction rate at 590 nm was determined using the TMPD extinction coefficient of 0.00826 μM^{-1} .

G. Experimental protocols

G.1 Determination the effect of ovariectomy and estrogen supplementation on the contractile function and muscle damage in the exercise-induced muscle injury.

It has been reported that estrogen deprivation could augment the level of the injury in skeletal muscle and reduce in functional recovery after injury. These effects, however, were reversed or attenuated by estrogen supplementation. In this experiment, we designed to determine the contractile properties to indicate the functional capacity level during the recovery time periods at immediately after, 3 and 14 days post-exercise induced injury. In addition, we determined the levels of biochemical markers of muscle damage which were activities of enzymes creatine kinase and myeloperoxidase activities. They both were accumulated after injury in blood and soleus muscles.

Following one week of acclimation, animals were randomly assigned to sham unexercised (SHAMC), sham exercised (SHAME), ovariectomized non-exercised (OVXC), ovariectomized + exercise (OVXE), ovariectomized + exercise+ estrogen supplement (OVXES). These animals, except for the sham, were undergone ovariectomy to remove the major source of endogenous estrogen, whereas animals in the control groups were left intact. These OVX rats were rest for two weeks post-operation for recovery from surgical operation and were pair-fed to amount of food consumed by sham control to minimize confounding factors that might be attributed to differences in food intake. During this period, the rats were carefully monitored and handled daily. Following one day post-operation, the animals in OVX-ES rats were treated with estrogen (17β -estradiol, 2.5 $\mu\text{g}/\text{kg BW}$) by subcutaneous injection every the other day for 2 weeks. All animals were exposed to the rodent treadmill for 5-10 min on two separate occasions to acclimate them to downhill running. After 2 weeks post-estrogen treatment, the animals in OVX-E and OVX-ES groups were acutely exercised (four animals at a time) on a motorized rodent treadmill. Immediately after, and at day 3 or 14 days post-exercise, animals were anesthetized and hind limb muscles were removed for contractile properties measurement and biochemical assay of MPO activity. At the end of experiment, blood samples were collected from cardiac puncture for CK assay, and the animals were killed by cardiac incision (Figure 5).

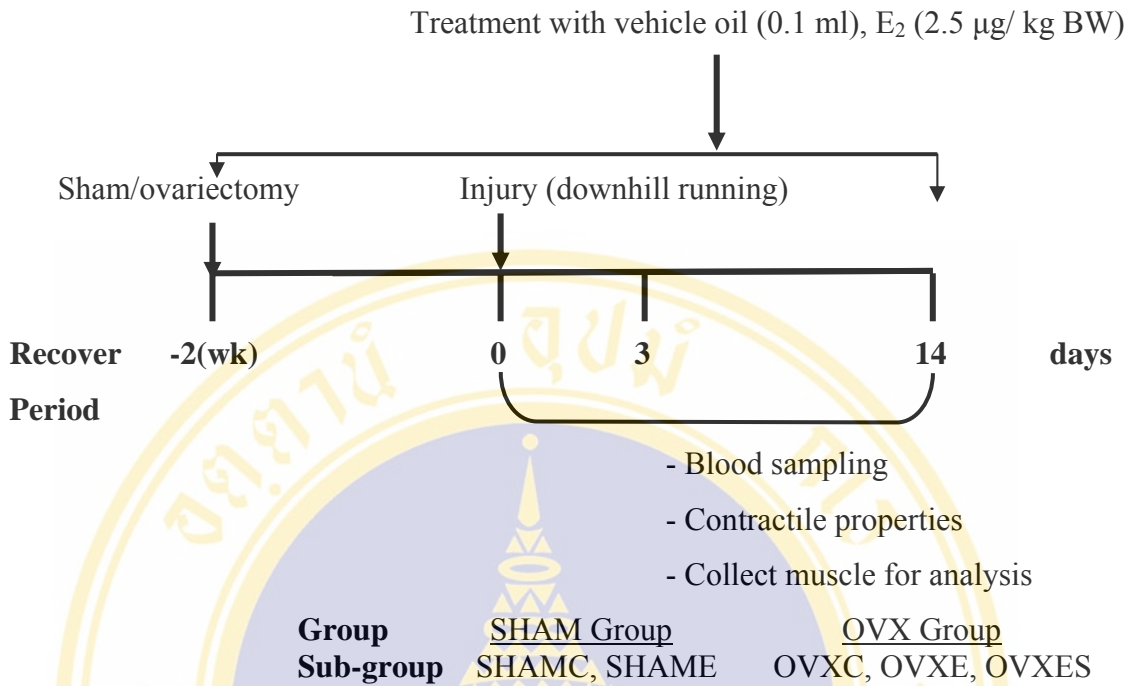


Figure 5 Schematic diagram showing the experimental protocol an samples collection.

G.2 Effect of ovariectomy and estrogen supplementation on satellite cells activation.

Skeletal muscle is a post-mitotic tissue. Therefore, the growth of skeletal muscle fiber during regeneration process is primarily dependent on satellite cells function. This experiment was designed to determine changes in satellite cell after injury at immediately after, and either at 3 or 14 days post-injury.

Following one week of acclimation, animals were randomly assigned in to different group and received treatment similar to those described in experiment G.1. At the end of experiment, hind limb muscles were removed and fixed in neutral buffered formaldehyde (4% paraformaldehyde) for 12-18 h and then embedded with paraffin for histological analysis and satellite cell identification.

G.3 Determination whether the estrogenic-dependent effect on satellite cells activation mediates via the COX-2 inflammatory pathway.

This experiment was designed to measure the levels of COX-2 activity in rat skeletal muscle using enzymatic analysis.

Experimental protocols were those similar as the described in experiment G.1. At the end of experiment, hind limb muscles were removed for COX-2 activity assay. (Figure 5).

H. Statistical analysis

Data were expressed as mean \pm SEM. Data analysis of group differences were accomplished via one way analysis of variance (ANOVA), followed by Tukey's *post-hoc* tests (GraphPad Prism 3.0; GraphPad Software, San Diego, CA, USA) when a significant ($P < 0.05$) difference between groups was noted.

CHAPTER 4

RESULTS

In the study, we examined the effect of estrogen on skeletal muscle injury which was induced by exercise, and the ability to repair. We hypothesized that estrogen deprivation (i.e. ovariectomy) would augment the level of damage in skeletal muscle and also delayed the functional recovery following exercise. These effects would be attenuated or restored by estrogen supplementation.

Effects on body weight, muscle mass, and uterine weight.

Figure 6-9 showed changes in body weights, SOL and EDL mass, and uterine weight in all experimental groups. The initial body weights of all experimental groups are comparable. They were significantly increased with time throughout the experimental period. Specifically, OVXE showed significantly increased in the final body weight when compared to the any of other groups. The final body weight of estrogen-treated animals (OVXES), however, was less than that of the OVX groups at 14 days of recovery (Fig. 6) in spite of having similar food intake (14g/day).

In the present study, the final uterine weight was used an indirect indicator of successful OVX and estrogenic activity (Branham et al., 1993, Berchtold et al., 2001). Our data showed that there was a significant decreased in uterine weight ($p < 0.01$) in OVX groups when compared to the control groups (Fig. 9). The SOL and EDL muscle wet weight were normalized and expressed in relative to body weight. Although ovariectomy caused an increase in body weight, the ratio of muscle weight to body weight in both SOL and EDL muscles were unaffected by either ovariectomy or exercise (Fig. 7-8). Estrogen supplement had also no effect on this ratio in both muscle examined immediately after, and at day 3 and 14 post injury.

Effect on contractile function and muscle damage after exercise-induced muscle injury.

Effects on contractile properties.

Twitch Kinetics

In this study, the *in vitro* contractile properties of soleus muscle including peak force, contraction time and one-half relaxation time were determined *in vitro* (Fig. 10-13). Twitch kinetics were compared following downhill running between unexercised and exercised groups. As shown in Fig. 10A, there is a significant reduction in soleus peak twitch tension of the exercised rats when compared to unexercised control rats at immediately post-exercise ($p < 0.05$). The mean value of peak twitch tension was 134.2 ± 6.9 , 114.6 ± 15.0 , 117.8 ± 8.6 , 185.8 ± 19.9 , and 163.7 ± 11.8 mN for OVXE, OVXES, SHAME, OVXC, and SHAMC respectively. These values, however, were gradually restored and return to basal level within 14 days of recovery. No significant differences in peak twitch tension were observed among groups at day 3 and 14 post-exercise (Fig. 10B-C). Similar results were obtained for specific twitch tensions (sP_t) (P_t divided by muscle physiological cross-sectional area) (Fig. 11). The mean values of sP_t in OVXES, SHAME and OVXE were 3.0 ± 0.4 , 2.9 ± 0.1 , and 3.2 ± 0.2 N/cm², respectively, whereas were 4.3 ± 0.3 and 4.5 ± 0.5 N/cm² in SHAMC and OVXC, respectively. In contrast, the contraction time (TPT) and one-half relaxation time ($RT_{1/2}$) were not affected, either by ovariectomy or by exercise at any time points of recovery (Fig. 12-13).

Tetanic Force

There were a combined effect of ovariectomized and exercise on force-generating capacity of soleus muscle. As shown in Fig. 14, immediately post-exercise, the absolute peak of maximum tetanic force of soleus muscles were significantly lower in exercised animals than that in unexercised animals ($p < 0.05$). The mean value of peak tetanic force was reduced by 22.0% for OVXE, by 27.2% for OVXES, and by 20.0% for SHAME when compared to controls. At day 3 post-exercise, these values were gradually restored and completely return to control value within 14 days of recovery, where there was no significant differences in specific P_o (P_o divided by muscle physiological cross-sectional area) between SHAME vs SHAMC and between

OVXES and SHAMC. Similar results were obtained for specific P_o (s P_o) (OVXE 25.4%, OVXES 30.5%, and SHAME 31.5%) (Fig. 15).

Myofibers cross-sectional area

Mean fiber size of soleus muscle was determined by H&E staining on the two consecutive sections and were presented in Fig. 16-17. There was a significant reduction in soleus muscle fiber cross-sectional area in OVXE (28.6 %), OVXES (25.5 %), and SHAME (25.7 %) at immediately post-exercise, as compared with the controls. These values, however, were not different between SHAMC and OVXC. The mean value of soleus muscle fiber size was 1444 ± 96.9 for OVXE, 1505 ± 109.0 for OVXES, and $1502 \pm 56.5 \mu\text{m}^2$ for SHAME, whereas were 2021 ± 130.2 for SHAMC and $1899 \pm 109.5 \mu\text{m}^2$ for OVXC, respectively. The mean muscle fiber cross-sectional areas, however, were not altered from control values at day 3 and 14 post-exercise (Fig. 17B-C).

Table 1 Changes in body weight in all animal groups throughout the experiment. Data were presented as mean \pm SEM, n=8-9/group. * and ** significant difference at $p < 0.05$ and $p < 0.01$, ^{a, b, c, d, e} significant from SHAMC OVXC, OVXE, OVXES, and SHAME group, respectively.

Groups	Initial BW(g) (6wks old)	Final BW (g)		
		0 day post-exercise	3 days post-exercise	14 days post-exercise
SHAMC	176.0 \pm 4.6	215.2 \pm 5.2	211.9 \pm 2.3	249 \pm 8.5
OVXC	174.2 \pm 4.8	220.4 \pm 6.4	223.1 \pm 4.6	276.9 \pm 6.4
OVXE	176.3 \pm 5.5	210.4 \pm 3.3	235.4 \pm 6. ^{3a**,d*,e**}	275.5 \pm 11.1
OVXES	175.6 \pm 4.0	209.1 \pm 1.9	210.3 \pm 2.5	235.3 \pm 5.3 ^{b**,c**}
SHAME	174.3 \pm 5.6	201.8 \pm 1.3 ^{b**}	214.8 \pm 3.5	256.6 \pm 5.5

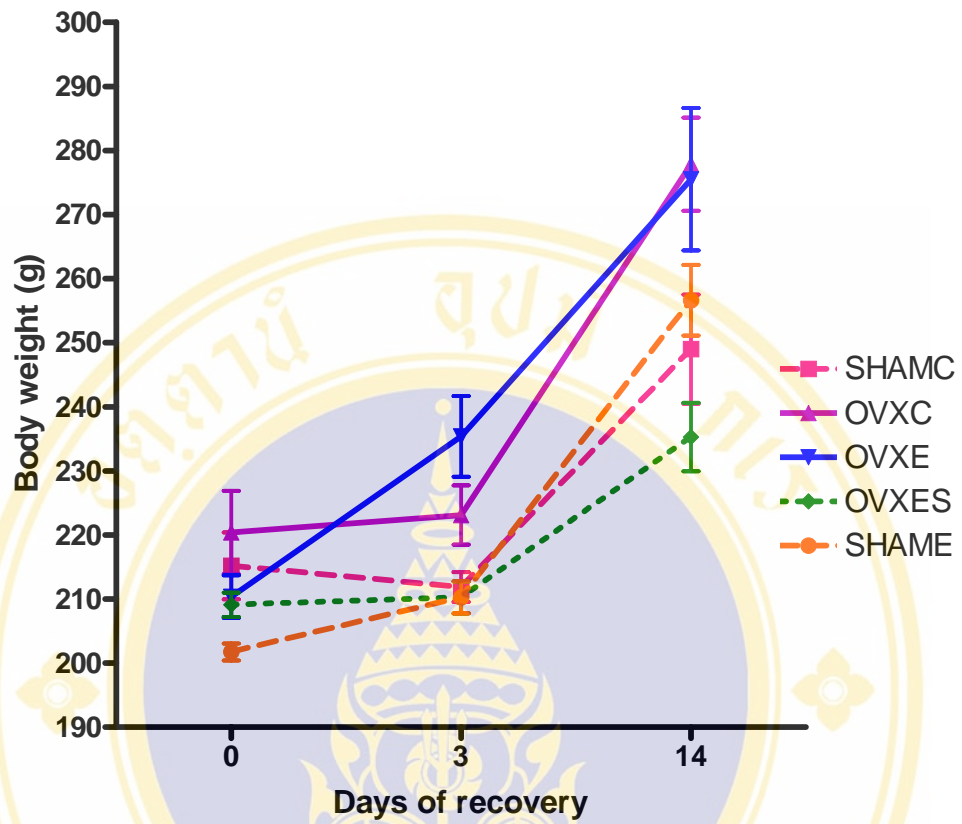
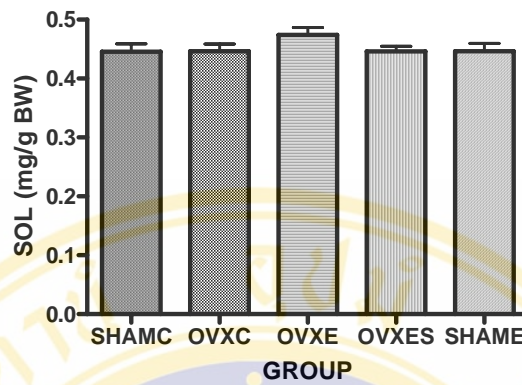


Figure 6 Changes in body weight in all animal groups throughout the experiment. Data were presented as mean \pm SEM, n=8-9/group.

A



B



C

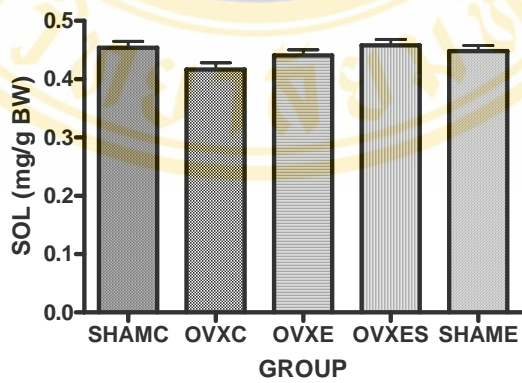
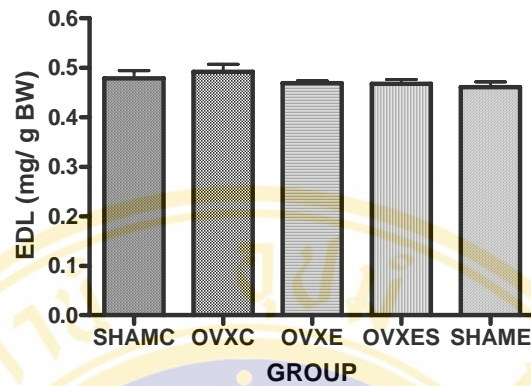


Figure 7 Changes in soleus (SOL) muscle wet weight per body weight ratio (A) at immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean± SEM, n=7-8/group. No significant difference ($p < 0.05$) in soleus mass were observed between groups.

A



B



C

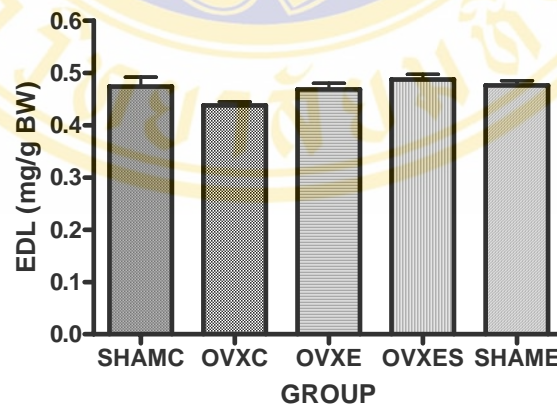
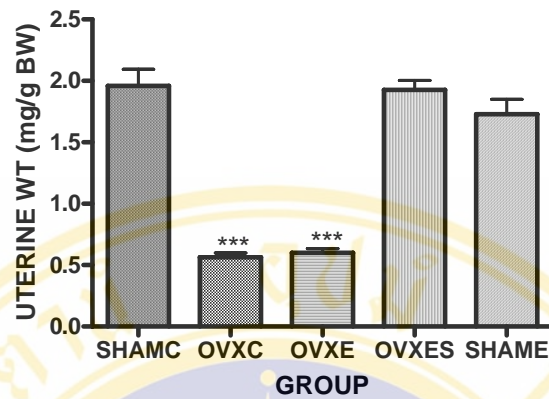


Figure 8 Changes in extensor digitorum longus (EDL) muscle wet weight per body weight ratio (A) at immediately (0-1h) after, (B) at 3 days, and (C) 14 days after injury. Data were presented as mean± SEM, n=8/group. No significant difference ($p < 0.05$) in EDL were observed between groups.

A



B



C

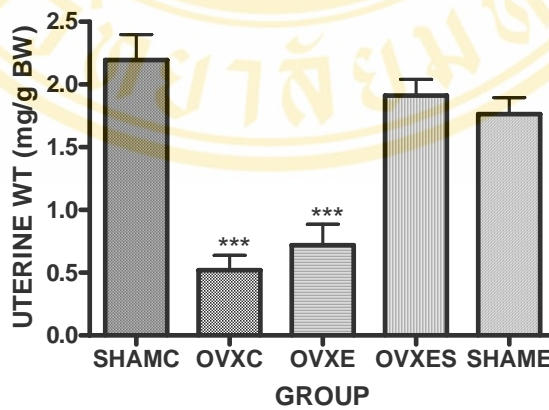
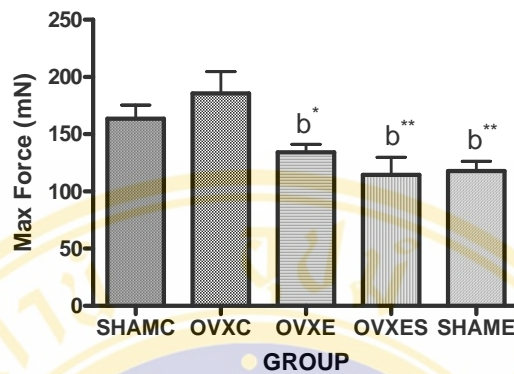
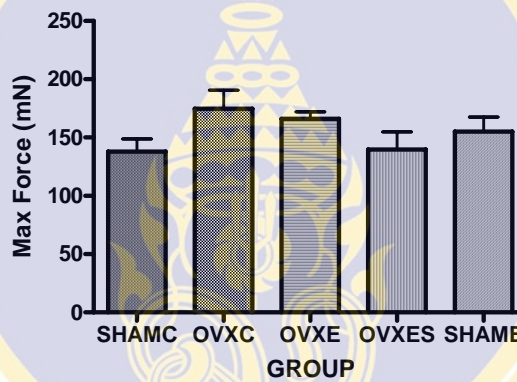


Figure 9 Changes in uterine weight per body weight ratio (A) at immediately (0-1h), (B) at 3 days, and (C) 14 days after injury. Data were presented as mean \pm SEM, n=8/group. *** Significant difference ($p < 0.001$) from control group (SHAM).

A



B



C

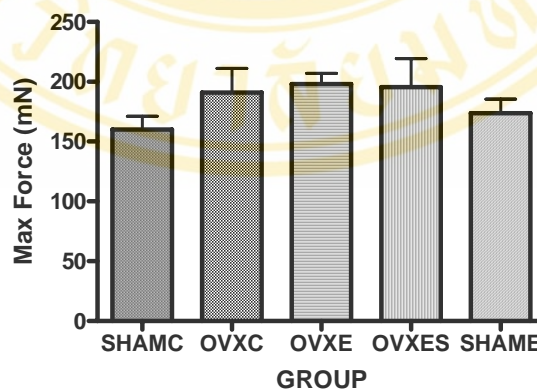
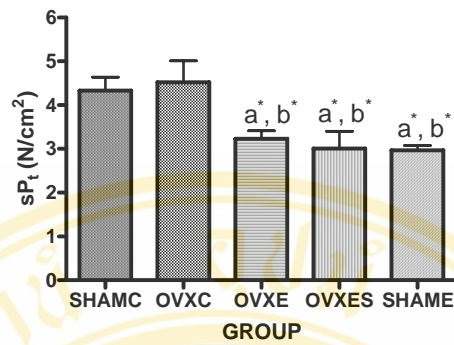


Figure 10 Changes in peak twitch force of soleus muscles at (A) immediately (0-1h) after, (B) at 3 days, and (C) 14 days after injury. Data were presented as mean \pm SEM n=7-8 rat/group. b** Significant difference ($p < 0.01$) compared to OVXC group.

A



B



C

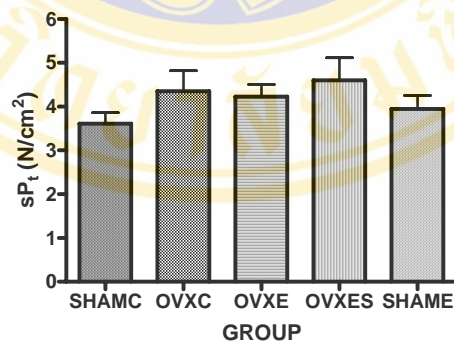
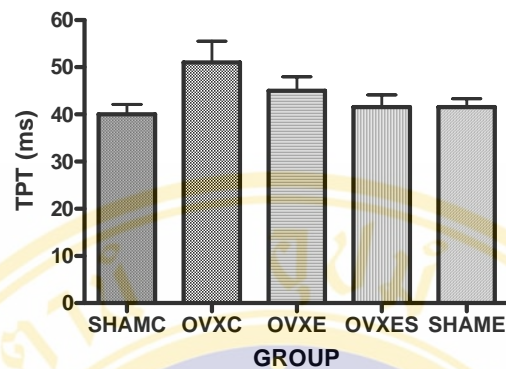
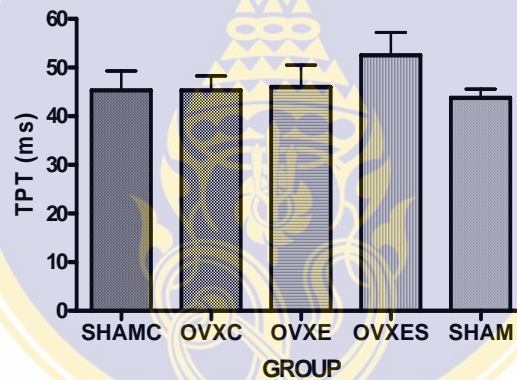


Figure 11 Changes in specific peak twitch tension (sP_t) of soleus muscle at (A) immediately (0-1h) after, (B) at 3 days, and (C) 14 days after injury. Data were presented as mean \pm SEM $n=7-8$ rats/group. * Significant difference at $p<0.05$. a, b significant from SHAMC and OVXC, respectively.

A



B



C

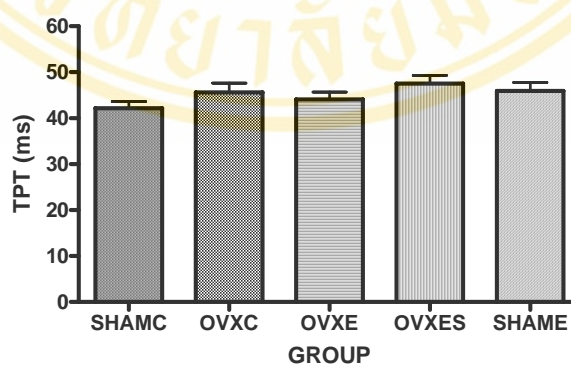
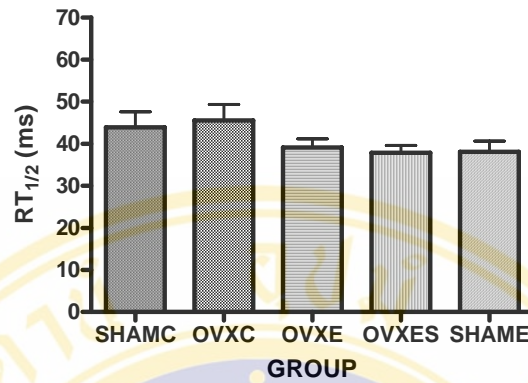
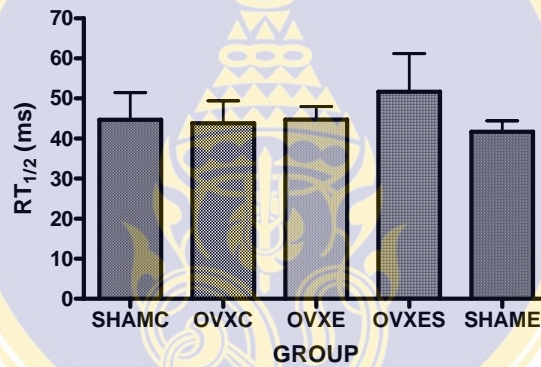


Figure 12 Changes in time to peak tension (TPT) of soleus muscle at (A) immediately (0-1h) after, (B) 3 days, and (C) 14 days after injury. Data were presented as mean \pm SEM n=5-8 rats/group. No significant difference ($p < 0.05$) in TPT were observed between groups.

A



B



C

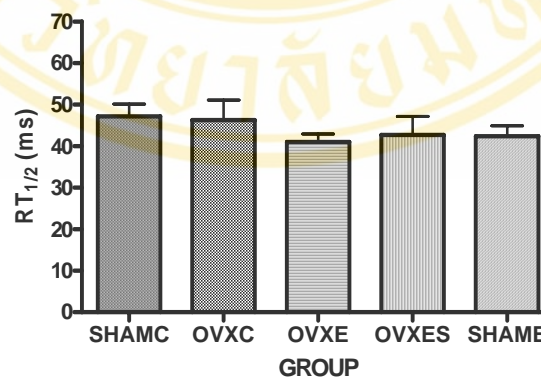
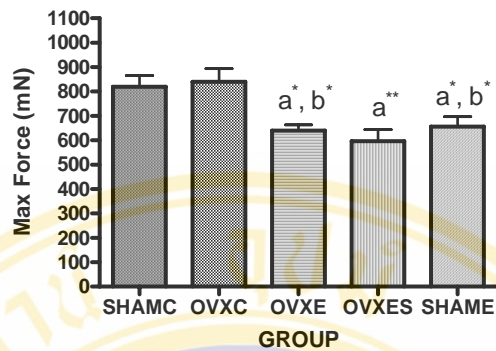
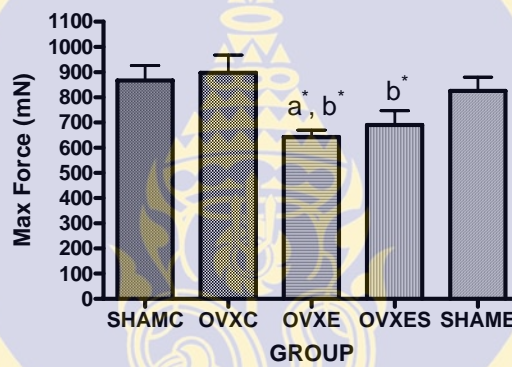


Figure 13 Changes in half relaxation time (RT_{1/2}) of soleus muscle at (A) immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean ± SEM n=5-8 rats/group. No significant difference (p<0.05) in RT_{1/2} were observed between groups.

A



B



C

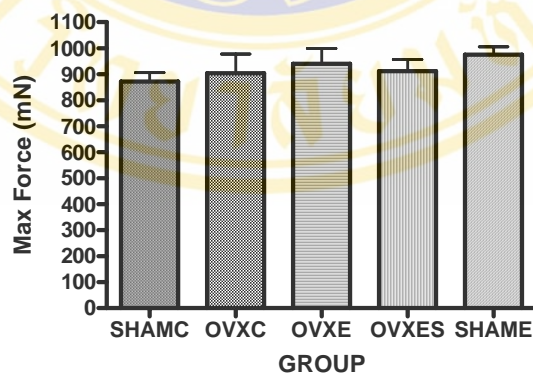
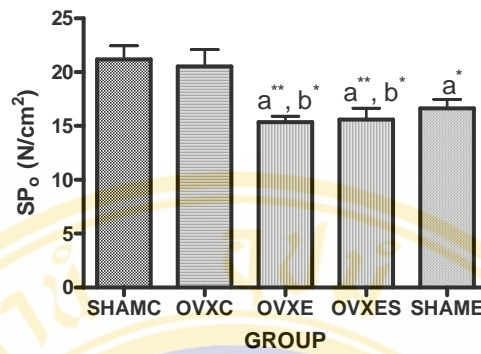
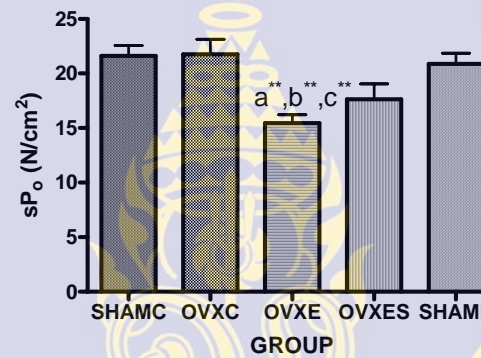


Figure 14 Changes in peak tetanic force of SOL muscles (mN) at (A) immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM n=8 rat/group. *, ** Significant difference at $p < 0.05$ and $p < 0.01$. a, b significant from SHAMC and OVXC, respectively.

A



B



C

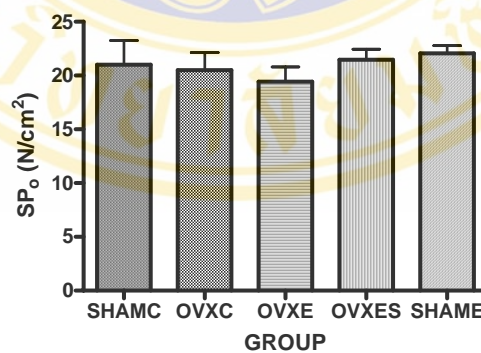
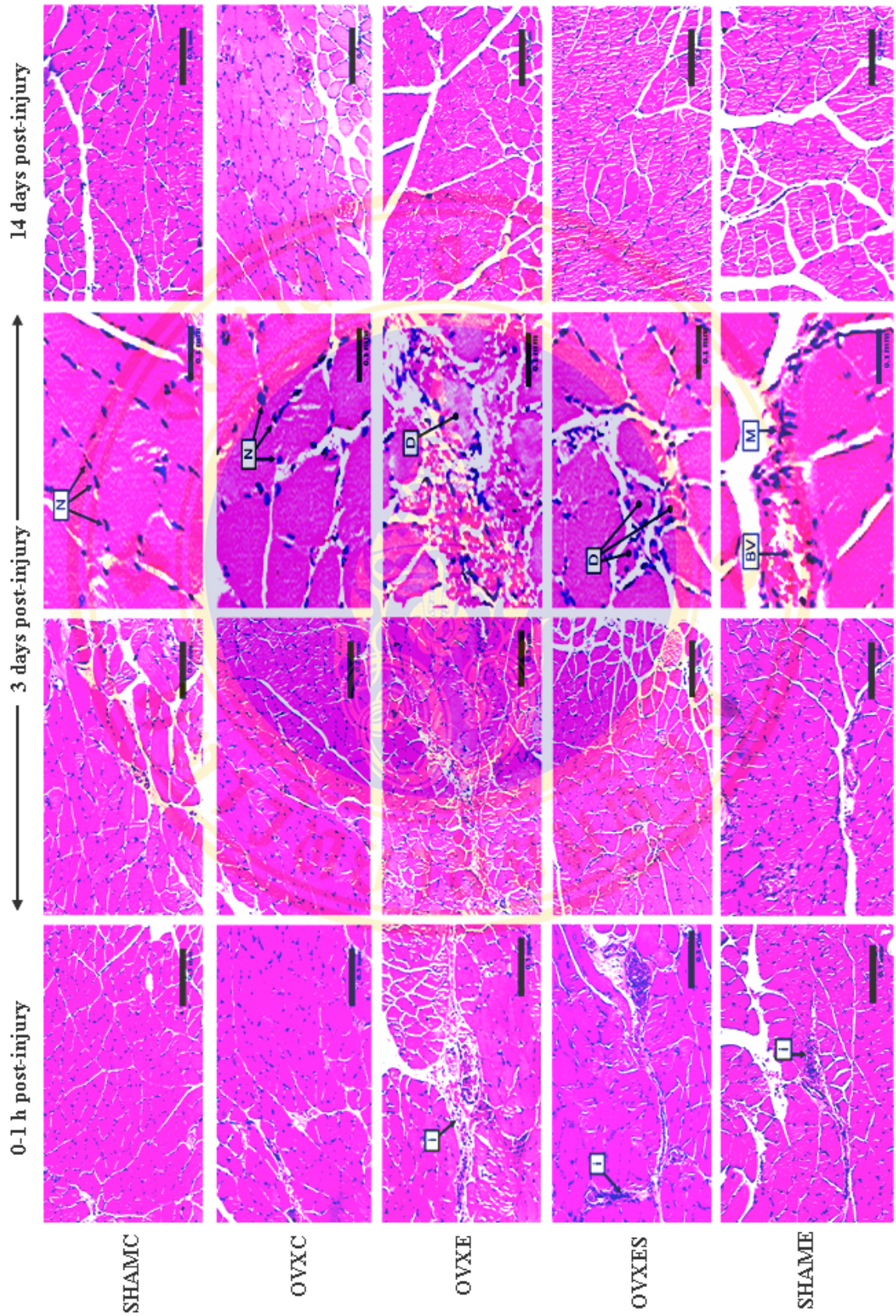


Figure 15 Changes in specific peak tetanic tension (sP₀) of soleus muscle at (A) immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean ± SEM n=8 rat/group. *, ** Significant difference at p<0.05 and p<0.01, a, b, c significant from SHAMC, OVXC, SHAME, respectively.



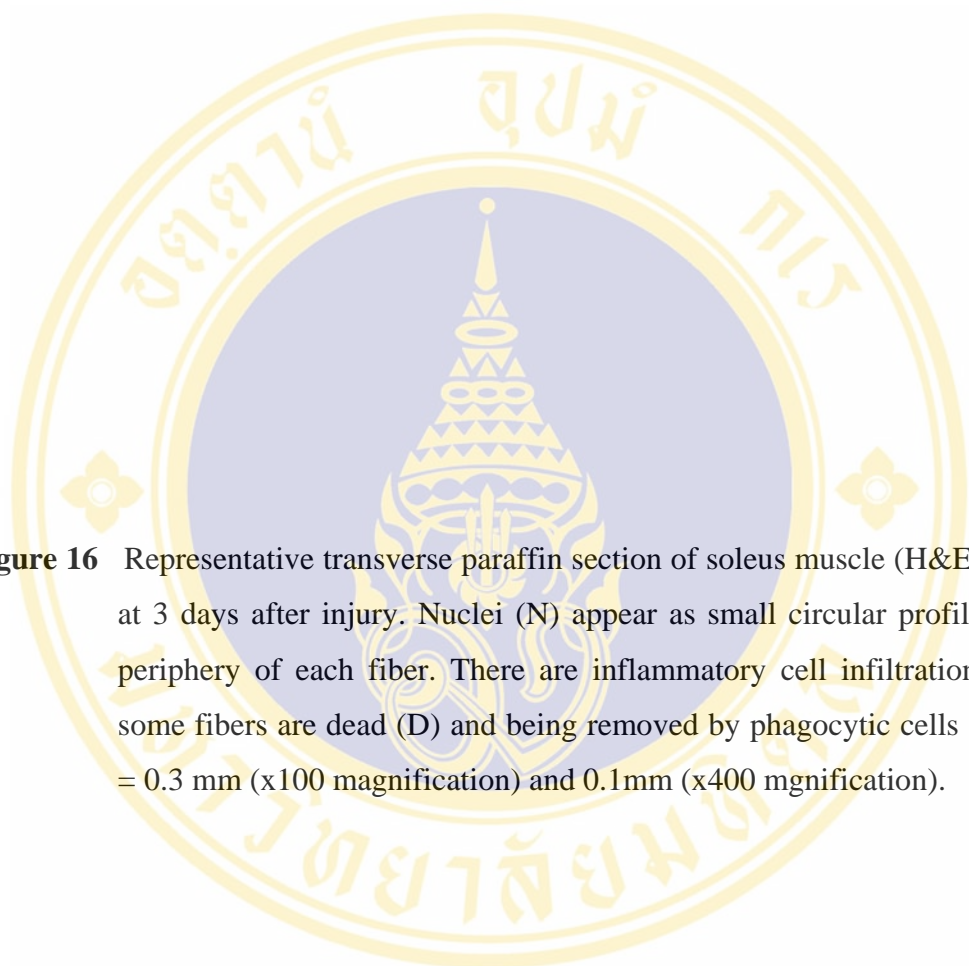
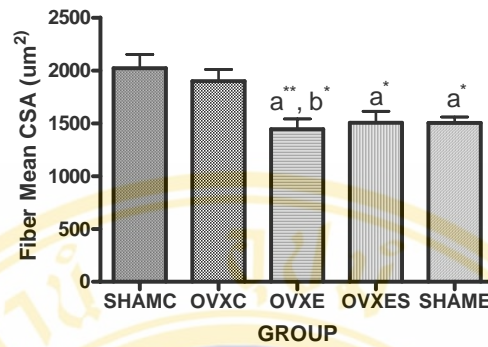
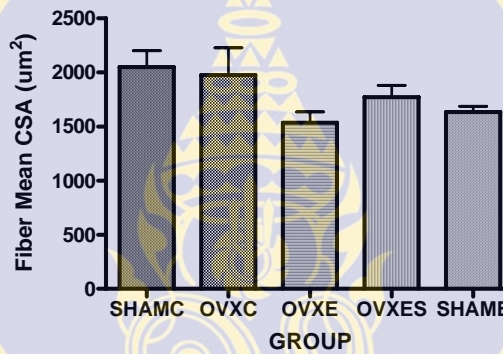


Figure 16 Representative transverse paraffin section of soleus muscle (H&E stained) at 3 days after injury. Nuclei (N) appear as small circular profiles at the periphery of each fiber. There are inflammatory cell infiltration (i) and some fibers are dead (D) and being removed by phagocytic cells (M). Bar = 0.3 mm (x100 magnification) and 0.1mm (x400 magnification).

A



B



C

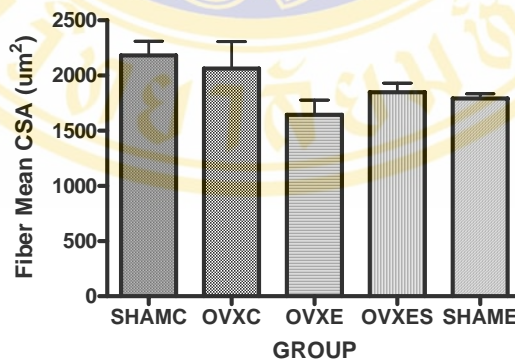


Figure 17 Changes in mean of soleus muscle fiber size. Soleus muscle fiber size at (A) immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM n=5-6 rat/group. *, ** Significant differences at $p < 0.05$, $p < 0.01$. a, b significant from SHAMC and OVXC, respectively.

Effects on muscle damage and inflammation.

Serum Creatine Kinase.

To determine whether muscle damage occurred after downhill running, we measured serum CK enzyme activity, an indirect biomarker of muscle damage, at immediately, 3, and 14 days post-exercise induced muscle injuries. Immediately following downhill running, there was significant elevations ($p < 0.05$) in serum CK activities only in the OVXE (3201.0 ± 499.5 U/L) group when compared to the OVXC (1445.0 ± 66.3 U/L) group (Fig. 18A). Although the level of CK activity was slightly increased in OVXES (2514.0 ± 506.6 U/L) and SHAME (2621.0 ± 424.2 U/L), but did not reach the statistical significance as compared with SHAMC (1849 ± 197.2). CK activity was partially recovered by 3 days of recovery period, and completely restored to control value by 14 days of recovery (Fig. 18B-C).

Myeloperoxidase (MPO) activity

MPO activities, a biomarker of neutrophil accumulation, in soleus muscle from either ovariectomized or sham-operated groups at immediately after, and at 3 and 14 days post-exercise were shown in Fig. 19. MPO activity was significantly elevated ($p < 0.05$) at immediately (Fig. 19A) post-exercise in OVXE (0.18 ± 0.04 U/mg protein) and SHAME (0.20 ± 0.03 U/mg protein) groups as compared with the controls. In contrast, there were no significant differences in MPO activities between SHAMC (0.05 ± 0.00 U/mg protein), OVXC (0.07 ± 0.00 U/mg protein), and OVXES (0.10 ± 0.00 U/mg protein). For all groups, however, MPO activities were returned to the control value within 3 days post-exercise (Fig. 19B-C).

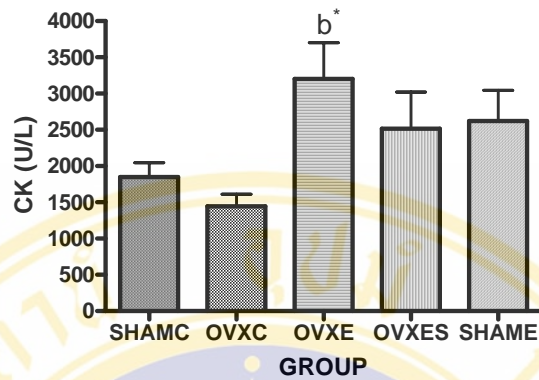
Muscle dry weight and water content

In the present study, downhill running caused a significant alteration in soleus dry weight and water content (Fig. 20-21). Immediately post-exercise, soleus water content from exercised rats (e.g. OVXE, OVXES and SHAME) were significantly higher ($p < 0.001$) than that in unexercised rats (e.g. SHAMC and OVXC) (Fig. 20A). The mean value of soleus water content was 81.3, 80.0, and 79.6% for OVXE, OVXES, and SHAME, respectively, and was 77.0 and 77.8 % for SHAMC and OVXC, respectively. In all groups, these values were restored to control values by 14 days of the recovery (Fig. 20B). Similar to changes in water content, there was an increase ($p < 0.001$) in muscle wet-to-dry weight ratio in all exercised animals at

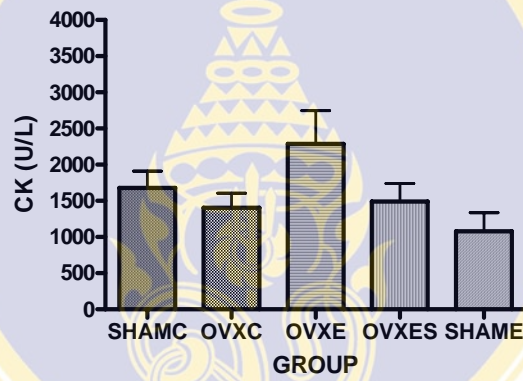
immediately following exercise when compared to unexercised animals (Fig. 21A). The mean values of muscle wet-to-dry weight ratio were 5.4 ± 0.1 for OVXE, 5.2 ± 0.1 for OVXES, and 4.9 ± 0.1 for SHAME, respectively, and were 4.4 ± 0.1 and 4.5 ± 0.1 for SHAMC and OVXC, respectively. These values, however, were restored to control value by 14 days of recovery (Fig. 21B).



A



B



C

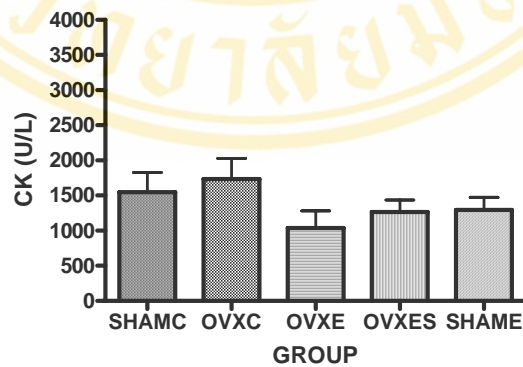
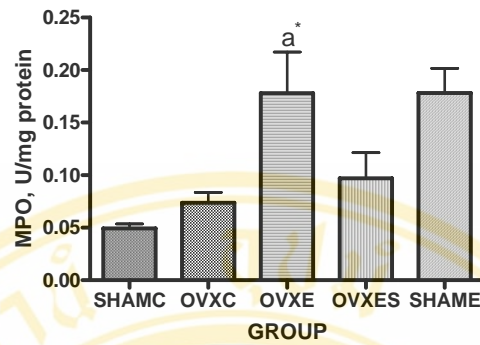
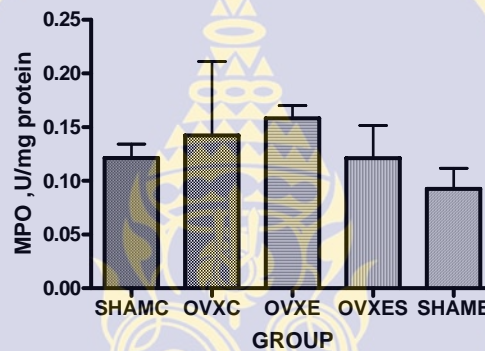


Figure 18 Changes in serum creatine kinase (CK) activity (A) at immediately (0-1h) after injury, and (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM, n=5-6/group. b* Significant difference ($p < 0.05$) from control group (OVXC).

A



B



C

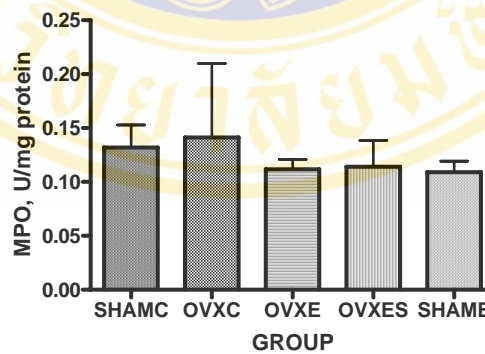
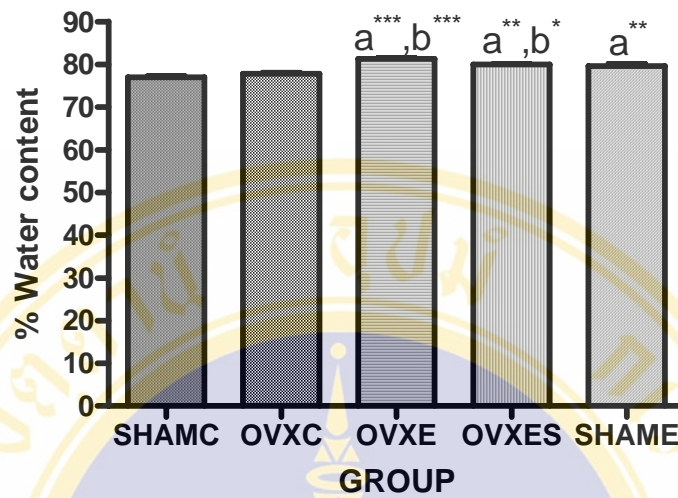


Figure 19 Changes in myeloperoxidase activity (MPO) of SOL muscle (A) at immediately (0-1h) after, (B) and at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM, n=5-6/group. * Significant differences at $p < 0.05$. a, b significant from SHAMC and OVXC, respectively.

A



B

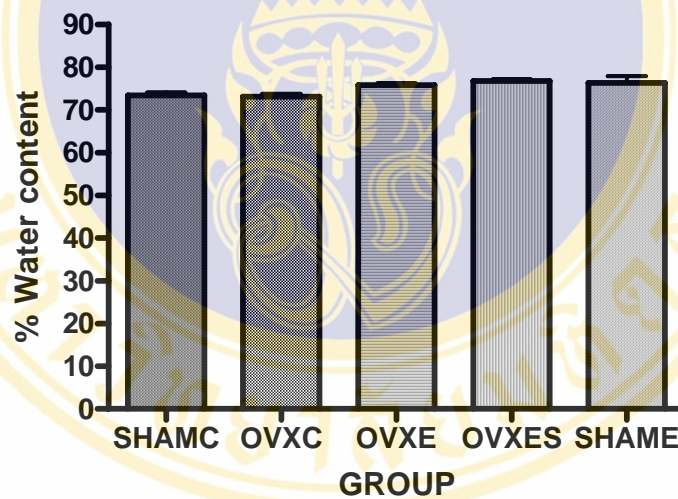
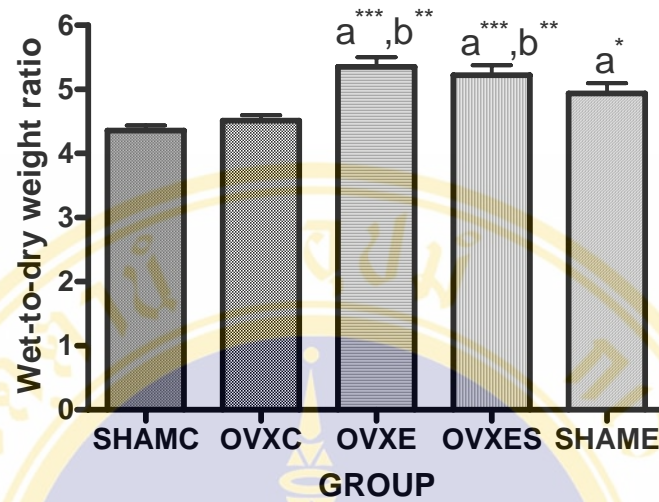


Figure 20 Changes in water content (%) of SOL muscle (A) at immediately (0-1h) after and at (B) 14 days after injury. Data were presented as mean \pm SEM n=5-6 rats/group. *, **, *** Significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. a, b significant from SHAMC and OVXC, respectively.

A



B

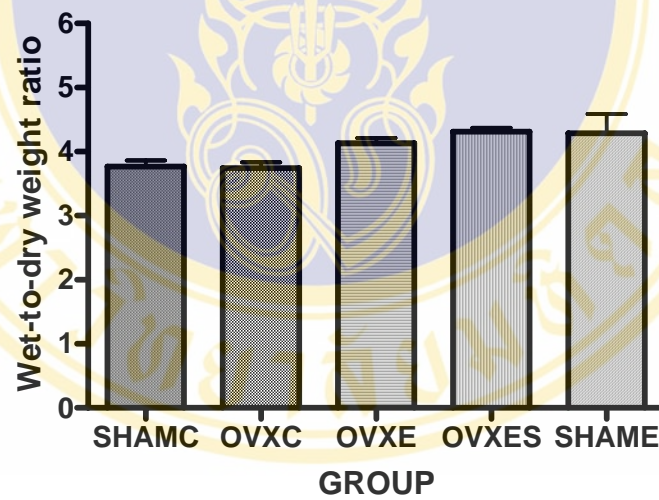


Figure 21 Changes in wet-to-dry weight ratio of SOL muscle (A) at immediately after (0-1h) and (B) 14 days after injury. Data presented as mean ± SEM n=5 rat/group. *, **, *** Significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. a, b significant from SHAMC and OVXC, respectively.

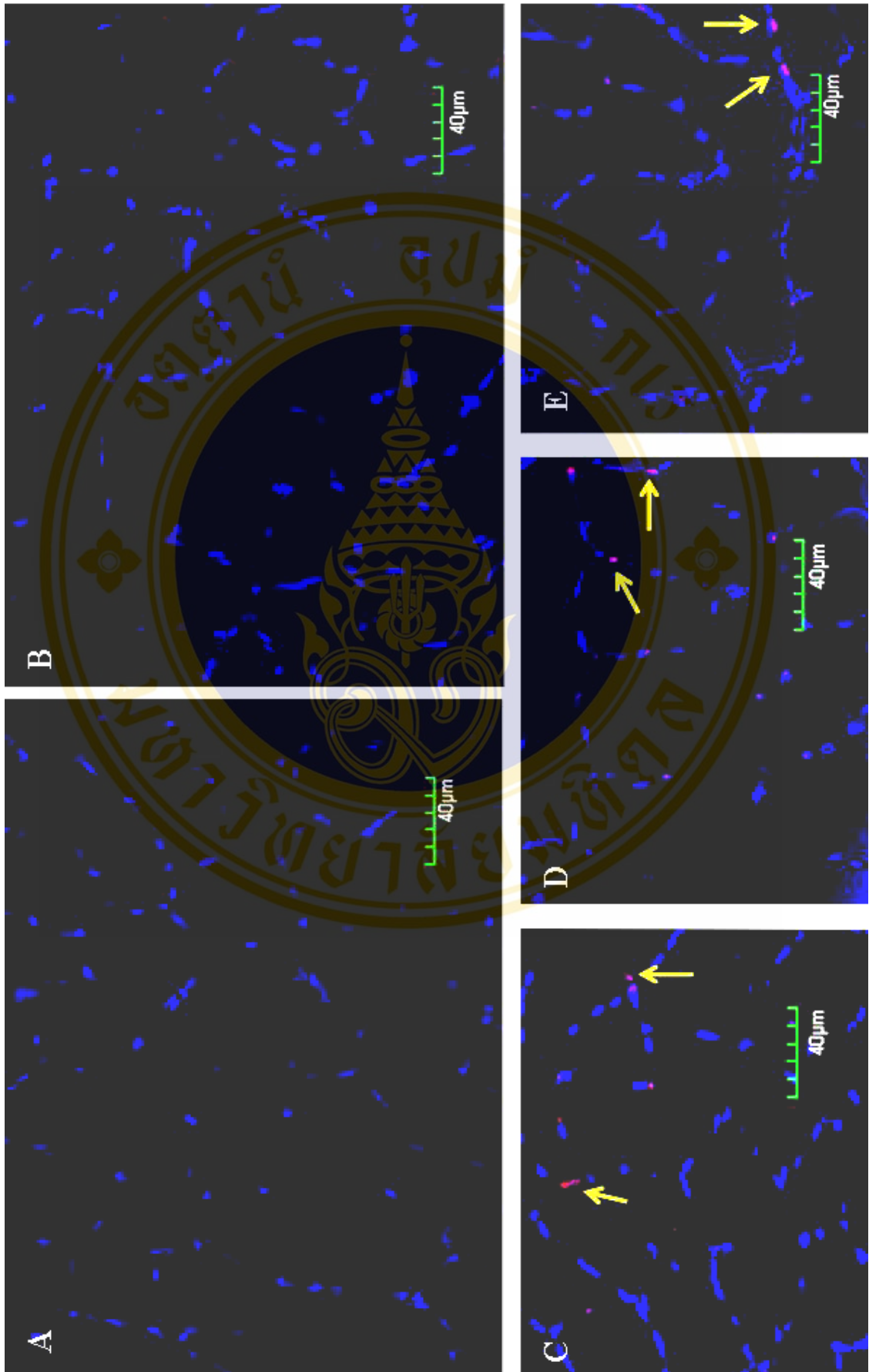
Effect on satellite cells activation.

Satellite cells and total myonuclei

The relative numbers of myofibers containing MyoD-positive satellite cells present in transverse sections from soleus muscles at immediately, and at 3 and 14 days post-exercise were shown in Fig. 22-23. Downhill running caused an increased numbers of myofibers containing satellite cells staining positive for MyoD in all groups following 3 days of recovery, but not at immediately or 14 days post-exercise. On average, the numbers of satellite cells in exercised animals were greater than that in unexercised animals. The relative numbers of satellite cells were 4.3 ± 0.3 % for SHAME and 4.3 ± 0.1 % for OVXES, respectively, which were significantly higher than that of OVXE (3.1 ± 0.4 %) animals. No significant difference was observed in the numbers of satellite cells between SHAMC and OVXC. The relative numbers of satellite cells were 1.5 ± 0.1 % for SHAMC and were 1.5 ± 0.1 % for OVXC. Interestingly, there were no differences in the numbers of myonuclei per fiber among groups (Fig. 24).

Cyclo-oxygenase-2 activity

To determine whether the estrogen's effect was mediated by the activation of COX-2 pathway, we measured the COX-2 activity of soleus muscles immediately after exercise, and at 3 and 14 days post-injury (Fig. 25-26). Immediately after downhill running, there were no significant differences in COX-2 activity among groups. By 3 day post-exercise, however, COX-2 activity was significantly elevated only in OVXE (12.2 ± 1.5 U/mg protein), but not in other groups (SHAMC 7.4 ± 0.3 , OVXC 8.6 ± 0.6 , OVXES 7.1 ± 0.9 , and SHAME 7.9 ± 0.3 U/mg protein). No differences in COX-2 activity were observed among groups at 14 day post-exercise (Fig. 25-26C).




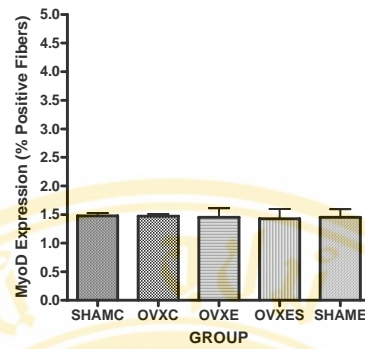
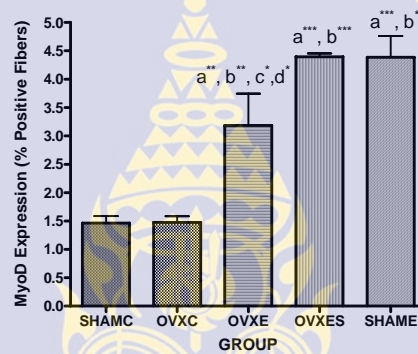


Figure 22 The myofibers expressing activated (MyoD) satellite cells markers (Alexa-Fluor 568; red) present in transverse sections from soleus muscles at 3 days post-exercise of SHAMC (A), OVXC (B), OVXE (C) and OVXES (D) group, and SHAME (E). Nuclei are visible by Tropo3 staining (blue). Bar = 40 μ m.

A



B



C

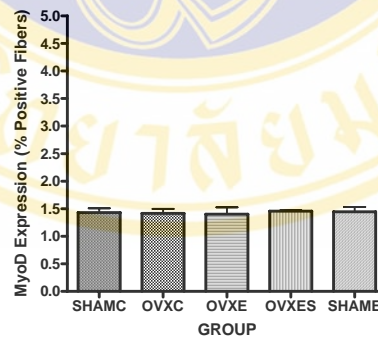
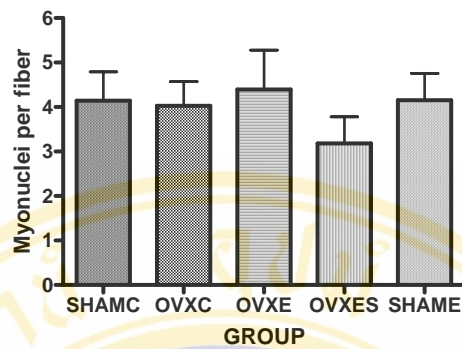
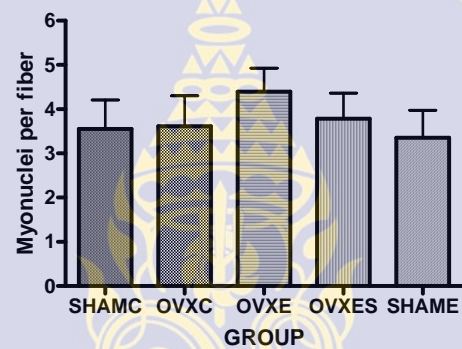


Figure 23 Changes in relative numbers of myofibers expressing activated (MyoD) satellite cells markers in rat soleus muscle (A) at immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data are presented as mean \pm SEM, n= 4/group. *, **, *** Significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, a, b, c, d significant from SHAMC, OVXC, OVXES, and SHAME group, respectively.

A



B



C

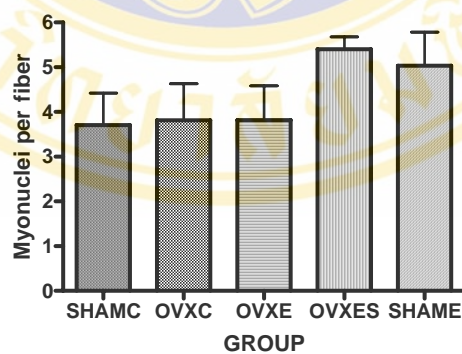
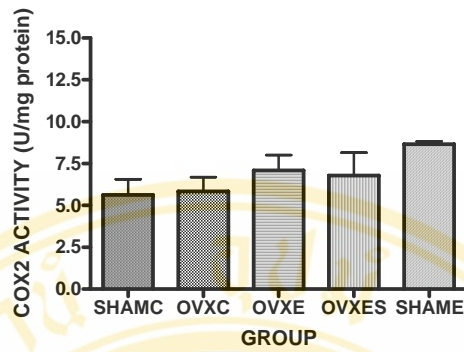
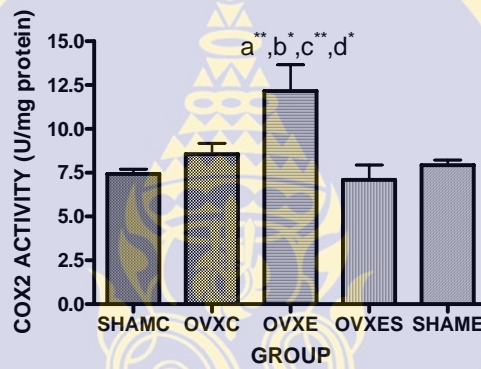


Figure 24 Changes in numbers of myonuclei per fiber in rat soleus muscle (A) at immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM, n= 4/group. No significant difference in myonuclei per fiber ($p < 0.05$) between groups.

A



B



C

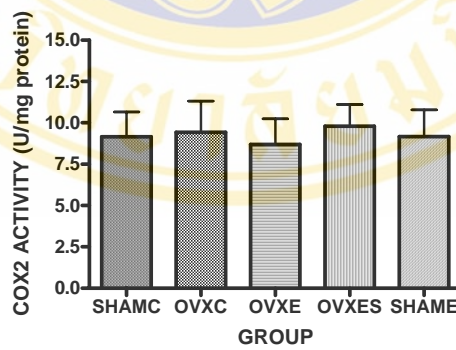
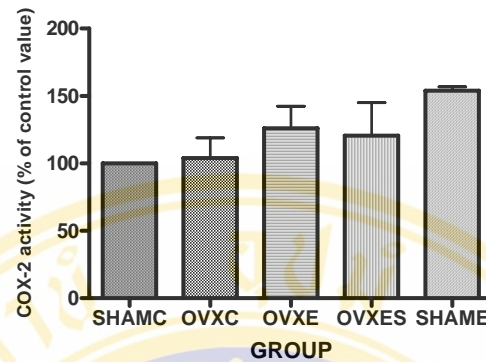
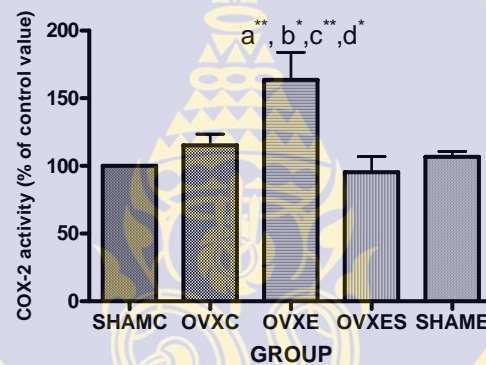


Figure 25 Changes in cyclo-oxygenase activity (COX-2) (A) at immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM, n=5-6/group. *, ** Significant difference at $p < 0.05$ and $p < 0.01$, a, b, c, d significant from SHAMC, OVXC, OVXES, and SHAME group, respectively.

A



B



C

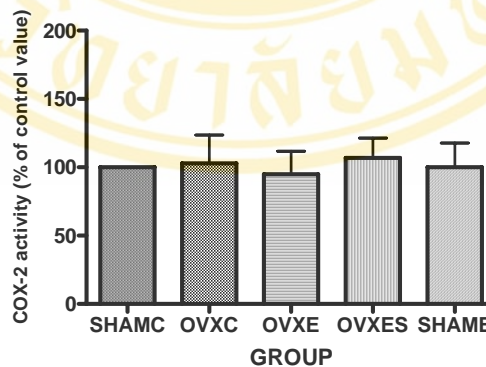


Figure 26 Changes in cyclo-oxygenase activity (COX-2) expressed as % of control value (A) at immediately (0-1h) after, (B) at 3 days and (C) 14 days after injury. Data were presented as mean \pm SEM, n=5-6/group. *, ** Significant difference at $p < 0.05$ and $p < 0.01$, a, b, c, d significant from SHAMC, OVXC, OVXES, and SHAME group, respectively.

CHAPTER V

DISCUSSION

The major finding of this study was that the loss of estrogen hormone caused a greater extent of muscle damage, greater muscle neutrophil infiltration, and less muscle strength following downhill running. Estrogen replacement partially attenuated these effects and also speeds up the recovery of muscle function as early as 3 days of recovery. This effect partly exerted through stimulating satellite cells and might not appear to involve the COX-2 pathway.

Effects on body weight and muscle mass.

In this study, although body weights in the OVX animals were significantly higher than that in the sham-operated groups, the loss of estrogen did not alter soleus muscle wet weights. This suggests that the differences in body weight between groups was not solely due to the changes in muscle mass. This finding is in contrast to the results from previous studies reporting that estrogen loss could cause an increased muscle mass in OVX rats (Borki et al., 1996; Jansson et al., 1984). Estrogen is known to have an indirect effect on skeletal muscle through the regulation of growth hormone (GH) and insulin-like growth factor I (IGF-I). After ovariectomy, GH release and IGF-I expression are both increased (Borki et al., 1996; Jansson et al., 1984). Additionally, there is evidence indicating that ovariectomy caused a decrease in spontaneous cage activity and an increase in food intake (Wade et al., 1982). These factors may contribute to increase muscle mass following ovariectomy.

Thus, it is likely that an increase in body weight in OVX rats observed in our study may stem from the increase in body fat mass, given that muscle mass was not change after ovariectomy. This finding is in keeping with previous studies demonstrating that OVX rodents become obese as a result of decreasing lipid metabolism-related gene expression in adipose tissue with increased body fat, and

estrogen administration decreased adiposity by promoting the use of lipid fuel and adipocyte size in OVX mice (D Eon et al., 2005; Kamai et al., 2005).

In the present study, the ovariectomized animals with estrogen-replacement had a lower in body weight, compared to any of other groups. In agreement with this finding, Christy Piccone et al (2005) reported that the estrogen-treated animals consumed 6% less food per day than the OVX groups. This small different amount in food intake was attributed to reduced body and plantaris muscle weights of the estrogen-treated animals.

However, when the muscle mass was normalized by body weight, the mass-to-body weight ratio were similar in control and OVX animals. This finding was in agreement with the previous reports (Booth et al., 1969; Fisher et al., 1998). Ovariectomy has been demonstrated to cause a generalized increase in body weight and organ weights (e.g. heart, kidney, spleen) (Booth et al., 1969). Therefore, the lack of alteration in muscle mass seen in OVX animals could be due to a change in hormone that regulates body growth rather than the direct effects of estrogen on skeletal muscle (Fisher et al., 1998).

Effects on contractile properties.

Although the release of intramuscular enzyme has been widely used to assess muscle injury in many studies that showed the protective effect of estradiol, the extent of muscle contractile impairment is considered to be a better marker of muscle injury (St. Pierre Schneider and Tiidus, 2007). In this study, we examined muscle contractile properties at immediately after, and at 3 days post-exercise. These time points were selected according to the earlier support that it reflects the two main stages of the muscle injury process, namely the autogenetic and phagocytic stages (Amstrong et al., 1990). Data in the current study also clearly demonstrated that downhill running exercise employed was effective in inducing injury in soleus muscle.

In the present study, although downhill running resulted in a decrement of peak twitch tension, however it caused no alterations in contraction and relaxation time in any of the groups. There are conflicting results in the literature regarding the effect of exercise-induced muscle injury and/or ovariectomy on single-twitch characteristics. In

one study, Sotiriadou et al (2006) reported that the administration of estrogen had a protective effect against muscle injury resulting from downhill running by preserving muscle strength at low stimulation frequencies and maintaining the ability of the muscle to sustain tension after fatigue. However, the soleus muscle isometric single-twitch characteristics (i.e. P_t , TPT, and $\frac{1}{2}$ RT) were unaffected by estrogen treatment. In contrast, McCormic et al (2004) reported that neither ovariectomy nor estrogen replacement affected the maximal muscle twitch kinetics while estrogen replacement slowed relaxation time. Moreover, ovariectomy was reported to reduce soleus muscle time to peak tension, the effect was reversed by estrogen replacement. On the other hand, Fisher et al (1998) also have demonstrated that TPT in EDL and $RT_{1/2}$ were increased in OVX animals, following hindlimb unloading. This finding was further supported by a study of Kadi et al (2002) showing that ovariectomy reduced fast MHC expression in the soleus muscle and thus leading to a reduced contraction speed. Although ovariectomy in the present study tended to cause a small increase in time to peak tension after 14 days of recovery, others reported no changes in TPT after 16 days of recovery (Kadi et al., 2002). These findings were consistent with some previous study showing that the lack of estrogen hormone did not alter soleus muscle fiber maximal shortening velocity (Wattanapernpool and Reiser., 1999). Twitch force responses were reported to depend on the ability of the sarcoplasmic reticulum to release and re-sequester Ca^{2+} , the affinity of the thin filament regulatory proteins for Ca^{2+} , the relationship between bound Ca^{2+} and tension, and the actomyosin cross-bridge kinetics. In addition, contraction speed in an isolated muscle preparation is well known to be affected by changes in calcium sensitivity and/or the rate of calcium release. Nonetheless, the previous work has shown that estrogen deficiency did not affect calcium sensitivity of single soleus muscle fibers (Wattanapernpool and Reiser., 1999). Recently, there is evidence suggesting that estrogen treatment may slow calcium uptake in ovariectomy (McCormic et al., 2004). Nevertheless, in intact animals, this estrogenic effect on calcium uptake may be influenced by another ovarian hormone such as progesterone. Therefore, further studies are needed to better understand how ovarian hormones affect all aspects of calcium handling in skeletal muscle.

In this study, peak tetanic contraction showed significantly lower in the exercise groups than did unexercised control immediately following downhill running and at 3 days of recovery. During tetanic stimulation, when the function is not limited by activation, force production becomes a property of the force-generating ability of the actomyosin cross-bridges and the number of sarcomere in series. Thus, the force deficit seen in this study may be related to the failure of the damaged sarcomeres to produce tension and/or the disruption of the membrane systems involve in E-C coupling (Warren et al., 1996).

Of interest, we have demonstrated that estrogen replacement could reverse or restored ovariectomy-induced contractile dysfunction of peak tetanic tension as early as 3 days of recovery. The underlying mechanism for this protective effect is not known, but one potential mechanism might involve the increased strong-binding of myosin (Moran et al., 2007). Following ovariectomy, several hormones are known to systematically alter. Since 17- β estradiol was only administered in this study, therefore, this suggests that estrogen status may be important for optimizing skeletal muscle contractility. This finding was agreed with the results from some studies (Fisher et al., 1998; Warren et al., 1996; Wattanapermpool and Reiser, 1999). However, others reported that maximal specific isometric force (P_o) was not different in either ovariectomy with or without estrogen replacement (McCormick et al., 2004; Brown et al., 2005). This discrepancy could be due to whether or not these studies normalized the force data to muscle CSA.

Apart from above mention, Wattanapermpool and Reiser (1999), in single-fiber study, showed that Ca^{2+} -activated forced by soleus and EDL muscle were reduced by 20% following ovariectomy in immature rodents. Recently, there has been increasing evidence that the maximal Ca^{2+} -activated force was lower by 25% in permeabilized EDL fibers from OVX mice compared with sham mice (Moran AL, 2005). These results were associated with reductions of the fraction of strong-binding myosin during contraction (i.e., myosin that is generating force during contraction) in muscle fibers from ovariectomized mice (Moran et al 2006; Moran et al., 2007). Based on these data, it implies that the major contractile protein is somehow affected by estrogen status and accounts for the ovariectomized-induced loss of force-generating capacity. Alternatively, the mechanism by which the loss of ovarian hormones leads to altered

myosin may be related to estrogen's antioxidant properties (Klinge, 2001; Klinge et al., 2004). Oxygen radical production after exercise-induced injury is low in mature female rats with normal estrogen levels. Moreover, evidences accumulating that female rats were less susceptible than male rats to exercise-induced membrane damage as indicated by lower increases in serum CK activity (Sotiriadou et al., 2003; Kendall and Ester., 2002; Carter et al., 2001) and TBARS (Joo et al., 2004) following acute exercise or by the lack of significant muscular microtrauma following exercise. Taken together, these data suggest that estrogen may offer a line of defense against exercise stress (Tiidus and Houston., 1993). Estrogen is well known to have a hydroxyl group on their phenolic ring, in the same configuration and position as tocopherol (vitamin E) and thyroxine, which posses the potent anti-oxidant activity, and thus could terminate peroxidation chain reactions, in similar manner as tocopherol (Kendall & Eston., 2002).

As expected, our data demonstrated that estrogen could attenuate the exercise-induced CK leakage and neutrophil infiltration. These changes were accompanied with the restoration of both maximal twitch and tetanic force production following downhill running relative to that produced in control rats. In this study, we also determined histological characteristics of soleus muscle section from each experimental group. Overall, the maximal force production was correlated with the individual values of CSA in the muscles. Immediately (0-1h) post-exercise, SOL muscle fibers in the exercised groups were evenly small in size, some fibers were dead and were being removed by phagocytic cell, and the amount of fluid (cellular infiltration) was consistently increased compared with unexercised groups. This suggests that muscle injury predominantly caused fiber damage and/or loss, and edema, both of which can ultimately lead to a reduction in force production. In the present study, while the cross-sectional areas of muscle fibers in exercised groups increased over time, most injured muscle fibers could regain their original size by 14 days post-injury, suggesting the fiber growth and regeneration following downhill running-induced injury.

In this study, it did not know whether ovarian hormone status did induce alterations in soleus muscle CSA or not because we did not include ovariectomized control with estrogen replacement in the present experiment. Nevertheless, many studies reported that, in hindlimb suspension model, loss of ovarian hormones could

impair the recovery of skeletal muscle fiber cross-sectional during the first 2 wk and estrogen replacement at physiologically relevant levels would restored the ability of muscle fibers to recover over this same 2 wk period (McClung et al., 2006). On the other hand, previous works showed that treating male and female rats with estrogen could reduce skeletal muscle mass and fiber size (Kobori and Yamamuro., 1989; McCormick et al., 2004; Ihemelandu et al., 1980; Piccone et al., 2004). Similarly, treating sexually immature male and female mice and rabbits with estrogen results in smaller muscles (Ihemelandu., 1981). Consistent with reduced muscle size, muscle protein synthesis (Toth et al., 2001) and whole body protein gain (Richard et al., 1987) were reported to reduce with estrogen replacement. More recently, there is evidence that ovariectomy caused a reduction in the fastest MHC (IIx) expression, such change was reversed with estrogen replacement (Piccone et al., 2004). Collectively, it is still unclear how estrogen hormone affects muscle mass and fiber size. Therefore, a further study is needed to determine the role that estrogen play in modulating muscle fiber size and mechanism involved.

Effects on muscle damage and inflammation.

A number of animal and human studies have demonstrated on sex differences in muscle damage and the inflammatory responses to exercise (MacIntyre et al., 2000; Stupka et al., 2000; Schneider et al., 1999; Tiidus and Bombardier., 1999). Specifically, male was reported to be more susceptible to muscle damage in response to intense exercise than female. Although the exact mechanism underlying such difference is not clear, estrogen is believed to play a significant anti-inflammatory role (Tiidus., 2000). We found that downhill running essentially induced injury as reflected by the reductions in both maximal twitch and tetanic force production in OVX rats subjected to downhill running at immediately post-exercise. These changes were accompanied with the marked increase in serum CK activity in OVXE rats. Interestingly, we also found that estrogen replacement partially attenuated these effects.

Additionally, we found that MPO activities in injured muscle were remarkably increased in unexercised muscle at immediately (0-1 h) after, but not at day 3 and day 14 post-injury. This finding was in agreement with the finding of other study (Morozov et al., 2006) reporting that only 48 h could resolve inflammatory component, as measured by MPO concentration in skeletal muscle. This change in time course of MPO activity suggests that at least 48-72 h after injury may be required for providing sufficient time to resolve an intramuscular component of inflammatory response.

In the present study, muscles were also analyzed for dry weight to wet weight ratio and percent water content to allow accurate description of alterations in the weight. We found a difference between the post-exercise involved in edema and tissue injury at immediately (0-1 h) post-exercise. This change was roughly correlated with a change observed in MPO activity. The resolution of inflammation and edema is considered to be critical in signaling associated with myofiber growth. During inflammatory process, inflammatory cells are rapid infiltration of damaged skeletal muscle and alterations in their temporal appearance are responsible for disruption of muscle healing process (Bondesen et al., 2004). This rapid sequence of inflammatory cells invasion observed after damage includes neutrophil, and macrophages (Frenette et al., 2002). Of these, neutrophil are considerate to be one of the first immune cell types to enter skeletal muscle in response to damage and are partly responsible for the stimulation of inflammation through phagocytosis and oxygen free radical release (Tidball., 2005). Macrophage infiltration increases and decreases within a 2-3 day during recovery and coincides with decreasing concentrations of neutrophils.

Interestingly, our result demonstrated that in the ovariectomized rat treated with estrogen (OVXES) in MPO activity immediately (0-1h) post-exercise were not significant increased. This finding was consistent with the findings from previous studies (Tiidus et al., 2001; Tiidus and Bombardier., 1999), indicating that estrogen administration did diminish exercise-induced muscle damage and lower post-exercise neutrophil and/or macrophage infiltration and consequently lower muscle MPO activity. However, this protecting effect of estrogen could be interpreted with caution since others physiological factors including circulating sex hormones, age, strain, stress-related hormones (e.g. catecholamins and corticosterone), acute stressors and

muscle fibre type could interfere the results (St. Pierre Schneider & Tiidus., 2007). Therefore, it is still unclear whether the attenuation of post-exercise tissue neutrophil infiltration is direct and/or indirect effect of estrogen. An additional study is needed to better understand the anti-inflammatory effect of estrogen.

In addition to the above factors, neutrophil adhesion to damaged tissues is influenced by numerous complex and interrelated factors including local production of histamine, tumour necrosis factor (TNF), interleukin-1 (IL-1), and nitric oxide as well as oxidative stress (Evans & Cannon., 1991; Dallegrì & Ottonello., 1997). Therefore, the control of tissue infiltration by neutrophils and macrophages is very complex and can be influenced by among other things, locally generated cytokines such as IL-1 and IL-8, oxygen radicals, and the degree of tissue damage (Cannon et al., 1990; Pyne et al., 1994; Dallegrì & Ottonello., 1997). Cytokines are well known to induce tissue prostaglandin E₂ (PGE₂) synthesis which in turn can influence further cytokine synthesis, tissue inflammation, as well as muscle proteolysis (Cannon et al., 1991). Neutrophils are generally believed to survive for at least 1-2 days after tissue infiltration and have been found as late as 5 days post-exercise in human muscle (Fielding et al., 1993). While exercise may temporarily increase the ability of circulating neutrophils to produce superoxide and active MPO immediately post-exercise (Pyne et al., 1994). Previous studies have suggested that estrogen may act as antioxidant and membrane stabilizer (Sugioka et al., 1987; Tiidus et al 1995; Bar et al., 1997) and hence diminish oxidative stress, its resultant and membrane damage induced by exercise. Similarly, the potential antioxidant and membrane stabilizing abilities of estrogen may act to diminish exercise-induced damage to the sarcoplasmic reticular membrane and hence act to preserve muscle calcium homeostasis during and following exercise (Raj et al., 1998).

It is well known that muscle inflammatory response is required for subsequently repair and that inhibition of inflammation may lead to further damage at a later post-exercise time point (Duarte et al., 1994, Mishra et al., 1995). Hence, it cannot yet be conclusively determined if a reduction in post-exercise muscle neutrophil infiltration is necessarily beneficial. Thus, the effects of estrogen on the immune system in remediation muscle injury post-injury remain to be determined.

Effect on satellite cells activation.

Skeletal muscle is considered to be post-mitotic tissue. Therefore, the growth of skeletal muscle fiber during regeneration process is primarily depends on satellite cells function. When a muscle is injured, quiescent satellite cells begin to proliferate and generate myogenic cells. The activation of satellite cells is a prerequisite for their proliferation and subsequent subsequent differentiation which is indicated by increased expression of the myogenic regulatory factor MyoD and/or Myf-5 that can transactivate muscle-specific gene expression (Canitini et al., 1995; Koishi et al., 1995; Seale and Rudnicki., 2000). Studies have reported that MyoD (mRNA and/or protein) was up-regulated by overt muscle injury induced by muscle trauma (i.e. crush, freeze, or bupivacaine-induced injury) (Grounds et al., 1992; Koishi et al., 1995; McLoon et al., 1998) and by a single bout of weight-lifting exercise (Tamaki et al., 2000). Typically, the general accepted sequence of events during muscle injury and repair suggested that most inflammation and satellite cell activation occur within the first 72 h after injury (Hawke & Garry., 2001, Grounds et al., 1992, Horme & Kalimo., 1992, Deborah et al., 2007). So, we have chosen the time point following downhill running that the number of MyoD+ cells would be at peak level. As expected, our result revealed an increase in numbers of MyoD+-stained cells within 3 days of recovery in response to downhill running, suggesting that satellite cells were activated to re-enter the cell cycle and proliferated after exercise at this time period. Interestingly, the greater numbers of activated satellite cells were detected in section samples of soleus muscle from estrogen treated and sham rats at 3 days following downhill running exercise (90 min, 16°graded, 16 m/min) when compared to OVX rats and these values were similar to SHAME rats. This finding is consistent with the findings from previous studies (Tiidus., 2005; Schutz et al., 2006; Deboran et al., 2007) even using a different downhill running protocol. These findings suggested that estrogen may play a role in muscle repair process by influencing activation of satellite cell. Further, we also found that the number of MyoD-positive cells in the post-exercised muscle decrease to the control level within 14 days of recovery. This implies that there was no further net increase of satellite cells in these periods of recovery and that MyoD-positive cells may have differentiated to MyoD-negative cells during this

interval. Differentiation beyond the stage at which MyoD was expressed would render the cells fusion-complement and able to contribute to fibre growth (Smith et al., 1994; Friday et al., 2003), and/or that the responsiveness of satellite cells to exercise stimulus was reduced.

Despite the increased number of activated satellite cells, the mechanism by which estrogen may be able to exert myogenic satellite cell proliferations following downhill running induced injury has not been determined. Previous studies have reported sex-based differences in response to exercise-induced muscle injury with respect to attenuated levels of creatine kinase activity (Komulainen et al., 1999), neutrophil infiltration (Schneider and Tiidus et al., 2007), and greater muscle strength (Sotiriadou et al., 2006) during recovery. These changes have been attributed to the influence of estrogen because a removal of estrogen (i.e., via ovariectomy) also removed this effect. Thus, ovarian hormones may function in other capacities to mediate recovery processes in response to myofibre damage, including secondary damages or the subsequent immune response. Many studies have demonstrated that neutrophil/macrophage are an important source of inflammatory cytokines; both immune and non-immune cells are capable of producing cytokines involved in inflammation during skeletal muscle recovery (Schneider et al., 1999). Muscle inflammatory cytokines such as interleukin- 1β expression have been reported to increase at the onset of the immune response phase of skeletal muscle repair and these cytokines subsequently stimulated the expression of other muscle related cytokines such as IL-2, IL-3, IL-6 and TNF- α (Tidball., 1995). These cytokines are capable of inducing prostaglandin synthesis, which are reported to mediate inflammatory process and induce satellite cell activation (Herman et al., 1995; Bondesen et al., 2004; Shen et al., 2005; Shen et al., 2008). Of these, it has recently been reported that the COX-2 pathway products including PGE₂ and PGF₂ are important inflammatory mediators that induce regeneration in skeletal muscle healing (Shen et al., 2005). Null mutation of cyclo-oxygenase 2 or COX-2 inhibition studies revealed a reduction in muscle fibre size in areas of muscle repair following injury and decreases in MyoD mRNA in injured muscle (Bondesen et al., 2004). Moreover, there is good evidence indicating that satellite cell proliferation, differentiation, and/or fusion was inhibited with NSAID treatment in rodent satellite cell culture (Mendias et al., 2004). Hence, in present study, we expected that the COX-

2 activity, a rate limiting enzyme in PG synthesis, would increase in accordance with an increase in a number of satellite cells in the injured muscle, especially in estrogen present animals, in the first few days following injury. Surprisingly, we found that even though estrogen supplementation and sham-operated rats illustrated significantly increased in satellite cell number especially at 3 days post-injury relative to unexercised groups. COX-2 activity in these animal groups, however, did not altered when compared to those in the control. This suggests that there is no causal effect of COX-2 activity in the activation of muscle satellite cell in estrogen-presented rats. This finding was further supported by other models of muscle damage, including hindlimb suspension, in which cyclo-oxygenase-2 expression was significantly elevated during recovery regardless of ovarian hormone status. These data suggest that the initial myofiber damage and inflammatory gene expression induced during muscle recovery are independent of ovarian hormone status (McClung et al., 2006). Additionally, previous studies reported that satellite cell and fibroblast proliferation were unaffected by the NSAID, a non-selective COX inhibitor, treatment (Thorsson et al., 1998, Vignaud et al., 2005). Based on these data, it is less likely that COX-2 pathway may directly involve in the enhancement of satellite cell activation and proliferation in response to exercise in estrogen present status. Further studies are necessary to determine whether the activation of satellite cells following exercise-induced injury is the COX-2 dependent-mechanism.

Alternatively, numerous other factors have been implicated in influencing muscle satellite cell activation and proliferation (Hawke & Garry., 2001). Studies in animal models have suggested that nitric oxide (NO) may be a critical effector of estrogen-mediated protection. Anderson and Wozniak (2004) have documented the important influence of muscle NO and nitric oxide synthase (NOS) in satellite cell activation and proliferation. Estrogen has been demonstrated to influence NOS activity and NO levels in a number of tissues (Node et al., 1997; Prorock et al., 2003). In skeletal muscle, NO is produced by the NOS-1 isoform at the sarcolemma, and nonspecific inhibition of NOS with *N*-nitro-L-arginine methyl ester before muscle injury has been shown to prevent satellite cell activation (Anderson et al., 2000). Moreover, the release and localization of hepatocyte growth factors, a known regulator of satellite cell activation (Tatsumi et al., 1998), is an NO-dependent process during conditions of

muscle fiber stretch and injury (Tatsumi et al., 2002). Thus it is possible that estrogenic influence on post-damage satellite cell proliferation may be mediated through its effects on muscle NOS activity and NO levels.

Another factor which may potentially participate in the regulation of muscle satellite cell activity is circulating/ muscle IGF-1 levels (Hawke & Garry., 2001; Stupka & Tiidus., 2001). Recent work (Tsai et al., 2007) showed that ovariectomy increased IGF-1 mRNA expression of weight-bearing skeletal muscles and this increase was reversed by estrogen replacement. However, one week of estrogen replacement significantly decreased IGF-1 protein level in all muscle examined, suggesting that reduced the levels of IGF-1 may mediate estrogenic effect on growth of immature, ovariectomized rats (Tsai et al., 2007). Additionally, an important consideration is the estrogenic effect on the GH/IGF-1 axis. Estrogen has been shown to antagonize several metabolic actions of GH (Wolthers et al., 2001). Thus, ovarian hormones may, therefore, function in other capacities to mediate recovery processes in response to myofiber damage. Future work is necessary to determine whether ovarian hormones such as estrogen regulate other cytokines (i.e. TNF, IL1, IL6) related to satellite cell activation during recovery from exercise-induced damage, and whether neutrophil/macrophage behavior after muscle injury is a mechanism for these potential alterations.

In summary, the present study revealed the role of estrogen hormone on skeletal muscle damage, regeneration, and the restoration of skeletal muscle function following exercise-induced injury. Based on our finding, we suggest that estrogen hormone have a protective effect against downhill running-induced muscle injury, and accelerates the restoration of skeletal muscle function partly through stimulating satellite cells. These effects are not correlated with an alteration in COX-2 activity. Deliberation of ovarian hormone-sensitive mechanisms related to muscle recovery will aid in the development of future pharmaceutical therapies for rapid and efficient recovery of skeletal muscle from muscle damage.

CHAPTER VI

CONCLUSIONS

Our results demonstrated that ovariectomy caused a greater exercise- induced muscle damage, as reflected by a significant loss of force production and increased indices of muscle injury including serum creatine kinase and MPO activities, following downhill running, and the administration of 17- β estradiol partially attenuated these effects especially during the first few day of recovery. Downhill running resulted in an increased the number of activated satellite cells in ovariectomized rats as early as 3 days post-exercise, the effect was accentuated by estrogen replacement. These changes were correlated with the functional recovery of skeletal muscle function after injury. However, the estrogen's effect did not appear to associate with an alteration in COX-2 activity.

REFERENCES

- Aarima V, Rantanen J, Best T, Schultz E, Corr D, Kalimo H. Mild eccentric stretch injury in skeletal muscle causes transient effect on tensile load and cell periferation. *Scand J Med Sci Sports* 2004; 14: 367-372.
- Amelink G, Van der Waal W, Wokke J, et al. Exercise induced muscle damage in the rat: the effect of vitamin E deficiency. *Eur J Physiol* 1991; 419: 304-309.
- Amelink GJ and Bar PR. Exercise-induced muscle protein leakage in the rat. Effects of hormonal manipulation. *J Neurol Sci.* 1986 Nov; 76(1): 61-8
- Anderson JE, Wozniak AC. Satellite cell activation on fibers: modeling events in vivo-an invited review. *Can J Physiol Pharmacol.* 2004 May; 82(5):300-10.
- Anderson JE. A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. *Mol Biol Cell.* 2000 May; 11(5):1859-74.
- Aoi W, Naito Y, Takanami Y, Kawai Y, Sakuma K, Ichikawa I, Yoshida N, and Yoshikawa T. Oxidative stress and delayed-onset muscle damage after exercise. *Free radical Biology & Med* 2004; 37: 480-487.
- Armstrong RB, Ogilvie RW, and Schwane JA. Eccentric exercise-induced injury to rat skeletal muscle. *J Appl Physiol.: Respirat Environ Exercise Physiol* 1983; 54(1): 80-93.
- Armstrong RB. Initial events in exercise-induced muscular injury. *Med Sci Sports Exerc.* 1990 Aug; 22(4): 429-35.
- Ashley CW, Jiming K, Erika B, Orest P and Judy EA. Signaling satellite-cell activation in skeletal muscle: markers, models, stretch, and potential alternate pathway. *Muscle Nerve* 2005; 31: 283–300.
- Bar PR, Amelink GJ. Protection against muscle damage exerted by oestrogen: hormonal or antioxidant action? *Biochem Soc Trans.* 1997 Feb; 25(1): 50-4.
- Bassey EJ, Mockett SP, Fentem PH. Lack of ovarian in muscle strength with menstrual status in healthy women aged 45-54 years: data from a national

- survey. *Eur j Appl Physiol* 1996; 73: 382-6 Belcastro AN, Arthur G, Alblisser T and Raj DA. Heart, liver and skeletal muscle myeloperoxidase activity during exercise. *J Appl Physiol* 1996; 80: 1331-1335.
- Bemben DA, Langdon DB. Relationship between estrogen use and musculoskeletal function in postmenopausal women. *Maturitas* 2002; 42: 119-27.
- Berchtold NC, Kesslak JP, Pike CJ, Adlard PA, Cotman CW. Estrogen and exercise interact to regulate brain-derived neurotrophic factor mRNA and protein expression in the hippocampus. *Eur j Neu* 2001; 14: 1992-2002.
- Bondesen BA, Mills ST, and Pavlath GK. The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms. *Am J Physiol* 2006; 290: C1651-1659.
- Bondesen BA, Mills ST, Kegley KM, and Pavlath GK. The COX-2 pathway is essential during early stages of skeletal muscle regeneration. *Am J Physiol Cell Physiol* 2004; 287: C475-C483.
- Booth FW and Tipton CM. Effects of training and 17- β estradiol upon heart rates, organ weights, and ligamentous strength of female rats. *Int. Z. Angew. Physiol.* 1969; 27: 187-197.
- Borski, R. J., W. Tsai, R. DeMott-Friberg, and A. L. Barkan. Regulation of somatic growth and somatotrophic axis by gonadal steroids: primary effect on insulin-like growth factor I gene expression and secretion. *Endocrinology* 1996; 137: 3253-3259.
- Borycki AG, Li J, Jin F, Emerson CP, and Epstein JA. Pax3 functions in cell survival and in pax7 regulation. *Development* 1999; 126: 1665–1674.
- Branham WS, Zehr DR, Sheehan DM. Differential sensitivity of rat uterine growth and epithelium hypertrophy to estrogens and antiestrogens *Proc. Soc. Exp. Biol. Med* 1993; 203: 297-303.
- Brown M, Foley A, Ferreria JA. Ovariectomy, hindlimb unweighting, and recovery effects on skeletal muscle in adult rats. *Aviat Space Environ Med.* 2005 Nov; 76(11): 1012-8.
- Bunt J. Metabolic actions of estradiol: significance for acute and chronic exercise response. *Med Sci Sports Exerc* 1990; 22: 286-90.

- Cannon JG, Meydani SN, Fielding RA, Fiatarone MA, Meydani M, Farhangmehr M, et al. Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis. *Am J Physiol*. 1991 Jun; 260(6 Pt 2): R1235-40.
- Cannon JG, Orencole SF, Fielding RA, Meydani M, Meydani SN, Fiatarone MA, et al. Acute phase response in exercise: interaction of age and vitamin E on neutrophils and muscle enzyme release. *Am J Physiol*. 1990 Dec; 259(6 Pt 2): R1214-9.
- Carter A, Dobridge J, Hackney AC. Influence of estrogen on markers of muscle tissue damage following eccentric exercise. *Fiziol Cheloveka*. 2001 Sep-Oct; 27(5): 133-7.
- Cantini M, Massimino ML, Rapizzi E, Rossini K, Catani C, Dalla Libera L, et al. Human satellite cell proliferation in vitro is regulated by autocrine secretion of IL-6 stimulated by a soluble factor(s) released by activated monocytes. *Biochem Biophys Res Commun*. 1995 Nov 2; 216(1):49-53.
- Carlson BM and Faulkner JA. Muscle transplantation between young and old rats: age of host determines recovery. *Am J Physiol Cell Physiol* 1989; 256: C1262–C1266.
- Chambers RL and McDermott JC. Molecular Basis of Skeletal Muscle regeneration. *Can J Appl Physiol* 1996; 21(3): 155-184.
- Chevion S, Moran DS, Heled Y, Shani Y, Regev G, Abbou B, Berenshtein E, Stadtman ER, and Epstein Y. Plasma antioxidant status and cell injury after severe physical exercise *PNAS* 2003; 100(9): 5119-5123.
- Chung HY, Kim HJ, Kim KW, Choi JS, and Yu BP. Molecular Inflammation Hypothesis of Aging Based on the Anti-Aging Mechanism of Calorie Restriction. *Microscopy Research and Technique* 2002; 59:264-272.
- Clarke MS, Khakee R, and McNeil PL. Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. *J Cell Sci* 1993; 106: 121–133.
- Clarkson PM and Hubal MJ. Are women less susceptible to exercise-induced muscle damage. *Curr Opin Clin Nutr Metab Care* 2001; 4: 527-531.

- Clarkson PM, Hubal MJ. Exercise-induced muscle damage in humans. *Am J Phys Med Rehabil* 2002; 81(Suppl): S52–S69.
- Close GL, Ashton T, Cable T, Doran D, MacLaren DPM. Eccentric exercise, isokinetic muscle torque and delayed onset muscle soreness: the role of reactive oxygen species *Eur J Appl Physiol* 2004; 91: 615–621.
- Close GL, Kayani A, Vasilaki A and McArdle A. Skeletal muscle damage with exercise and aging. *Sports Med* 2005; 35(5): 413-427.
- Dallegri F, Ottonello L. Tissue injury in neutrophilic inflammation. *Inflamm Res*. 1997 Oct; 46(10): 382-91.
- Darr KC, Schultz E. Exercise-induced satellite cell activation in growing and mature skeletal muscle. *J Appl Physiol*. 1987 Nov; 63(5):1816-21.
- D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem*. 2005 Oct 28;280(43):35983-91.
- Dietrich S, Schubert FR, Gruss P, and Lumsden A. The role of the notochord for epaxial myotome formation in the mouse. *Cell Mol Biol (Oxf)* 1999; 45: 601–616.
- Duarte JA, Carvalho F, Bastos ML, Soares JM, Appell HJ. Do invading leucocytes contribute to the decrease in glutathione concentrations indicating oxidative stress in exercised muscle, or are they important for its recovery? *Eur J Appl Physiol Occup Physiol*. 1994; 68(1): 48-53.
- Dubois RN, Abramson SB, Crofford L, et al. Cyclooxygenase in biology and disease *FASEB J*. 12: 122-129, 1998.
- Dupouy, V. M., P. J. Ferre, E. Uro-Coste, and H. P. Lefebvre. Time course of COX-1 and COX-2 expression during ischemia-reperfusion in rat skeletal muscle. *J Appl Physiol* 2006; 100: 233–239.
- Enns DL, Tiidus PM. Estrogen influences satellite cell activation and proliferation following downhill running in rats. *J Appl Physiol*. 2008 Feb; 104(2):347-53.
- Evans WJ, Cannon JG. The metabolic effects of exercise-induced muscle damage. *Exerc Sport Sci Rev*. 1991; 19: 99-125.

- Felice B, Paul G, Ryall JG, Plant DR, Sillence MN, and Lynch GH. Beta2-adrenoceptor agonist fenoterol enhances functional repair of regenerating rat skeletal muscle after injury. *J Appl Physiol* 2004; 96: 1385–1392.
- Fielding RA, Manfredi TJ, Ding W, Fiatarone MA, Evans WJ, Cannon JG. Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle. *Am J Physiol*. 1993 Jul; 265(1 Pt 2): R166-72.
- Fisher JS, Hasser EM, Brown M. Effects of ovariectomy and hindlimb unloading on skeletal muscle. *J Appl Physiol*. 1998 Oct; 85(4): 1316-21.
- Frenette J, Chbinou N, Godbout C, Marsolais D, Frenette PS. Macrophages, not neutrophils, infiltrate skeletal muscle in mice deficient in P/E selectins after mechanical reloading. *Am J Physiol Regul Integr Comp Physiol*. 2003 Oct; 285(4): R727-32.
- Friday BB, Mitchell PO, Kegley KM, Pavlath GK. Calcineurin initiates skeletal muscle differentiation by activating MEF2 and MyoD. *Differentiation*. 2003 Apr; 71(3):217-27.
- Fride'n J, Kjorell U, Thornell LE. Delayed muscle soreness and cytoskeletal alterations: An immunocytological study in man. *Int J Sports Med* 1984; 5: 5–8.
- Garrett WE. Muscle strain injuries: clinical and basic aspects. *Med Sci Sports Exerc* 1990; 22: 436-443.
- Grounds MD, Garrett KL, Lai MC, Wright WE, Beilharz MW. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res*. 1992 Jan; 267(1):99-104.
- Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*. 2001 Aug; 91(2):534-51.
- Ihemelandu EC. Comparison of effect of oestrogen on muscle development of male and female mice. *Acta Anat (Basel)*. 1981; 110(4): 311-7.
- Ihemelandu EC. Effect of oestrogen on muscle development of female rabbits. *Acta Anat (Basel)*. 1980; 108(3): 310-5.
- Jacobs SC, Wokke JH, Bar PR, Bootsma AL. Satellite cell activation after muscle damage in young and adult rats. *Anat Rec*. 1995 Jul; 242(3):329-36.

- Jansson JO, Ekberg S, Isaksson OG, Eden S. Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrinology*. 1984 Apr; 114(4): 1287-94.
- Joo MH, Maehata E, Adachi T, Ishida A, Murai F, Mesaki N. The relationship between exercise-induced oxidative stress and the menstrual cycle. *Eur J Appl Physiol*. 2004 Oct;93(1-2):82-6. Epub 2004 Jul 8.
- Kadi F, Karlsson C, Larsson B, Eriksson J, Larval M, Billig H, et al. The effects of physical activity and estrogen treatment on rat fast and slow skeletal muscles following ovariectomy. *J Muscle Res Cell Motil*. 2002; 23(4): 335-9.
- Kamei Y, Suzuki M, Miyazaki H, Tsuboyama-Kasaoka N, Wu J, Ishimi Y, et al. Ovariectomy in mice decreases lipid metabolism-related gene expression in adipose tissue and skeletal muscle with increased body fat. *J Nutr Sci Vitaminol (Tokyo)*. 2005 Apr;51(2):110-7.
- Kasemkijwattana G, Menetrey J, Bosch P, Moreland MS, Fu F, Buranapanikit B, Watkins SC, Huard J. The use of growth factors to improve muscle healing after strain injuries. *Clin Orthop* 2000; 370: 272-285.
- Kendall B and Eston R. Exercise-induced muscle damage and the potential protective role of estrogen. *Sport Med* 2002; 32(2): 103-123.
- Klinge CM, Jernigan SC, Mattingly KA, Risinger KE, Zhang J. Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. *J Mol Endocrinol*. 2004 Oct; 33(2): 387-410.
- Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res*. 2001 Jul 15; 29(14): 2905-19.
- Kobori M, Yamamuro T. Effects of gonadectomy and estrogen administration on rat skeletal muscle. *Clin Orthop Relat Res*. 1989 Jun; (243): 306-11.
- Koishi K, Zhang M, McLennan IS, Harris AJ. MyoD protein accumulates in satellite cells and is neurally regulated in regenerating myotubes and skeletal muscle fibers. *Dev Dyn*. 1995 Mar; 202(3):244-54.
- Komulainen J, Koskinen SOA, Kallioski R, Takala TES, Vihko V. Gender differences in skeletal muscle damage after eccentrically biased downhill running in rats. *Acta Physiol Scand* 1999; 165: 57-63.

- Komulainen J, Kytola J and Vihko V. Running-induced muscle injury and myocellular enzyme release in rats. *J Appl Physiol* 1994; 77: 2299-2304.
- Kurina LM, Gulati M, Everson-rose SA, et al. The effect of menopause on grip and pinch strength: results from the Chicago, Illinois, site of the study of women's health across the nation. *Am J Epidemiol* 2004; 160: 484-91.
- LeRoith D, McGuinness M, Shemer J, Stannard B, Lanau F, Faria TN, Kato H, Werner H, Adamo M, and Roberts CT. Insulin-like growth factors. *Biol Signals* 1992; 1: 173-181.
- Mackey AL, Kjaer M, Dandanell Jurgensen S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koshinen SO, Jensen CH, Schoder HD, Langburg H. The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol Inpress*: doi: 10.1152/jappphysiol.00157. 2007.
- MacIntyre DL, Slawnych MP, Reid WD, McKenzie DC. Fatigue of the knee extensor muscles following eccentric exercise. *Electromyogr Clin Neurophysiol*. 1998 Jan-Feb; 38(1): 3-9.
- McClung JM, Davis JM, Carson JA. Ovarian hormone status and skeletal muscle inflammation during recovery from disuse in rats. *Exp Physiol*. 2007 Jan; 92(1):219-32.
- McClung JM, Davis JM, Wilson MA, Goldsmith EC, Carson JA. Estrogen status and skeletal muscle recovery from disuse atrophy. *J Appl Physiol* 2006; 100: 2012-2023.
- McCormick KM, Burns KL, Piccone CM, Gosselin LE, and Brazeau GA. Effects of ovariectomy and estrogen on skeletal muscle function in growing rats. *J Muscle Res Cell Motil* 2004; 25: 21-27.
- McCully KK and Faulkner JA. Injury to skeletal muscle fibers of mice following lengthening contractions. *J Appl Physiol* 1985; 59(1): 119-26.
- McLoon LK, Nguyen LT, Wirtschafter J. Time course of the regenerative response in bupivacaine injured orbicularis oculi muscle. *Cell Tissue Res*. 1998 Dec; 294(3):439-47.
- Mendias CL, Tatsumi R, and Allen RE. Role of cyclooxygenase-1 and -2 in satellite cell proliferation, differentiation, and fusion. *Muscle Nerve* 2004; 30: 497-500.

- Mishra DK, Friden J, Schmitz MC, Lieber RL. Anti-inflammatory medication after muscle injury. A treatment resulting in short-term improvement but subsequent loss of muscle function. *J Bone Joint Surg Am*. 1995 Oct; 77(10): 1510-9.
- Moran AL, Nelson SA, Landisch RM, Warren GL, Lowe DA. Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice. *J Appl Physiol*. 2007 Apr; 102(4): 1387-93.
- Moran AL, Warren GL, and Lowe DA. Removal of Ovarian hormones from mature mice detrimentally affects muscle contractile function and myosin structural distribution. *J Appl Physiol* 2006; 100: 548-559.
- Moran AL, Warren GL, Lowe DA. Soleus and EDL muscle contractility across the lifespan of female C57BL/6 mice. *Exp Gerontol*. 2005 Dec; 40(12): 966-75.
- Morozov VI, Tsyplenkov PV, Golberg ND, Kalinski MI. The effects of high-intensity exercise on skeletal muscle neutrophil myeloperoxidase in untrained and trained rats. *Eur J Appl Physiol*. 2006 Aug; 97(6): 716-22.
- Nikolaou PK, MacDonald BL, Gilsson RR, Seaber AV, Garrett WE. Biomechanical and histological evaluation of muscle after controlled strain injury. *Am J Sports Med* 1987; 15: 9-14.
- Node K, Kitakaze M, Kosaka H, Minamino T, Funaya H, Hori M. Amelioration of ischemia- and reperfusion-induced myocardial injury by 17beta-estradiol: role of nitric oxide and calcium-activated potassium channels. *Circulation*. 1997 Sep 16; 96(6):1953-63.
- Orchard J and Best TM. The management of muscle strain injuries: an early return versus the risk of recurrence. *Clin J Sport Med*. 2002 Jan; 12(1): 3-5.
- Otis JS, Burkholder TJ, and Pavlath GK. Stretch-induced myoblast proliferation is dependent on the COX pathway. *Exp Cell Res*. 2005 Nov 1; 310(2): 417-25.
- Pansarasa O, Castagna L, Colombi B, Vecchiet J, Felzani G, Marzatico F. Aged and sex difference in human skeletal muscle: role of reactive oxygen species. *Free Radic Res* 2000; 33: 287-293.
- Pansarasa O, Castagna L, Colombi B, Vecchiet J, Felzani G, Marzatico F. Aged and sex difference in human skeletal muscle: role of reactive oxygen species. *Free Radic Res* 2000; 33: 287-293.

- Piccone CM, Brazeau GA, McCormick KM. Effect of oestrogen on myofibre size and myosin expression in growing rats. *Exp Physiol*. 2005 Jan; 90(1): 87-93.
- Pizza FX, Peterson JM, Baas JH, Koh TJ. Neutrophils contribute to muscle injury and impair its resolution after lengthening contractions in mice. *J Physiol*. 2005 Feb 1; 562(Pt 3): 899-913.
- Powers SK, Kavazis AN, and DeRuisseau KC. Mechanism of disuse muscle atrophy: role of oxidative stress. *Am J Physiol Integr Comp Physiol* 2005; 288: R337-R344.
- Prisk V and Huard J. Muscle injuries and repair: The role of prostaglandins and inflammation. *Histol Histopathol* 2003; 18: 1243-1256.
- Prorock AJ, Hafezi-Moghadam A, Laubach VE, Liao JK, Ley K. Vascular protection by estrogen in ischemia-reperfusion injury requires endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol*. 2003 Jan; 284(1):H133-40.
- Proske U and Allen TJ. Damage to Skeletal Muscle from Eccentric Exercise. *Exerc Sport Sci Rev* 2005; 33: 298–104.
- Pyne DB. Regulation of neutrophil function during exercise. *Sports Med*. 1994 Apr; 17(4): 245-58.
- Radak Z, Pucsok J, Mecseki S, Csont T, Ferdinandy P. Muscle soreness-induced reduction in force generation is accompanied by increased nitric oxide content and DNA damage in human skeletal muscle. *Free Radic Biol Med* 1999; 26: 1059–1063.
- Raj DA, Booker TS, Belcastro AN. Striated muscle calcium-stimulated cysteine protease (calpain-like) activity promotes myeloperoxidase activity with exercise. *Pflugers Arch*. 1998 May; 435(6): 804-9.
- Richard D, Rochon L, Deshaies Y. Effects of exercise training on energy balance of ovariectomized rats. *Am J Physiol*. 1987 Nov; 253(5 Pt 2): R740-5.
- Roth S, Martel G, Ivey F, et al. High-volume, heavy-resistance strength training and muscle damage in young and older women. *J Appl Physiol* 2000; 88: 1112-1118.
- Schmalbruch H and Hellhammer U. The number of nuclei in adult rat muscles with special reference to satellite cells. *Anat Rec* 1977; 189: 169–175.
- Schneider BS, Tiidus PM. Neutrophil infiltration in exercise-injured skeletal muscle: how do we resolve the controversy? *Sports Med*. 2007; 37(10):837-56.

- Schultz E and McCormick KM. Skeletal muscle satellite cells. *Rev Physiol Biochem Pharmacol* 1994; 123: 213–257.
- Schultz E, Chamberlain C, McCormick KM, Mozdziak PE. Satellite cells express distinct patterns of myogenic proteins in immature skeletal muscle. *Dev Dyn*. 2006 Dec; 235(12):3230-9.
- Seale P and Rudnicki MA. A new look at the Origin, Function, and “Stem-Cell” status of Muscle Satellite cells. *Developmental Biology* 2000; 218: 115-124.
- Seeley DG, Cauley JA, Grady D, et al. Is postmenopausal estrogen therapy associated with neuromuscular function or falling elderly women? *Arch Intern Med* 1995; 155: 293-9.
- Shen W, Li Y, Tang Y, Cummins J, Huard J. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. *Am J Pathol*. 2005 Oct; 167(4): 1105-17.
- Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-beta1, and the COX-2 pathway during the inflammatory phase of skeletal muscle healing after injury. *J Cell Physiol*. 2008 Feb; 214(2):405-12.
- Shen WL, Yong L, Tang Y, Cummins J, Huard J. NS-398, a cyclooxygenase-2-specific inhibitor, delays skeletal muscle healing by decreasing regeneration and promoting fibrosis. *Am J Pathol* 2005; 167: 1105-1107.
- Smith CK, 2nd, Janney MJ, Allen RE. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *J Cell Physiol*. 1994 May; 159(2): 379-85.
- Smith HK, Maxwell L, Martyn JA, Bass JJ. Nuclear DNA fragmentation and morphological alterations in adult rabbit skeletal muscle after short-term immobilization *Cell Tissue Res* 2000; 302: 235–241.
- Smith JK, Grisham MB, Granger ND and Korthuis RJ. Free radical defense mechanisms and neutrophil infiltration in post-ischemic skeletal muscle. *Am J Physiol* 1989; 256 (Heart Circ Physiol 25), H789-793.
- Snow MH. The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res* 1977; 185: 399–408.

- Soltow QA, Betters JL, Sellman JE, Lira VA, Long JH, Criswell DS. Ibuprofen inhibits skeletal muscle hypertrophy in rats. *Med Sci Sports Exerc* 2006; 38: 840-846.
- Sotiriadou S, Kyparos A, Albani M, Arsos G, Clarke MS, Sidoras G, et al. Soleus muscle force following downhill running in ovariectomized rats treated with estrogen. *Appl Physiol Nutr Metab.* 2006 Aug; 31(4): 449-59.
- Sotiriadou S, Kyparos A, Mougios V, Trontzos CH, Sidoras G, Atziari CH. Estrogen Effect on Some Enzymes in Female Rats after Downhill Running. *Physiol. Res.* 2003; 2: 743-748.
- Squadrio F, Altavilla D, Squadrito G, et al. 17-oestradiol reduces cardiac leukocyte accumulation in myocardial ischemia reperfusion injury in rat. *Eur J Pharmacol* 1997; 335: 185-192.
- St Pierre Schneider B, Correia LA, Cannon JG. Sex differences in leukocyte invasion in injured murine skeletal muscle. *Res Nurs Health.* 1999 Jun; 22(3): 243-50.
- Stauber WT, Clarkson PM, Fritz VK, et al. Extracellular matrix disruption and pain after eccentric muscle action: *J Appl Physiol* 1990; 69: 868-74.
- Steiner SM, Hu Y and Steiner MR. Regulation of Prostaglandin H synthase 1 and 2 in MyoD transfected cells. *Exp Cell Res* 1995; 218: 389-393.
- Stinick M, Foley AM, Brown M, and Spangenburg EE. Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. *J Appl Physiol* 2006; 100: 286-293.
- Stupka N, Lowther S, Chorneyka K, et al. Gender differences in muscle inflammation after eccentric exercise. *J Appl Physiol* 2000; 89: 2325-32.
- Stupka N, Tiidus PM. Effects of ovariectomy and estrogen on ischemia-reperfusion injury in hindlimbs of female rats. *J Appl Physiol.* 2001 Oct; 91(4):1828-35.
- Sugioka K, Shimosegawa Y, Nakano M. Estrogens as natural antioxidants of membrane phospholipid peroxidation. *FEBS Lett.* 1987 Jan 1; 210(1): 37-9.
- Suzuki S and Yamamuro T. Long-term effects of estrogen on rat skeletal muscle. *Exp Neurol* 1985; 87: 291-299.
- Tamaki T, Uchiyama S, Uchiyama Y, Akatsuka A, Yoshimura S, Roy RR, et al. Limited myogenic response to a single bout of weight-lifting exercise in old rats. *Am J Physiol Cell Physiol.* 2000 Jun; 278(6):C1143-52.

- Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev Biol*. 1998 Feb 1; 194(1):114-28.
- Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, Allen RE. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. *Mol Biol Cell*. 2002 Aug; 13(8): 2909-18.
- Teixeira CF, Landucci EC, Antunes E, Chacur M, Cury Y. Inflammatory effects of snake venom myotoxic phospholipases A2. *Toxicon* 2003; 42: 947–962.
- Tero AH Järvinen, Teppo LN Järvinen, Kääriäinen M, Kalimo H, and Järvinen M. Muscle Injuries Biology and Treatment. *The American Journal of Sports Medicine* 2005; 33: 5.
- Thorsson O, Rantanen J, Hurme T, and Kalimo H. Effects of Nonsteroidal Antiinflammatory Medication on Satellite Cell Proliferation During Muscle regeneration. *The American Journal of Sports Medicine* 1998; 26: 2.
- Tidball JG. Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc*. 1995 Jul; 27(7):1022-32.
- Tidball JG. Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol*. 2005 Feb; 288(2): R345-53.
- Tiidus PM, Bombardier F. Oestrogen attenuates post exercise myeloperoxidase activity in skeletal muscle of male rats. *Acta Physiol Scand* 1999; 166: 85-90.
- Tiidus PM, Deller M, Liu XL. Oestrogen influence on myogenic satellite cells following downhill running in male rats: a preliminary study. *Acta Physiol Scand*. 2005 May; 184(1):67-72.
- Tiidus PM, Holden D, Bombardier E, Zajichowski S, Enns D, Belcastro A. Estrogen effect on post-exercise skeletal muscle neutrophil infiltration and calpain activity. *Can J Physiol Pharmacol* 2001; 79: 400-406.
- Tiidus PM, Houston ME. Vitamin E status does not affect the responses to exercise training and acute exercise in female rats. *J Nutr*. 1993 May; 123(5): 834-40.
- Tiidus PM. Can estrogens diminish exercise induced muscle damage? *Can J Appl Physiol*. 1995 Mar; 20(1): 26-38.
- Tiidus PM. Estrogen and gender effects on muscle damage, inflammation, and oxidative stress. *Can J Appl Physiol* 2000; 25: 274-287.

- Tiidus PM. Oestrogen and sex influence on muscle damage and inflammation: evidence from animal models. *Curr Opin Clin Nutr Metab Care* 2001; 4:509-513.
- Toth MJ, Poehlman ET, Matthews DE, Tchernof A, MacCoss MJ. Effects of estradiol and progesterone on body composition, protein synthesis, and lipoprotein lipase in rats. *Am J Physiol Endocrinol Metab*. 2001 Mar; 280(3): E496-501.
- Trappe TA, Fluckey JD, White F, Lambert CP, Evans WJ. Skeletal muscle PGF2 and PGF2 in response to eccentric resistance exercise: Influence of ibuprofen and acetaminophen. *J Clin Endocrinol Metab* 2001; 86: 5067-5070.
- Tsai WJ, McCormick KM, Brazeau DA, Brazeau GA. Estrogen effects on skeletal muscle insulin-like growth factor 1 and myostatin in ovariectomized rats. *Exp Biol Med (Maywood)*. 2007 Nov; 232(10): 1314-25.
- Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* 2003; 189: 41-54.
- Vignaud A, Cebrian J, Martelly I, Caruelle JP, and Ferry A. Effect of anti-inflammatory and antioxidant drugs on the long-term repair of severely injured mouse skeletal muscle. *Exp Physiol* 2005; 90(4): 487-495.
- Wade GN. Gonadal hormones and behavioral regulation of body weight. *Physiol. Behav.* 1972; 8: 523-534.
- Warren GL, Lowe DA, Inman CL, Orr OM, Hogan HA, Bloomfield SA, et al. Estradiol effect on anterior crural muscles-tibial bone relationship and susceptibility to injury. *J Appl Physiol*. 1996 May; 80(5): 1660-5.
- Warren GL, Williams JH, Ward CW, Matoba H, Ingalls CP, Hermann KM, and Armstrong RB. Decreased contraction economy in mouse EDL muscle injured by eccentric contractions. *J Appl Physiol* 1996; 81: 2555–2564.
- Wattanapernpool J, Reiser PJ. Differential effects of ovariectomy on calcium activation of cardiac and soleus myofilaments. *Am J Physiol*. 1999 Aug; 277(2 Pt 2): H467-73.
- Widrick JJ, Maddalozzo GF, Lewis D, et al. Morphological functional characteristics of skeletal muscle fibers from hormone-replaced and nonreplaced postmenopausal women. *Gerontol A Biol Sci Med* 2003; 58: 3-10.

- Wiik A, Glenmark B, Ekman M, Esbjornsson-Liljedahl M, Johansson O, Bodin K, Enmark E, Jansson E. Oestrogen receptor beta is expressed in adult human skeletal muscle both at the mRNA and protein level. *Acta Physiol Scand*. 2003 Dec; 179(4): 381-7.
- Wolthers T, Hoffman DM, Nugent AG, Duncan MW, Umpleby M, Ho KK. Oral estrogen antagonizes the metabolic actions of growth hormone in growth hormone-deficient women. *Am J Physiol Endocrinol Metab*. 2001 Dec; 281(6): E1191-6.





APPENDIX I

GENERAL SOLUTIONS

A. Immunohistochemistry Washing Buffers

Phosphate Buffered Saline (PBS)

10X PBS (0.1M PBS, pH 7.2):

Na ₂ HPO ₄ (anhydrous)	10.9 g
NaH ₂ PO ₄ (anhydrous)	3.2 g
NaCl	90 g
Distilled water	1000ml

Mix to dissolve and adjust pH to 7.2

Store this solution at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary.

10X PBS-Tween 20 (0.1 PBS, 0.5% Tween 20, pH 7.2):

Na ₂ HPO ₄ (anhydrous)	10.9 g
NaH ₂ PO ₄ (anhydrous)	3.2 g
NaCl	90 g
Distilled water	1000 ml

Mix to dissolve and adjust pH to 7.2 and then add 5 ml of Tween 20.

Store this solution at room temperature. Dilute 1:10 with distilled water before use and adjust pH if necessary

B. Fixation

NaH ₂ PO ₄	1.86 g
NaOH	0.42 g
Formadehyde (from 37% formaldehyde)	10 ml
H ₂ O	90 ml

C. Homogenizing Buffer 100 ml

10 mM Tris	0.121 g
10 mM NaCl	0.058 g
0.1mM EDTA	3.724 mg

Mix to dissolve, then made up to 100 ml with distilled water and adjust pH to 7.6 and store this solution at 4°C.

D. MPO Assay**Homogenizing Buffer**50 mM Potassium Phosphate Buffer w/0.5% HTAB

K ₂ HPO ₄	0.871 g
KH ₂ PO ₄	0.6805 g
HTAB	0.05 g

Then made up to 100 ml with distilled water and adjust pH to 6.0.

Store this solution at 4°C.

Solution A50 mM Potassium Phosphate Buffer (pH 6.0)

K ₂ HPO ₄	0.871 g
KH ₂ PO ₄	0.6805 g
O-diasinidine	0.0167 g
H ₂ O ₂	2 µl

Then made up to 100 ml with distilled water.

Store this solution at 4°C.

Procedure

- Add 10 µl of supernatant to 290 µl of solution A
- Measure the changes in absorbance per minute at 480 nm, 25°C.
Use kinetic protocol, run for 5 minutes and read every 30 seconds.
- 1 Unit MPO defined as change in absorbances of 1.0 per minute. Normalize per gram of weight.

D. Reference

Smith JK, Grisham MB, Granger ND and Korthuis RJ. Free radical defense mechanisms and neutrophil infiltration in post-ischemic skeletal muscle. *Am J Physiol* 1989; 256 (Heart Circ Physiol 25), H789-793.

E. Krebs's Helesleit Buffer

Krebs-Henseleit buffer	4.8g
D-glucose	2.0 g/L
Magnesium Sulfate [anhydrous]	0.141 g/L
Potassium Phosphate Monobasic	0.16 g/L
Potassium Chloride	0.35 g/L
Sodium Chloride	6.9 g/L
Sodium Bicarbonate	1.05 g
CaCl ₂	0.147 g

Then made up to 500 ml with distilled water and adjust pH to 7.4.
Store this solution at 4°C.

E. Reference

Felice B, Paul G, Ryall JG, Plant DR, Sillence MN, and Lynch GH. Beta2-adrenoceptor agonist fenoterol enhances functional repair of regenerating rat skeletal muscle after injury. *J Appl Physiol* 2004; 96: 1385–1392.

APPENDIX II

CONTRACTILE CHARACTERISTICS

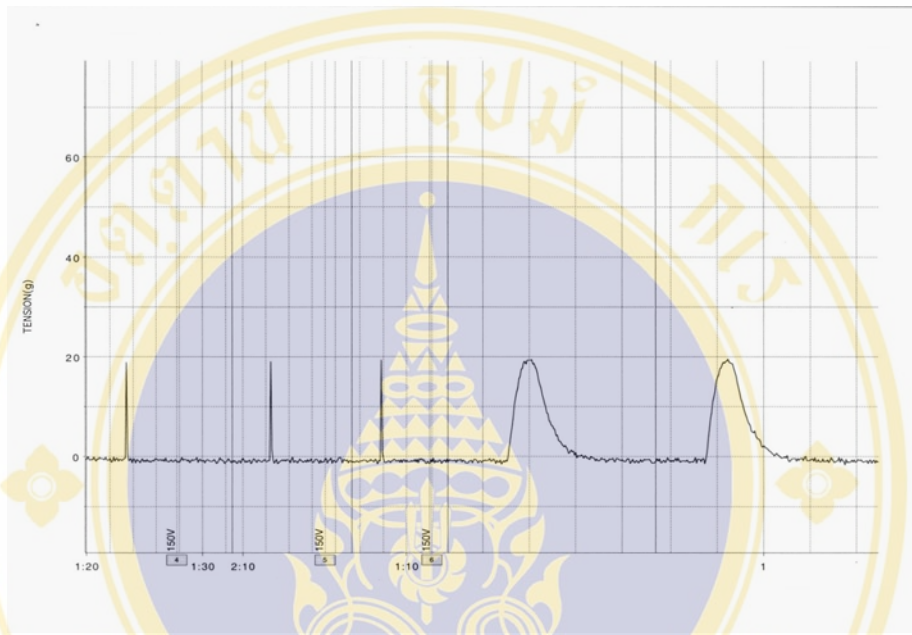


Figure 30 An example of peak twitch tension (g)

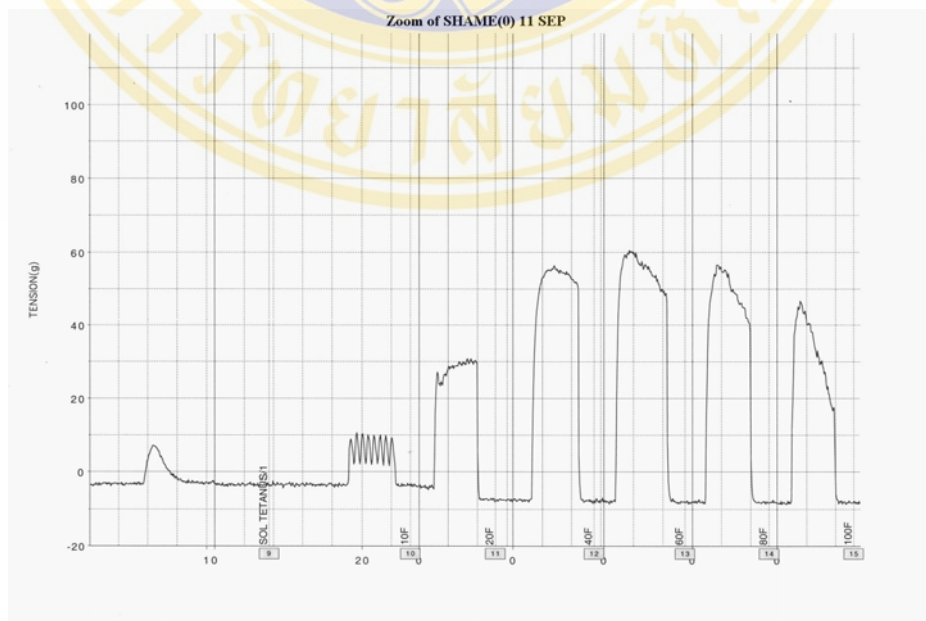


Figure 31 An example of peak tetanic tension (g)

BIOGRAPHY



NAME:	Miss Khajirat Netnee
DATE OF BIRTH:	19 August 1982
PLACE OF BIRTH:	Udonthani, Thailand
INSTITUTIONS ATTENDED:	Mahidol University, Bachelor of Physical Therapy Mahidol University, Master of Science (Exercise Physiology)
RESEARCH GRANT:	King Prajadhipok and Queen Rambhai Barni Memorial Foundation. Thesis Scholarships, Mahidol University for the research grant.
HOME ADDRESS:	12 Ponepisai Rd, Muang, Udonthani. Thailand