Thesis of Master of Science

Effects of aerobic and resistance exercise training on leukemia inhibitory factor in skeletal muscles of aging mice

노화쥐의 골격근에서 유산소 운동 및 저항성 운동이 leukemia inhibitory factor 발현에 미치는 영향

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February 2017

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Effects of aerobic and resistance exercise training on leukemia inhibitory factor in skeletal muscles of aging mice

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A Thesis submitted in partial fulfillment of
The requirement for the degree of
MASTER OF SCIENCE

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February 2017

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ABSTRACT

Introduction

Aging is an inevitable phenomenon in all living creature, deteriorating the body composition and physical activity capacity leading to mortality. Specially, age-related loss of muscle mass and strength are defined as Sarcopenia. Leukemia inhibitory factor, LIF, is not only known as a newly discovered myokine but also, an important player in skeletal muscle hypertrophy and regeneration following exercise and injury status. However, there is no study that reports a change of LIF expression in a chronic and regular exercise. As such, there was no relating study the expression of LIF during aging. Therefore, the purpose of this study is to investigate 12 weeks of resistance and aerobic exercise on expression of LIF in skeletal muscle of aging mice.

Methods

Twenty 19 months old male C57BL/6 mice were randomly assigned to each of the following groups: OLD-CON, sedentary aging mice group (n=6); OLD-AEX, 12 weeks of treadmill exercise group (n=7), OLD-REX, 12 weeks of resistance ladder climbing exercise group (n=7). Following 1 week of adaptation, OLD-AEX was performed for 12 weeks of forced treadmill exercise 3 days per week, and OLD-REX was performed for 12 weeks of resistance ladder climbing 3 days per week. During the intervention period, food intake was measured every week and grip strength and hanging tests were measured every two weeks. Body composition was measured by DXA (Dual Energy X-ray Absorptiometry) and LIF protein level was detected by using ELISA in each of muscle, including soleus (SOL), gastrocnemius (GAS), tibialis anterior (TA), and extensor digitorum longus (EDL). Western blotting analysis were performed to measure muscle pro-inflammatory factor, tumor necrosis factor-alpha (TNF- α) and

interleukin-1 beta (IL-1β) in soleus muscle. Moreover, physical activity test was measured at the end of period.

Results

There was no significant difference between groups in the body composition and weekly body weight change. The change of grip strength was significantly increased in OLE-AEX compare to OLD-CON. Muscle cross sectional area was significantly increased in OLD-AEX and OLD-REX compare to OLD-CON. The free weight hanging test was no significant difference between each groups, but weighted bearing hanging test was significantly increased in OLD-AEX compare to OLD-CON. Also, physical activity test was significantly increased in both of OLD-AEX and OLD-REX compare to OLD-CON. Both of OLD-AEX and OLD-REX LIF protein levels in SOL, EDL, and GAS were significantly decreased compare to OLD-CON. However, TA LIF protein level was significantly decreased in OLD-REX. In addition, there was a negative correlation between SOL LIF protein level and hindlimb lean mass. TNF- α and IL-1β protein expression in soleus muscle were no significant difference between each groups. Negative correlation was found between soleus muscle LIF protein level and hindlimb lean mass.

Conclusion

12 weeks of aerobic and resistance exercise training increased the grip strength, muscle cross sectional area, weighted hanging time, and physical activity. However, 12 weeks of aerobic and resistance exercise training decreased LIF protein levels in hindlimb muscle, and there was a negative correlation between soleus muscle LIF protein level and hinblimb lean mass.

Keywords: Aging, LIF, treadmill exercise, Progressive resistance ladder climbing

LIST OF ABBREVIATIONS

LIF	leukemia inhibitory factor
OLD-CON	aging sedentary group
OLD-AEX	aging aerobic exercise group
OLD-REX	aging resistance ladder climbing exercise group
TNF- α	tumor necrosis factor alpha
IL-1 β	interleukin 1 beta
SOL	soleus muscle
GAS	gastrocnemius muscle
TA	tibialis anterior muscle
EDL	extensor digitorum longus muscle
CAS	cross sectional area

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I. INTRODUCTION

1. Significance of the study

The phenomenon of aging is progressive and unavoidable in all living creatures. Despite the number of research and effort continues to overcome aging, no clue has not been found. Aging causes defective mitochondrial energetics, reduces skeletal muscle mass, and increase metabolic dysfunction (Cartee, Hepple, Bamman, & Zierath, 2016). Among all the symptoms of aging, age-related loss of skeletal muscle mass and function is one of the most widespread symptoms in aging. In 1988 at a meeting in Albuquerque, Rosenberg referred to the phenomenon as "Sarcopenia" (Rosenberg, 1989). To protect and delay age-related loss of skeletal muscle mass, regular exercise is one of the effective way to counteract aging-related skeletal muscle loss (Landi, Marzetti, Martone, Bernabei, & Onder, 2014). Repeating endurance exercise, low-resistance load performed at least three to five times per week for several weeks way can improve insulin sensitivity and reduce body fat (Cartee et al., 2016). Also, resistance exercise can slow the decline in skeletal muscle and strength in an aging state, and stimulate muscle hypertrophy that is included in low load intensity activity and regular resistance exercise of a single or few repetitions performed at least three to five times weekly for several weeks (Cartee et al., 2016).

Exercise reduces the risk of death by preventing metabolic diseases and protects against chronic diseases (Rogers, King, Hagberg, Ehsani, & Holloszy, 1990). Among the variable benefits of exercise, a well known impact are the prevention of harmful effects of pro-inflammatory adipokines through skeletal muscle-secreted

proteins (Shetty, Kusminski, & Scherer, 2009). Recently, a number of studies demonstrated that the endocrine effects of muscle fiber derived cytokines or peptides produced and secreted during skeletal muscle contraction (B. Pedersen et al., 2003). Given that muscle is as a secretory organ, LIF is a newly discovered myokine (Christa Broholm & Pedersen, 2010). Leukemia inhibitory factor has a variable array of actions, including acting as a stimulus for formation of platelet, bone formation, neural survival and formation (Broholm & Pedersen, 2010; Metcalf, 2003).

The important roles of LIF have been found in skeletal muscle hypertrophy and regeneration by enhancing cell proliferation though the common signaling mediators janus kinase (JAK), signal transducer and activator of transcription-3 (STAT3), and phosphoinositiede-3 kinase (Alter, Rozentzweig, & Bengal, 2008; Diao, Wang, & Wu, 2009; Spangenburg & Booth, 2002). The number of studies showed that LIF mRNA is upregulated exogenously after muscle injury, including muscle crush and contusion injury (Kurek et al., 1997). Among its various roles of LIF in muscle satellite cell, LIF has a role in proliferation for proper muscle hypertrophy and regeneration and inducing anti-inflammatory effect in regenerating skeletal muscle (Hunt, Upadhyay, Jazayeri, Tudor, & White, 2013).

Exercise-related LIF expression have been studied during the past two decades, however, its expression during exercise has been controversially remained. LIF mRNA levels increased after a single bout of both cycle ergometer exercise and heavy resistance exercise in human skeletal muscle, whereas LIF protein levels were not significantly changed (Broholm et al., 2011; Broholm et al., 2008). Despite of LIF promoting survival of myoblasts in dystrophic muscle, LIF mRNA levels were found to decrease after 2 weeks of voluntary wheel running in mdx mice (Hunt et al., 2011).

Numerous studies have attempted to find and explore the potential beneficial effects from exercise-related LIF expression. Nevertheless there remains an unexplained aspect including expression of protein level and exercise duration or type.

Therefore, the purpose of this study is to examine how a different type of exercise, which is resistance or aerobic exercise, stimulates the expression of LIF levels in skeletal muscle and to confirm the change of physiological characteristics in aging groups.

2. Purpose of the study

The purpose of this study is to provide the effect of resistance training and aerobic training on muscle strength and physiological fitness in old mice and to investigate the age-related difference muscle LIF protein level in aging mice.

3. Research hypothesis

1) 12 weeks of resistance ladder climbing exercise would induce an increase in muscle strength and skeletal muscle hypertrophy in old mice.

2) 12 weeks of aerobic and resistance exercise would induce increase skeletal muscle endurance capacity.

2) 12 weeks of aerobic and resistance exercise would induce increase physical activity

3) The cumulative exercise-induced LIF expression levels would be expressed in greater amount in the exercise groups than OLD-CON group.

4) There would be an association between exercise-induced LIF and lean body mass.

II. LITERATURE REVIEW

1. Aging

1.1 Skeletal muscle and aging

Aging is associated with a significant decrease in variable neuromuscular function and physical performance from adult to senescence(Doherty, 2003; Doherty, Vandervoort, & Brown, 1993; Vandervoort, 2002). The remarkable feature of these decline is the inevitable reduction in skeletal muscle mass which by 1-2% each year beyond the age of 50 and associated loss of muscle strength. Per Resenberg, age-related loss of skeletal muscle mass and strength firstly coined the term Sacropenia (Rosenberg, 1989).

Given that skeletal muscle mass accounts for up to 60% of body mass, loss of skeletal muscle mass is induced a pathological consequence. The consequences of sarcopenia are often severe in older adults, as the strength and functional decline associated with sarcopenia can in turn contribute to several adverse health outcomes, including loss of function, disability, and frailty (Dufour, Hannan, Murabito, Kiel, & McLean, 2013; Fried et al., 2001; Walston, 2012; Xue, Walston, Fried, & Beamer, 2011). Skeletal muscle was physiological and morphological changes with advancing age are characterized by decreasing in size and number of skeletal muscle fibers. An increase in type, or fast-twitch muscle fibers, increased with a marked infiltration of fibrous and an adipose tissues into the skeletal muscle as a result of aging (Lexell, 1995). In addition, aging-related loss of skeletal muscle mass is also exacerbated indirectly by obesity, with a vicious cycle between an increased fat mass and the concomitant a decrease in skeletal

muscle mass (Heber et al., 1996). These deleterious cycles can lead the elderly to an increased risk for a variety of disease and disabilities.

Aging deteriorates the immune system and induce the chronic low-grade inflammation system which like such as type 2 diabetes mellitus (Cesari et al., 2005; Paolisso et al., 1998; Pedersen et al., 2003), dementia (Bruunsgaard et al., 1999), and rheumatologically disease (Peake, Della Gatta, & Cameron-Smith, 2010). Many studies shown that aging can cause inadequate repair and maladaptation to injury might also contribute to a decline in muscle mass with age. Also, Peake J et al. reported that inappropriate inflammatory responses to muscle injury could explain long-term deterioration muscle mass and function in the aging (Peake et al., 2010).

1.2 Aging and exercise

For the past three decades, there has been many studies to elucidate the impact of exercise as a countermeasure for muscle atrophy in aging (Cartee et al., 2016). Progressive resistance exercise training is considered the most accepted strategy to promote myofiber hypertrophy and have shown remarkable gains in strength and power in atrophied older adults, with more proven efficacy than pharmacologic or nutritional alternatives (Bamman, Petrella, Kim, Mayhew, & Cross, 2007; Landi et al., 2014). Also, resistance exercise program impacts mainly on the muscle fiber cross-sectional area which is increased the number of myofibrils, with the fast-twitching fiber type such as Type IIa and IIx(Kim, Kosek, Petrella, Cross, & Bamman, 2005; Kosek, Kim, Petrella, Cross, & Bamman, 2006).

On the other hand, aerobic exercise generally maintains and improves maximum aerobic power as a consequence of the benefits to the cardiovascular system and trained skeletal muscle (Landi et al., 2014). In addition, the skeletal muscle mitochondrial compartment can rapidly be expanded with aerobic exercise and muscle capillarity are increased to enhance the oxygen uptake into muscle mitochondria (Landi et al., 2014). Strasser et al. study showed that the exercise program was divided into endurance and resistance training to compare each of exercise effect (Strasser, Keinrad, Haber, & Schobersberger, 2009). Forty-two health patients living in the community participated in the program and were randomized to receive 6 months of endurance exercise and performed on an ergometer or 6 months of resistance exercises. The resistance training group showed a significant increase in maximum strength, but the endurance group improved the aerobic power resulting in a significant deduction of body fat (Strasser et al., 2009). In order to more fully account for preventing loss of muscle mass in aging, it is necessary to study the potent therapeutic target for sarcopenia and to perform the exercise.

2. Leukemia Inhibitory Factor (LIF)

2.1 Leukemia inhibitory factor

Leukaemia inhibitory factor (LIF) is a newly discovered myokine (Broholm et al., 2008) and belongs to the IL-6 cytokine superfamily which consists of structurally and functionally related proteins named neuropoietins or gp130 cytokines (Broholm & Pedersen, 2010). The neuropoietins exhibit pleiotropy and redundancy in biological activities, and they all share the gp130 receptor component, a common transducer of their receptor complexes.

Hilton et al. identified LIF as a protein secreted from ascites tumour cells in 1988 (Hilton, Nicola, & Metcalf, 1988). The initial reported function of LIF was induced terminal differentiation of myeloid leukaemic cells, hence its name LIF. LIF has multiple biological functions, such as acting as a stimulus for platelet formation, proliferation of haematopoietic cells, bone formation, neural survival and formation, muscle satellite cell proliferation and acute phase production by hepatocytes (Metcalf, 2003). Recently, among its various roles, a new light was shed on including acting for proper muscle hypertrophy and regeneration. It has been recently discovered that LIF has an anti-inflammatory role through LIF receptor signaling in regenerating skeletal muscle (Hunt et al., 2013).

Whether LIF has a role in muscle hypertrophy and regeneration or not, Spangenburg et al. performed that LIF null (-/-) mice were unable to enlarge their muscle size in response to increase muscle load. Following these work, Spangenburg suggested that LIF was an important factor in muscle hypertrophy. In addition, skeletal muscle regeneration in an aspect, LIF stimulates muscle regeneration in mice suffering from

muscle dystrophy, and LIF null (-/-) mice show decreased muscle regeneration following muscle injury (Spangenburg & Booth, 2002).

When skeletal muscles responded to physical trauma, injury, or exercise, LIF mRNA was up-regulated as a responses. LIF knockout mice showed a decrease in the size of regenerated myofibers after crush injury compared to wild type mice (Kurek et al., 1997). However, targeted infusion of LIF in both normal and LIF knockout model stimulated muscle regeneration, but the stimulation observed was much greater in the mutant animals than in control. In addition, Donato R et al. demonstrated that LIF has a beneficial effect on skeletal muscle mass following nerve repair (Donato et al., 1995; Tham et al., 1997). In order to confirm the role of muscle-derived LIF, Austin et al. demonstrated LIF stimulated myoblast proliferation in culture (Austin, Bower, Kurek, & Vakakis, 1992). A further experiment confirmed that the LIF was induced to satellite cell and myoblast proliferation through the common signaling mediators janus kinase, signal transducer and activator of transcription-3 (STAT-3), and phosphoinositide-3-kinase (Alter et al., 2008; Diao et al., 2009; Spangenburg & Booth, 2002).

The inflammation associated with muscle injury necessary for the removal of damaged tissue and can also promote regeneration by interaction with myoblasts that promote myogenic differentiation (Hunt et al., 2013). Hunt. et al. study showed that the predominant role of LIF in skeletal muscle regeneration may be to regulate the inflammatory response (Hunt et al., 2013). However, further research is needed to investigate a role of LIF in relation to the inflammatory phase. Although a number of works suggested that LIF is an important role of muscle hypertrophy and regeneration, there is no studies that has investigated the expression of LIF in aging.

2.1 Leukemia inhibitory factor and exercise

Although LIF is a newly discovered myokine, few studies have attempted to investigate about the exercise-induced LIF expression in skeletal muscle. There are two studies that suggest that LIF is regulated by exercise. One of the studies investigated the exercise-induced LIF expression through aerobic exercise in human skeletal muscles. Eight males conducted cycle ergometer exercise for 3 hours at 60% VO2max. This study showed that LIF mRNA expression in their muscle was up-regulated after cessation of exercise and gradually decreased throughout the post-exercise (Broholm et al., 2008). In the other study, heavy resistance exercise was performed to induce expression of LIF in the human vastus medialis. Eight males conducted heavy resistance exercise for 20 minutes. LIF mRNA expression showed a 9 fold up-regulated after cessation of exercise and gradually decrease throughout the post-exercise (Broholm et al., 2011).

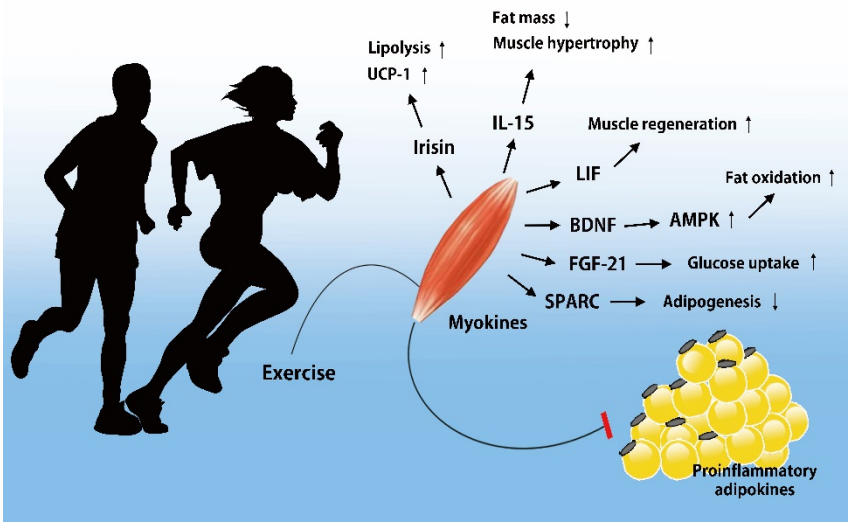


Figure 1. The role of exercise myokines **(So. et al., 2014)**

However, LIF mRNA level were found to decrease after 2 weeks of voluntary wheel running in wild-type muscle, but it was not demonstrated in mdx mice. This study suggested supports the role of LIF in normal muscle biology in response to exercise, and was demonstrated that the potential for anti-inflammatory actions of LIF that promote survival of transplanted myoblasts in dystrophic muscle.

To date, there has been minimal research regarding exercise in relation to LIF expression in skeletal muscle. Also, little is known of exercise-induced LIF expression in skeletal muscle in aging status.

III. METHODS

1. Animal

Nineteen-month old male C57BL/6 mice (Biomedical Mouse Resource Center, Korea) were purchased in order to use for this study. All mice were housed in a controlled adequate environment the temperature (23℃) and humidity (60%) control with a 12 hour light / 12 hour dark cycle. All mice were provided a chow (Purina rodent chow 5057, Purina, Korea) and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. Approved by IACUC number is SNU-130529-1-2. The researcher for this study finished “Animal experiment workshop” by Institute of Laboratory Animal Resources (ILAR) of Seoul National University. The certification number is IAR 13-02-061. In this study, all of experiments were performed to minimize the number of animals used and the suffering caused by the procedures used in the present study.

2. Experimental design

Nineteen-month old male C57BL/6 mice (Biomedical Mouse Resource Center, Korea) were used for this study (N=20). Twenty 19-month-old mice were randomly assigned to each of the following three groups: OLD-CON, control group (n=6); OLD-REX, 12 weeks of resistance ladder climbing exercise group (n=7); OLD-AEX, 12 weeks of treadmill exercise group (n=7). Their food were provided the Purina rodent chow 5057 (Purina, Korea) ad libitum and food intake was measured twice a week. During the intervention, grip strength was measured in order to check the four-limb strength of mice every two weeks.

Before to the onset of intervention, 1 week of adaptation was followed for mice to be familiarized to resistance ladder climbing and treadmill exercise. After 1 weeks of adaption period, OLD-REX group was performed to climb vertical ladder, which is 1-m ladder with inclined at 85° and 1.5cm gird, by attaching weights to mice's tail with clip 3 times a week for 12 weeks and OLD-AEX was performed to running by forced treadmill machine. Following the 12 weeks of exercise intervention, all group of mice were taken to DXA scanning and sacrifice procedure was followed after 48 hour last exercise training in order to minimize effect of the last bout of exercise.

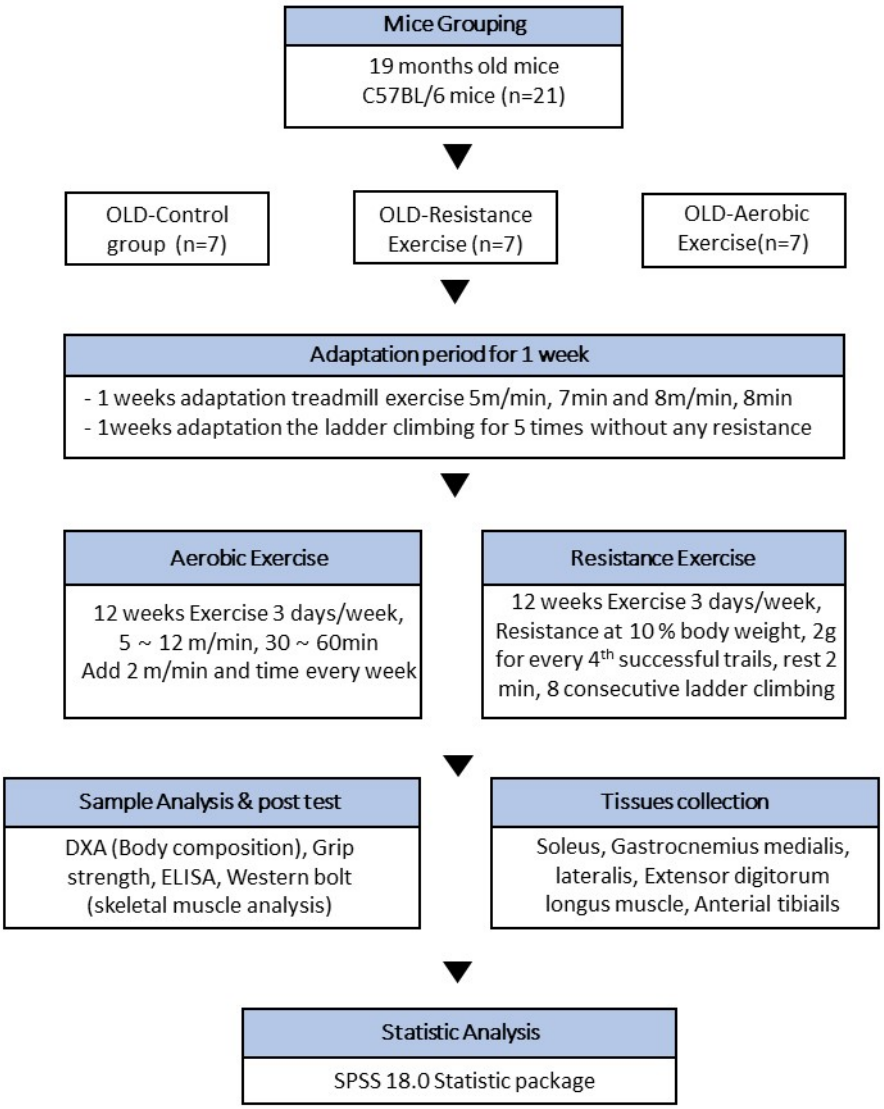


Figure 2. Study design

3. Exercise protocol

3.1 Resistance ladder climbing exercise

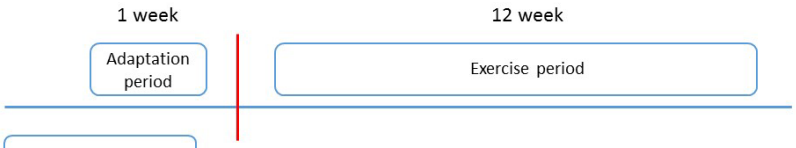
Resistance ladder climbing exercise was performed in the OLD-REX group to climb the vertical ladder 3 days per week for 12 weeks. Resistance ladder climbing exercise was performed by using 1 m ladder with 1.5cm grids and ladder was maintained 85 degree angle with ground during exercise. In order to progressively increase the intensity of resistance exercise, the weight load was gradually increased to mice tail. In order to minimize stress during exercise, stimulating such as food reward, electrical stimulation were not given to mice. The rest time was given to mice 2 minute between the exercise trial sessions after mice reached the top of ladder. The exercise trial was consisted with maximal 8 times consecutive climbing ladder.

For the adaptation period, OLD-REX performed ladder climbing 5 times without any weight attached to their tail and in order to weighted adaption tried to climb 2 times attached on 10g tail weight. After one week of the adaption period, the base of load was set 10 percent of their body weight. In order to progressively increase exercise intensity, 2 gram of additional weights were carried out four successful climbing trails. During each exercise session, the exercise intensity was carefully adjusted because of the mice were very old and fragile.

3.2 Aerobic exercise

Aerobic exercise was performed in the OLD-AEX group to forced treadmill running for 3days per week for 12 weeks. The treadmill machine was specially adapted for mice model by our laboratory. In order to reduce the stress during treadmill running, any outsources such as electrical stimulation was provide to encourage the performance.

For the adaptation period, all of OLD-AEX group mice putted the treadmill lane on to familiarize the environmental for 5 minutes. In order to adapt to the change of speed was carefully performed to increase the running speed from 5 m/min for 7 minutes to 8 m/min for 8 minutes. After the one week adaption period, the OLD-AEX group was performed to run from 30 minute to 1 hour per session and gave to warming up and down time for 3 minutes. The intensity of each exercise session was gradually increased time and intensity by every weeks. Also, we carefully supervised to reduce the risk of any problems during the exercise. The treadmill exercise protocol are presented in under the figure.



Resistance exercise

Training period	12 weeks
Frequency	3 days / week
Repetition	Maximal 8 times per day
Load	10% of BW + 2g for every 4th successful trials to 100% of BW
Rest	2 minutes

Aerobic exercise

Training period	12 weeks
Frequency	3 days / week
Repetition	30 minutes + 5 minutes every 2 weeks to 60 minutes
Load	Gradually increase of speed from 5m/min to maximum of 12 m/min

Figure 3. Exercise protocol

4. Measurements

Table 1. Measurements

Measurements	Method	Model	Company/Country
Body composition	DXA	Discovery W	Hologic, USA
Grip strength	Grip strength meter	Grip Strength Meter	Bioseb, France
Muscle LIF	ELISA	Mouse LIF ELISA kit	CUSABIO, USA
Skeletal muscle fiber Cross section area	Processing system	Innerview 2.0	Innerview, Korea
Skeletal muscle TNF-α	Western blot	Ab6671	Abcam, United Kingdom
Skeletal muscle IL-1β	Western blot	Ab9722	Abcam, United Kingdom

4.1 Body composition - DXA

In order to accurately analysis, in vivo DXA (Dual energy X-ray Absorptiometry) scanning was performed to measure whole body composition, fat percentage, lean mass, bone mineral density and contents of mice using a Hologic Discovery W model (Hologic, USA). Because detection range for small animal have to be greater than 150g, adults Sprague dawley rat was placed on the DXA together in order to fulfil the detection rage of DXA itself. Data collected and analyzed using by Standard Software (QDR for Windows XP Operating System, USA). By using this software, selecting carefully the sub-region to measure and analyze composition, muscle mass, bone mineral and contents was analyzed. All group of mice were anesthetized for DXA scanning and sacrifice was subsequently performed.

Figure 4. DXA (Dual Energy X-Ray Absorptiometry)

4.2 Grip strength

The grip strength was conducted using a Grip Strength Meter (Bioseb, France), and using method that was previously reported in modification of measuring (Meyer, Tilson, Byrd, & Riley, 1979). The grip strength test was performed in four-limb of mice. Four-limb grip strength was performed by allowing the animals to grasp a wired grill attached to the force gauge. This was followed by pulling the animal away from the gauge until the mice released the grill. It provides the value of force was recorded in unit of grams. The test was performed five times in each mice and tested in session of five trial separated by approximately 30 sec between each trail. Among the five measurements in each test, the maximal grip strength record was taken into analysis. The grip strength was measured once in every two weeks.

Figure 5. Grip strength

4.3 Skeletal Muscle Preparation

Following DXA measurement, anesthetized mice's soleus (SOL), extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius (GAS) muscle were rapidly removed and stored at -80 Celsius degree until the protein analysis. To extract the protein from muscle was homogenized in 500$\mu\ell$ of extraction buffer (RIPA). In order to eliminate insoluble materials, the extracts was then centrifuge at 13,000 rpm at 4℃ for 15 min. Insoluble materials was discarded and determination of protein concentrations in the supernatants quantification of protein assay was performed using the Pierce BCA protein assay kit (Thermo Scientific, USA).

4.4 LIF Protein analysis

The skeletal muscle level of LIF were measured by enzyme-linked immunosorbent assays (ELISA), according to the specifications of the manufacturer (Mouse leukemia inhibitory factor Kit; CUSABIO BIOTEC CO., LTD., USA). Detection rage was 1.56 pg/ml to 100 pg/ml for assay. And all samples were run within the range of the standard curve. The results were expressed as concentration of LIF (pg/ml) read from standard curves.

4.5 Hanging test

In order to confirm the skeletal muscle endurance capacity, hanging test was performed to divide a free weight hanging test and weight hanging test of 10g tail weight in mice for every two weeks. The start position that an animal being put on the top of grid and inverted the grid to measure the time until the mice fell in case.

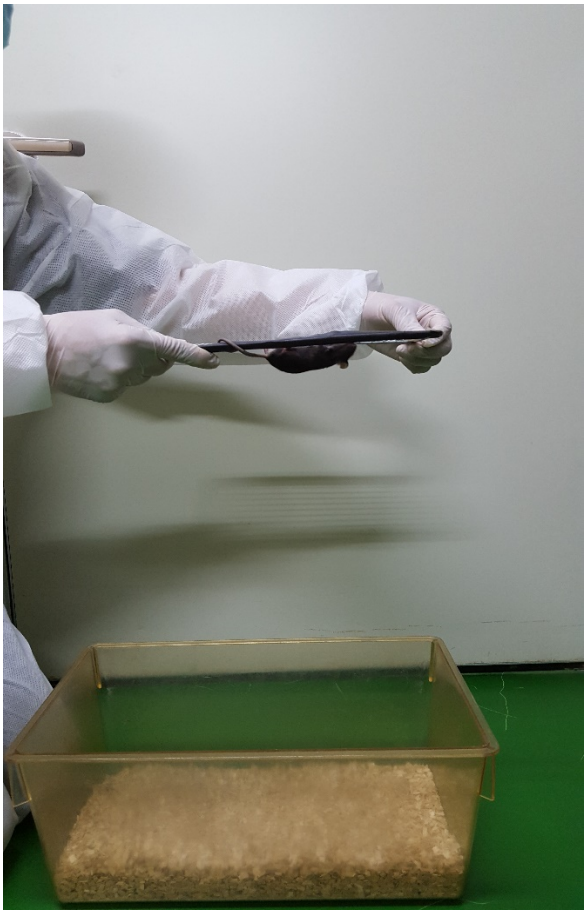


Figure 6. Hanging test

4.6 Mobility test

Mobility test was performed to check the physical activity capacity. The chamber was made 100 cm x 100cm x 16cm by with acrylic panels. In order to measure the activity scored, 10 cm x 10 cm square was set. For adaption the chamber, each group of mice were adapted for 1hour. The mobility test recorded a video camera fixed above that the device for 30 minute without any outsources in the same environment. The score was calculated by when the body of a mice crossed or moved the line drawn above the device.

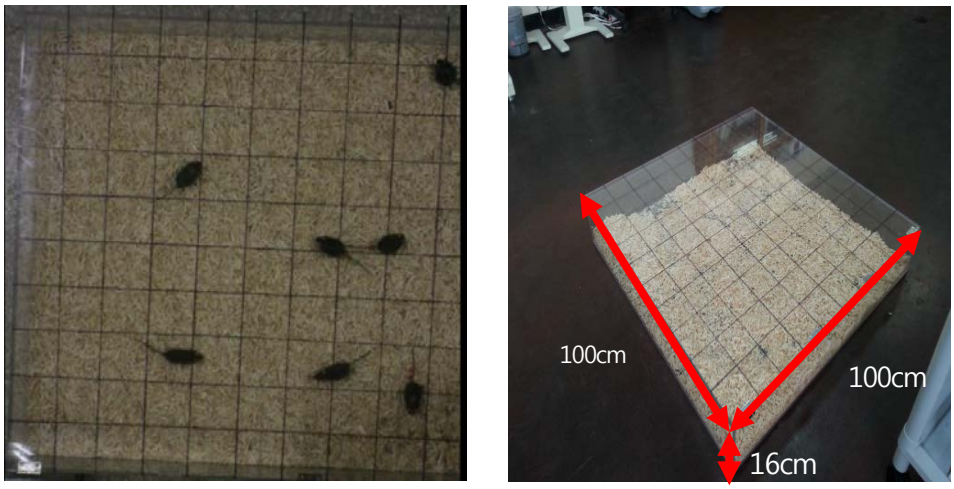


Figure 7. Mobility test

4.7 Skeletal muscle cross sectional area

Following sacrificed right of GAS muscle was measured. The muscle was preserved in 4 percent paraformaldehyde (PFA) for fixation for more than a week. In order to prepare the processing procedure, the muscle were moved on the cassette and washing off in distilled water for 15 minutes. All of cassettes were run in the processing machine (Shandon Citadel 1000, Thermo Fisher Co.) for dehydrate in graded ethanol. The samples were carried into embedded in paraffin using embedding machine (Tissue-TEK TEC 5 Tissue Embedding Consol System, Sakura) and cut in a cross section 4 um thickness to place on the slides using the Microtome (20 Microsystems, Leica). All of the slides were processed by the hematoxylin and eosin (H&E) staining which was a modification of the previously reported method (Stein, John M and Padykula, Hele A., 1962). All samples were captured by optical microscope (ECLIPSE E 100, Nikno) and analyzed the cross sectional area of myofibers in the GAS muscle using by innverview 2.0 software (innverview CO., LTD, Korea).

4.8 Skeletal Muscle TNF-alpha and IL-1 beta Analysis – Western Blot

Quantification and measurement of TNF-alpha and IL-1 beta protein in quantified soleus muscle sample with BCA assay was performed by using Western blot analysis. Briefly, total protein concentration was assayed with Pierce BCA protein assay kit (Thermo Scientific). After SDS-PAGE on 15% gels, protein was transferred to Immobilon™ transfer PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS-T containing 10% skim milk (Bio-Rad) for 1 h, washed 15 min with TBS-T. The antibodies were diluted 1:1000 with TBS containing 0.1% (v/v) Tween-20 (TBST, Biosesang, Seongnam, Korea) and then membranes were incubated with a goat polyclonal anti-TNF alpha antibody (abcam) and anti-IL1 beta antibody for overnight. Following the incubation for overnight, membranes were incubated in an anti-rabbit secondary antibody (Santa Cruz) for 2 h after washing with TBS-T. After incubation with antibodies, membranes were washed 15 minutes with TBS-T. The membrane were detected by using western blotting detection reagent kit (ab signal, Korea) and quantified using the microChemi 4.2 system (DNR bio Imaging Systems, Jerusalem, Israel). Band density was quantified by densitometry with ImageJ analysis program.

5. Data Analysis

Statistical analysis was performed using the SPSS 18.0 software (SPSS Inc.). Results were expressed as mean ± standard error of mean. Data were analyzed using one-way ANOVA to compare the food intake, grip strength, mobility test, hanging test, CSA and LIF level in this study. Tukey post-hoc were conducted to determine the existence of mean difference of each group. Also, Pearson correlation analysis was used to assess the correlation between hindlimb lean mass and muscle LIF protein level. The significance level was set at $p<0.05$.

IV. RESULTS

1. Body composition

1.1 Whole body composition

Table 2. Whole body composition using DXA

Whole body composition				
	OLD-CON	OLD-REX	OLD-AEX	p value
Total mass (g)	35.4±1.2077	35.6±0.7980	33.6±1.1596	0.408
Lean mass (g)	26.892±0.7714	26.439±0.6856	25.326±1.2165	0.477
Fat mass (g)	4.983±0.3790	5.400±0.3471	5.060±0.4874	0.717
Percent fat (%)	15.250±0.7637	16.543±0.9892	16.340±1.5449	0.664
Bone mineral contents (g)	0.6917±0.0440	0.7471±0.7124	0.694±0.0420	0.738
Bone mineral density (g/cm^2)	0.0970±0.1711	0.1160±0.0051	0.1114±0.0029	0.421

Data were presented as mean±S.E.M.

As table 2 shows that there was no significant difference in total mass, lean mass, fat mass, percent fat, bone mineral contents and bone mineral density among the OLD-CON, OLD-REX and OLD-AEX group.

1.2 Weekly body weight and food intake

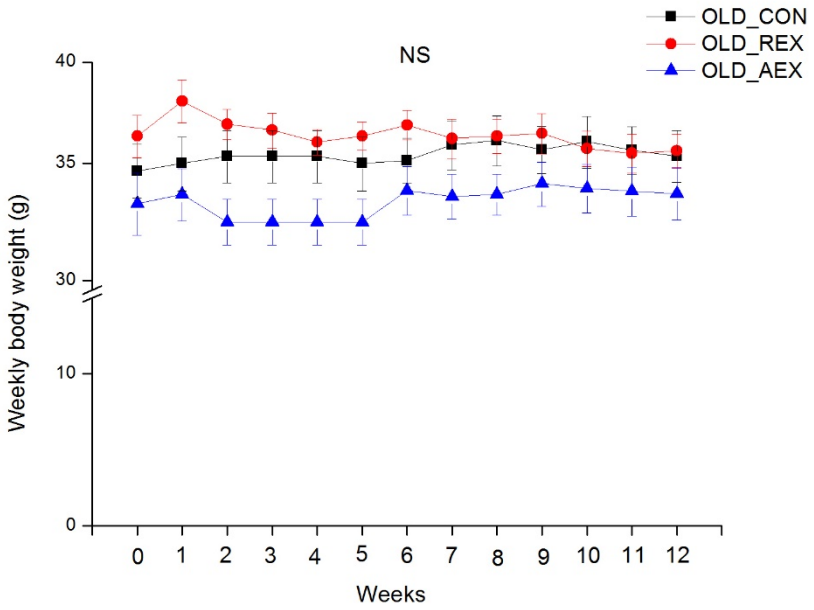


Figure 8. Weekly body weight. Data were presented as mean±S.E.M. $p<0.05$

In this study, the body weight was checked every week. Two-way ANOVA with repeated measurement was performed to examine the difference among the groups. However, there was no significant difference in body weight among the OLD-CON, OLD-REX and OLD-AEX group.

2. Food intake

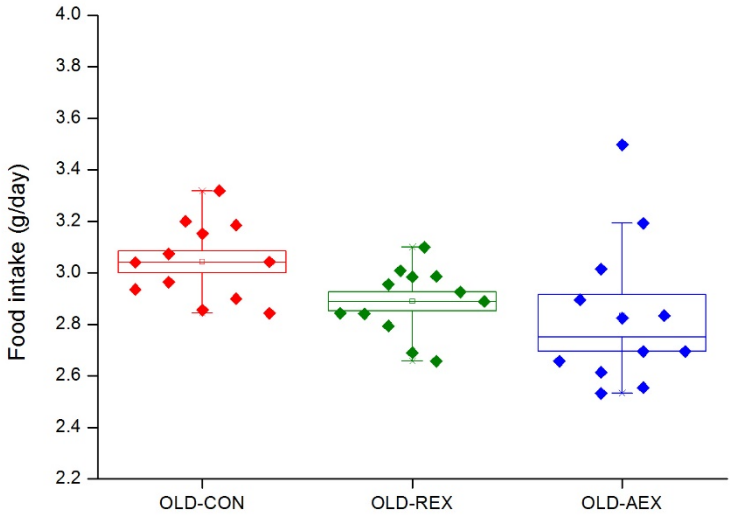


Figure 9. Food intake. Data were presented as mean±S.E.M. $p<0.05$

The Figure 9 shows daily food intake of all groups. There was a no significant difference in daily food intake among OLD-CON, OLD-REX and OLD-AEX group following 12 weeks of exercise training.

3. Grip strength

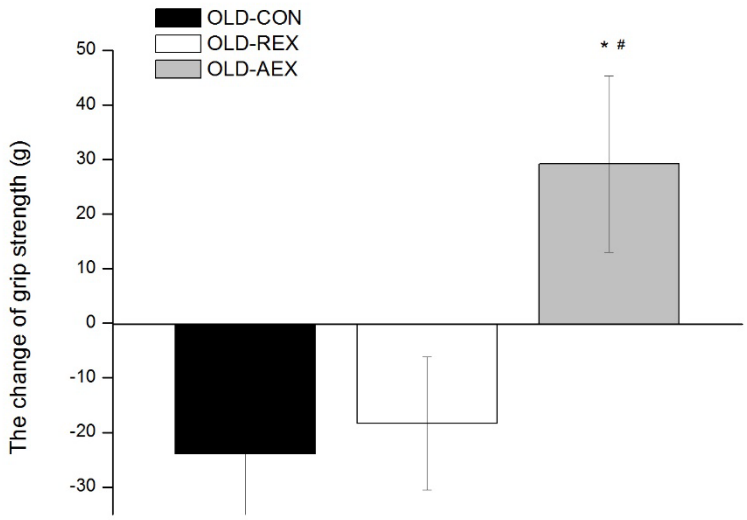


Figure 10. The change of grip strength

The change of grip strength. Data were presented as mean±S.E.M. *p<0.05 compared with OLD-CON, #p<0.05 compared with OLD-REX.

The change of grip strength was obtained by calculating the difference between the maximal grip strength record of post and pre period. The result shows that there was a significant difference in the change of grip strength in OLD-AEX group (29.16±7.201) compared to OLD-CON (-35.85±20.17) and OLD-REX group (-18.342±12.156).

4. Cross sectional area

4.1 Skeletal muscle cross sectional area

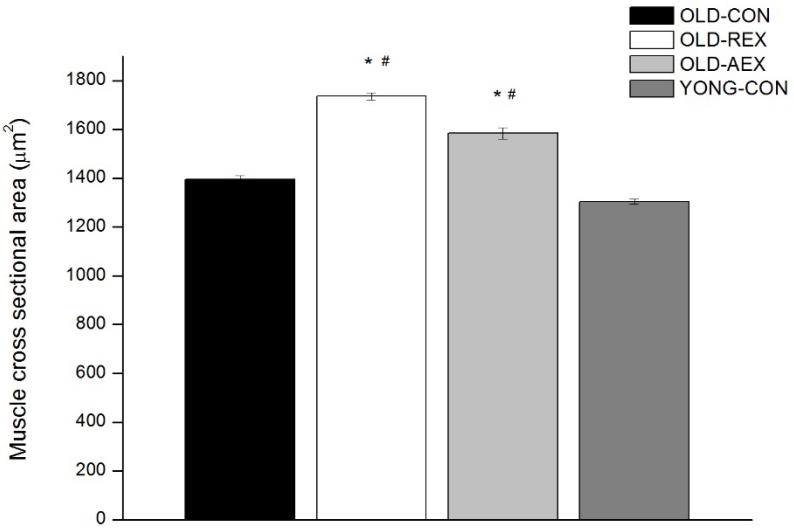


Figure 11. Effect of 12 weeks of resistance and aerobic exercise on myofiber cross sectional area. The mean of myofiber CASs in the gastrocnemius muscle each group. Data were represented as mean±S.E.M. *p<.001compared with OLD-CON, #p<.001 compared with YOUNG-CON

To observe the difference muscle fiber in each group, the histological alternation after exercise using H&E straining in gastrocnemius muscle was analyzed. There were significant differences among the OLD-CON (1394.6±16.1), OLD-REX(1735.8±15.3), and OLD-AEX group (1583.8±23.7). Also, there were significant differences between the YOUNG-CON (1303.8±10.6), OLD-REX and OLD-AEX group.

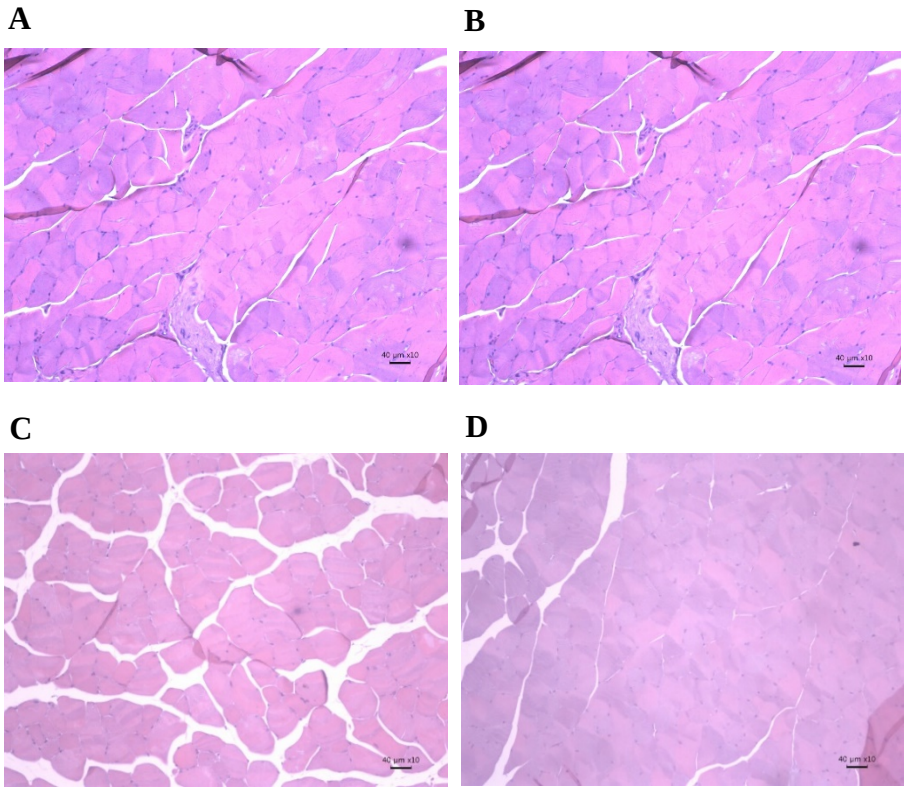


Figure 12. Representative micrographs of hematoxylin and eosin stained gastrocnemius muscle sections. A: OLD-EDX, B: OLD-REX, C: OLD-CON, D: YOUNG-CON. Scale bar = 40 µm.

4.2 Skeletal muscle fibers percent distribution

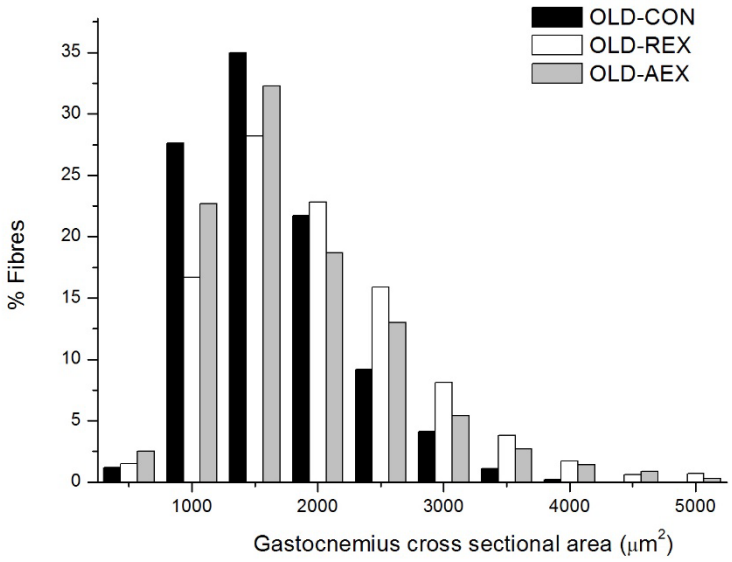


Figure 13. The distributions of muscle cross sectional area in OLD group in gastrocnemius muscle.

Figure 13 shows the distribution of cross section areas in each group difference in gastrocnemius muscle. The median percentage of fibers showed that in OLD-CON (1304.1 μm^2), OLD-REX (1584.9 μm^2), and OLD-AEX (1390.5 μm^2).

5. Hanging test

5.2 Free weight hanging test

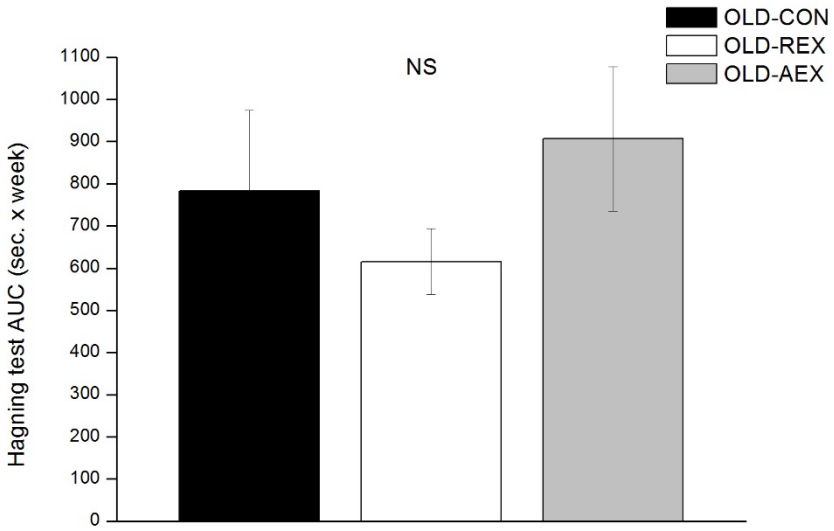


Figure 14. Free weight hanging test AUC. Data were presented as mean ± S.E.M. $p<0.05$

Free weight bearing hanging test was performed to confirm skeletal muscle endurance capacity for every 2 weeks. The values were presented as area under the curve (AUC). There was no significant difference between each group. However, the result showed a tendency of increased hanging AUC in OLD-AEX than other groups.

5.2 Weighted bearing hanging test

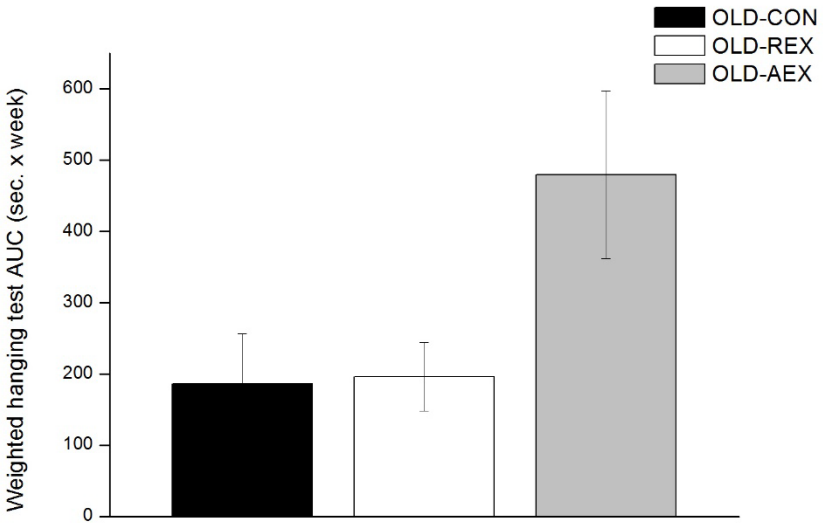


Figure 15. Weighted hanging test AUC. Data were presented as mean ± S.E.M. $p<0.05$

The weighted bearing hanging test was performed to confirm an improvement of skeletal muscle endurance capacity with 10g of tail weight. The values were presented as area under the curve. There was no significance difference between each groups. However, the OLD-AEX group showed an increased tendency of weighted bearing hanging time.

6. Mobility test

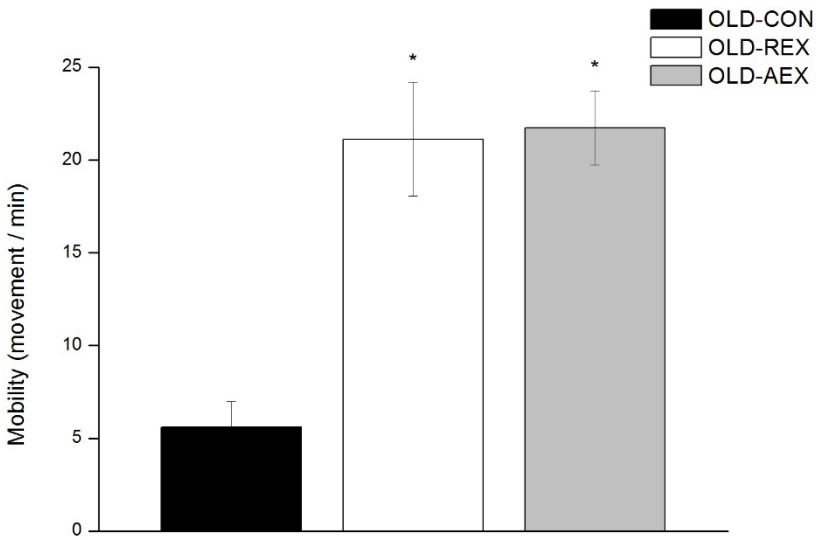


Figure 16. Mobility test**.** Data were represented as mean±S.E.M.

*p<0.001 compared with OLD-CON

The mobility test was performed to measure the movement of activity chamber during 28 minutes. The values were presented mean±SEM. There was a significant difference ($p<0.001$) between OLD-CON (5.59±1.395) and OLD-REX group (21.13±3.07). Also, in OLD-AEX group (21.74±1.99) showed a significantly higher ($p<0.001$) movement per minute than OLD-CON group.

7. LIF expression on skeletal muscle

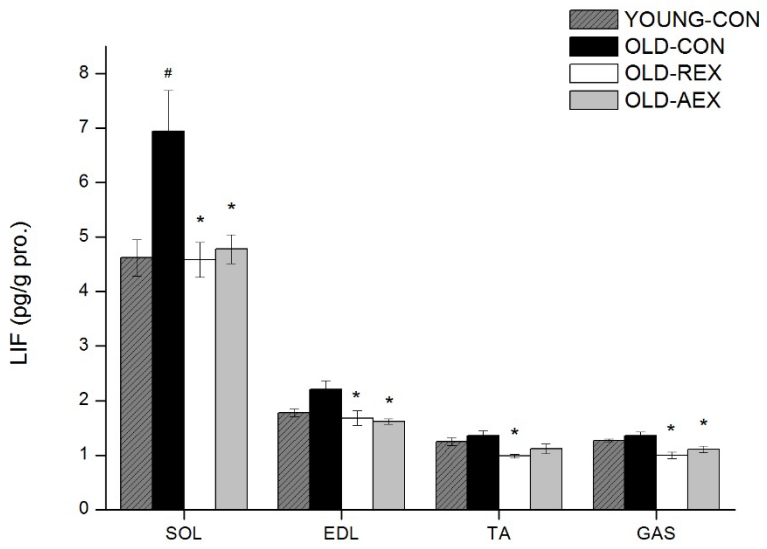


Figure 17. Effect of 12 weeks of aerobic and resistance exercise on LIF levels in skeletal muscle; SOL, EDL, GAS, and TA. Data were presented as mean±S.E.M. *p<0.05 compared with ODL-CON, #p<0.05 compared with YOUNG-CON

The levels of LIF in SOL, EDL, TA, and GAS were measured. There were significant differences on LIF levels between YOUNG-CON and OLD-CON in SOL muscle (p=0.06). Also, there were significant differences on LIF levels between OLD-CON and both of exercise group (OLD-REX, OLD-AEX) in SOL (p=0.006, p=0.024), EDL (p=0.021, p=0.017) and GAS (p=0.001, p=0.03) muscles, respectively.

8. Correlation between muscle LIF and hindlimb lean mass

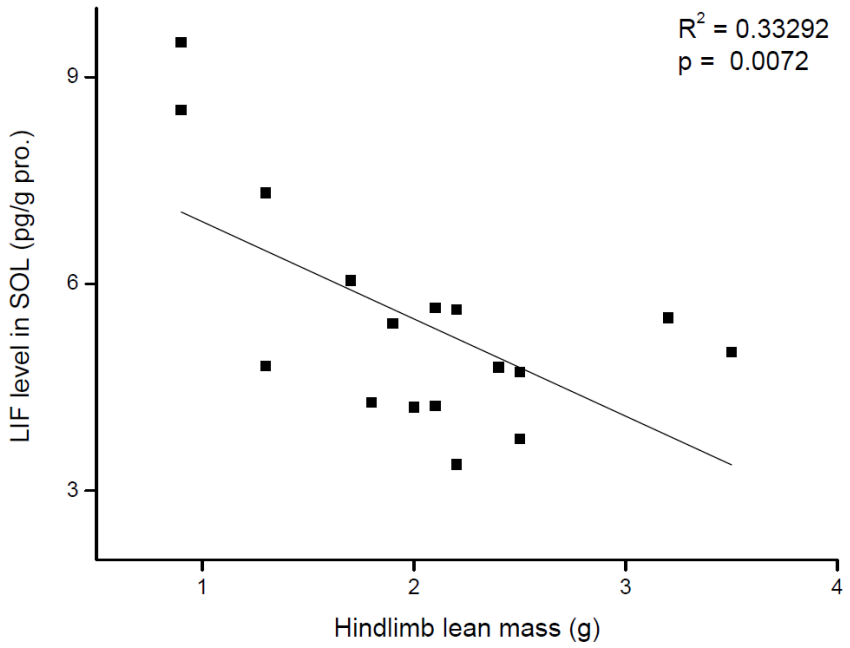


Figure 18. Correlation between muscle LIF protein and hindlimb lean mass

As figure 18 showed negative correlation between LIF protein level of soleus muscle and hindlimb lean mass (R^2=0.33292, p=0.0072). However, there were a no relationship of LIF level in GAS, TA, EDL muscles and hindlimb lean mass.

9. The quantification of IL-1 beta and TNF-alpha analysis

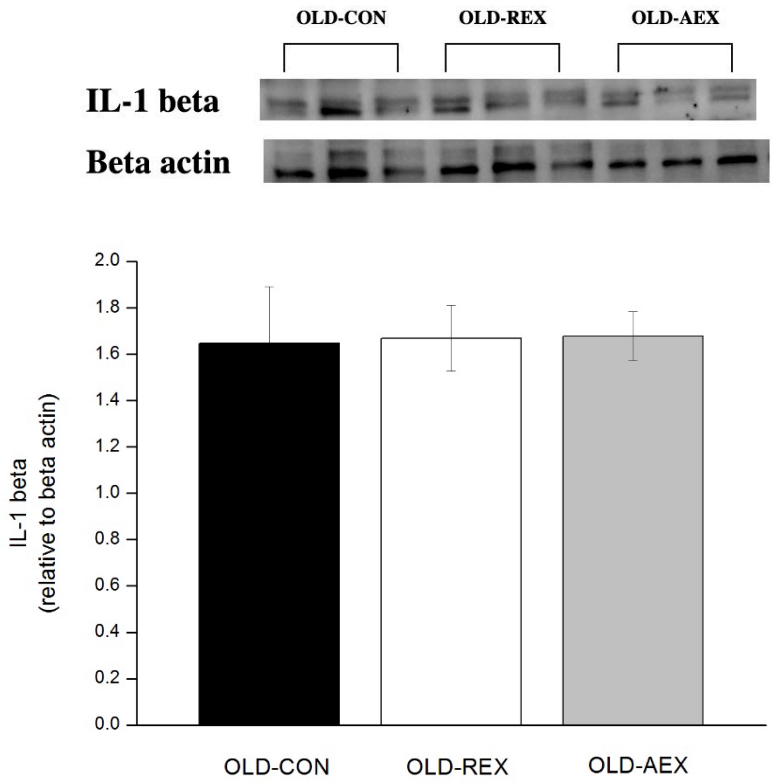


Figure 19. The quantification of IL-1 beta in soleus muscle.

Data were presented as mean±S.E.M. $p<0.05$

There was a no significant difference in the quantification of IL-1 beta analyzed between each group.

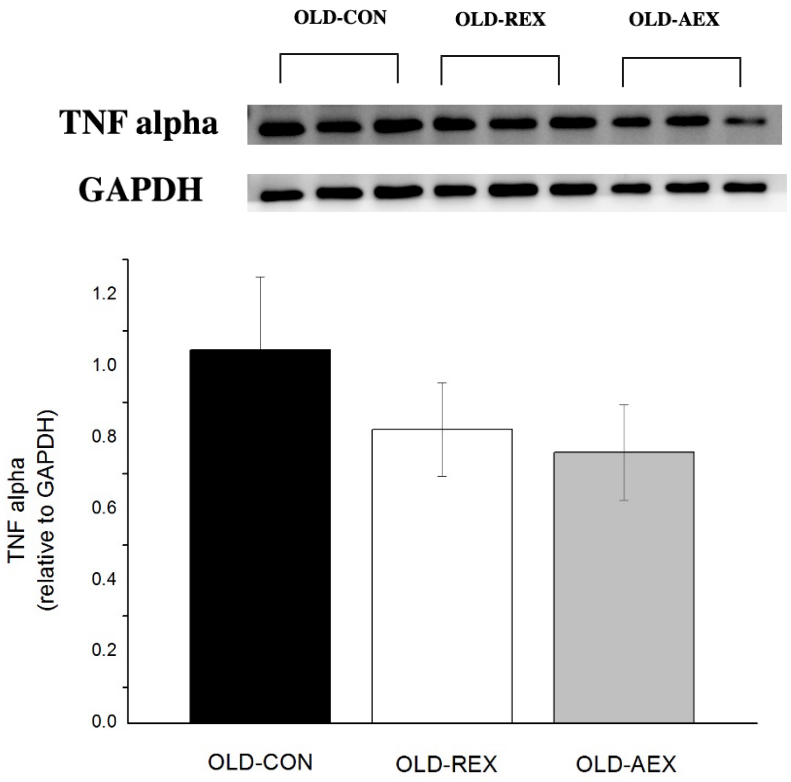


Figure 20. The quantification of TNF alpha in soleus muscle.

Data were presented as mean±S.E.M. $p<0.05$

There was a no significant difference the quantification of TNF alpha analyzed between each group. However, OLD-AEX showed a tendency to decline compared to OLD-CON.

V. DISCUSSION

The purpose of this study was to determine the change of LIF protein levels of skeletal muscle following 12 weeks of resistance ladder climbing and aerobic exercise in aging mice. Although a single bout of exercise increased the expression of LIF expression, no study has been focused on the effect of chronic and regular exercise that can induce the change of LIF protein levels in muscles. We found that there were no significant differences in body composition, weekly body weight, food intake among the groups following 12 weeks of resistance and aerobic exercise. On the other hand, the change of grip strength in aerobic exercise group was significantly increased when compared to resistance exercise and control group. In addition, the muscle cross sectional area was significantly increased in the both of exercise group in relation to the control group. There were no significant differences in the free bearing hanging test between the groups, but the weighed bearing hanging test was significantly increased in aerobic exercise group. Also, the movement per minute was significantly increased in the both of exercise groups than in the control group.

The major finding of this study was that LIF protein level in muscles was significantly decreased following 12 weeks in the resistance and aerobic exercise groups. Also, soleus muscle LIF protein levels showed a negative correlation with hindlimb lean mass. In addition, there was no significant difference between groups in TNF alpha and IL-1 beta in soleus, but TNF alpha expression showed a tendency to decrease following 12 weeks of aerobic exercise.

12 weeks of resistance and aerobic exercise increased grip strength and CSA without a change of body composition

It has been well known that resistance exercise is effective in increasing muscle mass and strength and endurance exercise is superior in maintaining and improving maximum aerobic power (Landi et al., 2014). Notwithstanding, the aerobic exercise group showed a significantly increased in the change of grip strength which is calculated by a delta of pre and post value without a change of body composition. Although the resistance exercise group was not significantly different between groups, the change of grip strength showed a tendency to maintain a strength. The number of studies have confirmed the ability of resistance exercise to improve physical function and reduce muscle mass or strength. However, few research investigates the ability of aerobic exercise to improve function. In this study, it markedly showed that even the weakest and most aging mice have the potential to restore grip strength with aerobic exercise.

Also, 12 weeks of resistance and aerobic exercise increased the muscle myofibers cross sectional area. Although it has been well known that resistance exercise-induced increased the muscle mass, strength, and physiological function in elderly people, aerobic exercise including brisk walking or jogging was often a more preferable mode of exercise due to its simplicity and practicality. This study showed that the both of the exercise groups was demonstrated significantly greater gastrocnemius CSA than in the control group. Per Lee study report, it has been shown that 8 weeks of progressively resistance ladder climbing resulted in significantly increased the hindlimb CSA in rodent model (Lee & Farrar, 2003). This study was performed by modifying these exercise methods for mice and it might be the reason for the similar result in gastrocnemius muscle CSA. Moreover, the gastrocnemius CSA was significantly increased in the

aerobic exercise group. In the case of human, moderate aerobic exercise training has been shown to increase the percentage and CSA of type IIa fibers in gastrocnemius in response to 10 months of aerobic exercise (Coggan et al., 1992; Williams, Higgins, & Lewek, 2002). In addition, 4 weeks of voluntary aerobic exercise was sufficient to induce positive physiological and morphological adaptations in mice and were consistent with the findings of the study (Graber, Ferguson-Stegall, Liu, & Thompson, 2014). These results were consistent with previous studies, which demonstrated the effectiveness of resistance and aerobic exercise in increasing muscle CSA compared to the control group.

12 weeks of resistance and aerobic exercise decreased LIF protein levels and pro-inflammatory factor in skeletal muscle.

Although previous studies showed that exercise-induced LIF protein levels were not significantly changed, the LIF protein levels at its analytic center was changed, which can be resulted from regular exercise for long periods. This study detected LIF protein level in skeletal muscle and the results showed that SOL, EDL, TA, GAS muscle were significantly decreased the LIF protein level when compared to the control group. In previous studies, it showed that LIF mRNA levels were increased after a single bout of cycle ergometer for 3h at 60% VO_2max and a single bout heavy resistance training increased LIF mRNA levels (Broholm et al., 2008). Unfortunately, both studies showed that LIF protein levels were not significantly changed following exercise. Because LIF protein level has a very short half-life of 6-8 minutes in serum, which makes it difficult to detect its circulating levels of LIF protein levels in human (Hilton, Nicola, Waring, & Metcalf, 1991). Brohomlm et al. has pointed out that LIF might be secreted to the

interstitial space between muscle fibers and never reaches circulation (Broholm & Pedersen, 2010). This point suggested that LIF does not function as a systemic myokine like IL-6. Putting previous studies together, LIF is more likely to affect skeletal muscle in an autocrine and/or a paracrine fashion.

Hunt. et al. investigated the expression and function of LIF using the antagonist MH35-BD during specific inflammatory and myogenic stages of notexin-induced muscle regeneration in mice (Hunt et al., 2013). LIF expression showed that the up-regulation coincided with the increased expression of pro-inflammatory cytokines such as TNF, IL-1 beta, and IL-6. Pro-inflammatory cytokines are known to be associated with muscle injury and aging. Also, indirect evidence for a role of inflammation in sarcopenia is that markers of systemic inflammation correlate with the loss of muscle mass and strength in the elderly (Peake et al., 2010). This study showed the LIF protein levels were significantly increased in control group compared to both of exercise groups. In comparison with previous studies, LIF protein levels were up-regulated in aging control group that might be consistent with inflammatory or/and aging milieu. We found that TNF alpha showed a tendency to be alleviated in aerobic exercise group compared to control group.

So far, no definitive answer has been given to address difference in the expression of LIF protein level in long term and/or different type of exercise training in aging model. This study attempted to confirm the exercise-induced LIF protein level in aging mice for the first time. Therefore, further study is required to investigate a change of LIF protein level in skeletal muscle.

12 weeks of resistance and aerobic exercise increased hanging time and the movement of physical activity.

A decrease in physical activity in age mice is a result of age-related loss of muscle mass and increased circulating levels of inflammatory cytokines (Hamrick et al., 2006). We found that the movement of physical activity was expressed as number of changes per minute in motion in the both of exercise groups and was significantly increased when compare to the control group. This study attempted to measure the difference in natural physical activity difference between groups using a transparent acrylic chamber. A previous study reported that a treadmill exercise not only delayed the age-dependent decrease of spontaneous physical activity, but also increased a higher degree of physical activity in the open field in aged rat model (Skalicky, Bubna-Littitz, & Viidik, 1996). The regular exercise group showed significantly increased the movement per minutes than the sedentary group. These phenomena could not be fully understood but it might be the result of the exercise group maintaining a higher level of endorphins in the exercise group (Walker, Berntson, Paulucci, & Champney, 1981).

In order to perform an endurance muscle capacity, this study was performed using the wire hanging test. There was no significant difference between the groups in free bearing but it was significantly higher than other group with an attachment of 10g on the tail in the aerobic exercise group. Aerobic exercise has been shown to increase the maximum aerobic capacity as shown by an increase hanging time which is also consistent with the grip strength test results. Thus, 12 weeks of aerobic exercise increased the endurance muscle capacity.

Conclusion

This study lays the foundation for future work on exercise-induced LIF expression studies that explores the change between aging and LIF. Numerous studies related with exercise-induced myokine indicated that skeletal muscle is a secretory organ that provides a beneficial effect to our bodies such as improving glucose tolerance, increasing skeletal muscle mass and strength. However, very few studies have been conducted to investigate exercise-induced LIF protein level in muscle and other cytokines in aging status. This study has attempted to establish a descriptive report of exercise-induced LIF protein level in muscle and the beneficial changes following 12 weeks of resistance and aerobic exercise in aging mice. Further studies are required to clarify the relationship between exercise-induced LIF and muscle regeneration.

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국문 초록

노화쥐의 골격근에서 유산소 운동 및 저항성운동이 leukemia inhibitory factor 발현에 미치는 영향

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노화 현상은 모든 생명체에 공통적으로 일어나는 자연적인 현상으로 노화의 진행은 신체조성을 약하게 만들며 약해진 신체조성은 신체활동을 떨어뜨려 궁극적으로 죽음에 이르게 한다. 특히 노화에 따른 근육의 양의 감소와 근력의 감소를 근감소증 (sarcopenia)이라 하며 이를 예방하고 막기 위한 연구가 전세계적으로 활발히 진행되고 있는 상황이다. 근감소증을 막기 위한 여러 인자 중 leukemia inhibitory factor, LIF가 새로운 마이오카인으로 제시되고 있다. LIF는 근비대에 긍정적인 영향을 미칠뿐만 아니라 운동 또는 근손상에 따른 근육 재생성에 핵심적인 인자로 보고되고 있다. 하지만 근비대와 근육 재생성에 긍정적인 영향을 미치는 것으로 알려진 LIF 발현에 대한 연구가 현재까지 부족한 실정이다. 일시적인 운동을 통한 LIF 발현에 대한 연구는 진행되었으나 장기적인 운동을 통한 LIF 발현의 변화에 대한 연구는 진행되지 않았으며, 특히 노화에서의 LIF 발현에 대한 연구는 전무한 실정이다. 따라서 본 연구에서는 12주간의 저항성 사다리 운동 및 유산소성 트레드밀 운동이

노화쥐의 골격근에서 LIF의 발현에 미치는 영향에 대해 알아보고자 한다.

본 연구에서는 19개월령 C57BL/6 마우스 20두를 실험 대조군 (n=6), 유산소성 트레드밀 운동군 (n=7), 저항성 사다리 운동군 (n=7)으로 할당하여 연구를 진행하였다. 각각의 운동군은 본 운동에 앞서 운동에 대한 거부반응을 최소화 하며 운동의 효과를 높이기 위해 1주간의 적응 훈련을 실시하였다. 유산소성 트레드밀 운동군은 주 3회, 12주간 실시하였으며 저항성 사다리 운동군 역시 주 3회, 12주간 실시하였다. 본 연구에서는 식이섭취량은 매주 1회 측정하였으며, 악력, 근지구력 테스트는 2주에 1회 측정하였다. 이중에너지 X-선 흡수계측법 (DXA)를 이용하여 마우스의 신체조성을 측정하였으며 LIF 단백질 수준을 확인하기 위해 효소면역측정법 (ELISA)를 이용하여 마우스 뒷다리 근육 비장근 (soleus), 비복근 (gastrocnemious), 전경골근 (anterior tibial), 긴발가락폄근 (extensor digitorum longus)에서 각각 확인하였으며, 근육 내 전염증성 인자로 알려진 tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β)를 단백질흡입법 (western blotting)을 이용하여 비장근에서 확인하였다. 또한 근형태적 변화를 확인하기 위해 H&E staining을 통한 근단면적 크기를 비장근에서 확인하였다.

12주간 유산소성 트레드밀 운동과 저항성 사다리 운동은 신체조성에서 각 그룹간 유의한 차이는 없었다. 하지만 악력 검사에서 트레드밀 운동군이 대조군에 비해 유의하게 증가한 것을 확인할 수 있었다. 근지구력을 평가한 결과 자가체중을 이용한 매달리기 테스트를 실시하였을 때 각 그룹간 유의한 차이가 없었으나, 10g 추를 꼬리에 매달아 매달리기 테스트를 실시한 결과 유산소성 트레드밀 운동군이

대조군에 비해 유의하게 오랫동안 매달렸다. 또한 신체활동량을 확인한 결과 각각의 운동군은 대조군에 비해 유의하게 증가하였다. 비장근의 근단면적 크기는 각각의 운동군에서 대조군과 비교하였을 때 유의하게 증가하였다. LIF 단백질 수준은 각각의 운동군에서 비장근, 비복근, 긴발가락폄근에서 대조군에 비해 유의하게 감소하였다. 비장근 내에서 전염증성 인자 (TNF-α, IL-1β)의 발현을 확인한 결과 각각의 운동군과 대조군에서 통계적인 차이는 나타나지 않았다.

12주간의 유산소성 트레드밀 운동과 저항성 사다리 운동은 악력, 근단면적, 근지구력, 신체활동량을 노화 마우스 모델에서 대조군에 비해 유의하게 증가시켰다. 하지만 12주간의 운동은 LIF 단백질 수준을 마우스 뒷다리 근육에서 감소하였다. 또한 LIF 단백질 수준은 노화 마우스 뒷다리 제지방과 음의 상관 관계를 나타내었다.

주요어 : 노화, LIF, 트레드밀 운동, 저항성 사다리 운동

학번 : 2012-21457