Thesis of Master of Science

Effect of Resistance Exercise and Endurance Exercise on Muscle and Bone FGF-2 Protein Level in Aged Mice

저항성운동과 유산소운동이 노화 쥐의 골격근과 뼈의 FGF-2에 미치는 영향

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Abstract

Background: Aging is natural phenomena which all living organism experience. With aging, muscle loss and bone loss occur, and induce functional and physiological impairments. Fibroblast growth factor 2, FGF-2, is potent growth factor which induce muscle regeneration via satellite cell activation and osteogenic differentiation. In normal status, FGF-2 is increased and have beneficial effects on muscle and bone regeneration and osteogenic differentiation. In aged status, FGF-2 in muscle is elevated as cell-autonomous response to repair the aged muscle fibers, however, it induce acceleration of muscle aging by depleting satellite cell, and effect of FGF-2 on bone is known. In spite of difference compared to the normal status, none of the studies investigate the effect of exercise on muscle and bone FGF-2 protein level in aged status. Therefore, in this study, we try to investigate the effect of exercise on muscle and bone FGF-2 protein level.

Method: Twenty 19 months old C57B/6 mice were randomly divided into 3 groups, OCON (old control group, n=7), ORT (old resistance exercise group, n=7) and OET (old endurance exercise group, n=6). 12 weeks of resistance ladder climbing exercise was conducted for ORT group and 12 weeks of treadmill exercise was conducted for OET group 3 days per week. Rough food intake was measured every week, and grip strength was measured every two weeks during intervention period. After the exercise intervention period, body composition was measured by DEXA (Duel Energy X-Ray Absorptiometry), and sacrificed. During sacrifice, soleus muscle (SOL), tibialis anterior muscle (TA) and femur were collected and weighted. Collected muscle tissues and bone tissues were frozen and stored in -70 °C right after the sample collection for protein analysis. ELISA protein assay were performed to measure the muscle and bone FGF-2 protein level. The data were analyzed by using one-way ANOVA, and the post-hoc test were conducted to analyze group difference. Also pearson correlation analysis was conducted to assess correlation between hindlimb lean mass and muscle FGF-2 protein level. The level of significance was set at $p<0.05$.

Results: Relative grip strength of ORT was increased compared to OCON group (OCON vs. ORT, 6.092±0.145 vs. 7.083±0.194, $p<0.05$). Appendicular lean mass of ORT were increased compared to OCON (forelimb; OCON vs. ORT, 5.25±0.360 vs 6.3±0.239, hindlimb; OCON vs. ORT, 1.52±0.01 vs. 2.15±0.141, $p<0.05$), only hindlimb lean mass was increased in OET compared to OCON (1.52±0.01 vs. 2.14±0.167, $p<0.05$). SOL wet weight of ORT was increased compared to OCON (OCON vs. ORT, 0.313±0.011 vs. 0.35±0.0215, $p<0.05$). Also appendicular BMC and BMD of ORT group were increased compared to OCON group (BMC; OCON vs. ORT, 0.166±0.00204 vs. 0.283±0.0425, BMD; OCON vs ORT, 0.1089±0.007 vs. 0.1329±0.0045, $p<0.05$), only BMC of forelimb was increased in OET compared to OCON group (OCON vs. ORT, 0.0152±0.002 vs. 0.423±0.0063, $p<0.05$). SOL FGF-2 protein level of both ORT and OET group were decreased compared to OCON group muscle (OCON vs. ORT, 1.35±0.1667 vs. 0.9434±0.035, $p<0.05$; OCON vs. OET, 1.35±0.1667 vs. 0.967±0.030, $p<0.05$), and only TA FGF-2 level of ORT group was decreased compared to OCON group (OCON vs. ORT, 0.400±0.027 vs. 0.268±0.027, $p<0.05$). However there was no change of femur FGF-2 protein level among all three groups. Negative correlation was found between SOL FGF-2 protein level and hindlimb lean mass ($r^2=0.48592$, $p=0.0012$).

Conclusion: 12 weeks of resistance exercise increased appendicular lean mass, BMC and BMD. Also 12 weeks of resistance exercise increased soleus muscle and tibialis anterior muscle wet weight. Also 12 weeks of resistance exercise reduced muscle FGF-2 protein level, but bone FGF-2 protein level. Lastly, there was negative correlation between soleus muscle FGF-2 protein level and hindlimb lean mass.

LIST OF ABBREVIATIONS

FGF-2	Fibroblast growth factor-2
IGF-1	Insulin like growth factor-1
OCON	Old control group
ORT	Old resistant exercise group
OET	Old endurance exercise group
SOL	Soleus muscle
TA	Tibialis anterior muscle
ELISA	Enzyme linked immune sorbent assays
DEXA	Dual Energy X-Ray Aborptimetry
IACUC	Institutional animal care and use committee

Contents

I. INTRODUCTION 1

1. Significance of the study 1

2. Purpose of the study 2

3. Research hypothesis 2

4. Limitations 3

II. LITERATURE REVIEW 4

1. Aging 4

1.1 Muscle quality and aging 4

1.2 Bone quality and aging 6

2. Fibroblast growth factor 2 (FGF-2) 8

2.1 FGF-2 and muscle 8

2.2 FGF-2 and bone 10

3. Aging and FGF-2 12

4. Exercise 13

III. METHOD 15

1. Animal 15

2. Experimental design 16

3. Exercise protocol 17

4. Measurements 19

4.1 Body composition 20

4.2 Grip-strength 21

4.3 Protein extraction 22

4.4 Protein analysis 23

5. Data Analysis 24

IV. RESULT 25

1. Whole body composition and Body Weight 25

2. Grip strength 26

3. Appendicular lean mass 27

4. Muscle wet weight 28

5. Appendicular BMC 29

6. Appendicular BMD 30

7. FGF-2 protein level in muscle 31

8. FGF-2 protein level in bone 32

9. Correlation between muscle FGF-2 and hindlimb lean mass 33

V. DISSCUSION 34

VI. REFERANCE 41

List of tables

Table 1. Measurements.....19

Table 2. Whole body scan of DEXA and body weight.....25

List of figures

Figure 1. Cell culture of human bone like cell with FGF-2/bFGF. Demonstrating increase of applied FGF-2 level, % of occupied dis surface increase (Noff et al., 1989).....10

Figure 2. FGF-2 mRNA level change in aged muscle fiber and histology of FGF-2 in single muscle fiber of aged, and adult muscle fiber (Chakkalakal, et al., 2012) 12

Figure 3. Experimental design.....16

Figure 4. Exercise protocol 18

Figure 5. DEXA (Dual Energy X-Ray Absorptiometry) 20

Figure 6. Grip strength meter 21

Figure 7. Relative grip strength of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group).....26

Figure 8. Appendicular lean mass of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb lean mass, B; hindlimb lean mass 27

Figure 9. Relative muscle wet weight of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; relative muscle mass of soleus muscle, B; relative muscle mass of tibialis anterior muscle 28

Figure 10. Appendicular BMC of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb BMC, B; hindlimb BMC.....29

Figure 11. Appendicular BMD of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb BMD, B; hindlimb BMD.....30

Figure 12. FGF-2 protein level in muscle of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; FGF-2 level in soleus muscle, B; FGF-2 level in tibialis anterior muscle. 31

Figure 13. FGF-2 protein level in femur 32

Figure 14. Correlation between muscle FGF-2 protein level and hindlimb lean mass. A; correlation between hindlimb lean mass and soleus muscle FGF-2 protein level, B; correlation between hindlimb lean mass and tibailis anterior muscle FGF-2 protein level 33

I. INTRODUCTION

1. Significance of the study

Aging is natural phenomena all living creatures are experience as getting old. With aging, various physiological changes occurs, such as decrease in metabolic capacity, muscle wasting and decrease of bone quality (Roubenoff & Hughes, 2000). Currently, Fiberblast growth factor-2(FGF-2), which is traditionally known to regulate venous growth, has been identified as one of the growth hormone which is increased in muscle by muscular stress, and regulates muscle regeneration and maintenance (Hamrick, 2011; Hamrick et al., 2010). At the same time, it has crucial impact on maintaining bone mineral density by increasing BMP-2, Bone morphogenic protein-2 (Agas, Sabbieti, Marchetti, Xiao, & Hurley, 2013).

However, currently, elevated FGF-2 level in muscle fiber has been demonstrated in aging status by several studies, and it was suggested that inhibition of FGF-2 signal in the muscle fiber is one of the strategies to slowing down the muscle aging (Chakkalakal et al., 2012). Also, traditionally, it has been proposed that the mechanical loading is one way to improve or maintain bone health. However, numerous studies demonstrated the importance of growth hormones (IGF-1 and FGF-2) not only in growth of bone, but also in maintenance of bone (Hamrick, 2011; Hamrick, McNeil, & Patterson, 2010). As aging processes, both muscle loss and bone loss occur significantly, and it is demonstrated that bone loss and muscle loss have influence to each other (Walston, 2012).

In spite of physiological changes caused by aging, no researches has been

demonstrated the effect of exercise on muscle and bone FGF-2 protein level in aged status. Therefore, it is necessary to study on FGF-2, the potent mitogenic and osteogenic factor, expression in muscle and bone of aged mice with after chronic exercise.

2. Purpose of the study

First purpose of this study is to investigate effects of different types of exercise on muscle in aged mice by showing muscle quality. Second purpose of this study is to investigate effects of exercise on FGF-2 in muscle and bone in aged mice.

3. Research hypothesis

1) Resistance exercise and aerobic exercise may increase both lean body mass and BMD in aged mice

2) Resistance exercise and aerobic exercise may reduce muscle FGF-2

3) Resistance exercise and aerobic exercise my increase Bone FGF-2

4. Limitations

This study cannot absolutely control foods intake. Nutrition is one of the most influential factor that effects the muscle and bone growth and maintenance. Although food intake will be measured weekly by weighing food supplements, only rough estimation of food intake per day can be measured. Also due to age of mice, the exercise should be conducted with careful observation of mice's condition. Every individuals will have different condition, intensity of exercise might be different at the end between each individuals. Lastly, the grip strength should be measured with careful attention, because grip strength itself requires animal's voluntary action. Resistance exercise group might feel more comfortable to the grip strength meter due to learning during exercise protocol (the action they conduct during grip strength test is similar to the ladder climbing exercise) compare to -CON and OET group, and it might causes error.

II. LITERATURE REVIEW

1. Aging

1.1 Muscle quality and aging

With aging, individual loss their muscle mass and function. Aging is related to the decrease of muscle mass, strength, and quality, and decrease of muscle starts during middle phase of human life, age of 30, as well and processes to Sarcopeina (Goodpaster et al., 2006; Hughes et al., 2001; Janssen, Heymsfield, & Ross, 2002).

The determinants of Sarcopenia are varied from genetic factors to environmental factors (Roubenoff & Hughes, 2000), it leads to reduced functional capacity and an increased risk of developing chronic metabolic syndromes, such as type II diabetes and osteoporosis (Nilwik et al., 2013). Age related muscle loss, Sarcopenia, is disorder of muscle cells, causing disruption of muscle homeostasis which is induced by intra and extra-cellular environment, and drive the decline of body cell mass. (Roubenoff & Hughes, 2000).

Aging is natural phenomena which cause harmonic changes of body and caused decline of cell mass (Cohn et al., 1980). Especially, decline in the quality of lean body mass, as cell mass declines faster than intercellular connective tissue and water (Kehayias, Fiatarone, Zhuang, & Roubenoff, 1997). In men aged 20 to 29 years, cell mass represents 59%of lean body mass, however, in men aged 80 to 89 years, cell mass is only 46% of lean mass (Ellis, 1990), and lean mass itself has also fallen

significantly (Roubenoff & Hughes, 2000).

Quantitative loss of muscle is caused by both loss of myocyte numbers and reduction in the protein content of the remaining muscle cells. As muscle quantity falls, it can cause decline of muscle quality, the functional term for muscle strength of muscle (Ivey et al., 2000; Tracy et al., 1999). With decrease muscle quality, it increases risk of having three or more disabilities, one or more balance abnormalities, using cane or walker, and falls, and ultimately increase risk of metabolic syndromes by restricting physical activities(Go, Cha, Lee, & Park, 2013; Iannuzzi-Sucich, Prestwood, & Kenny, 2002; Janssen et al., 2002; Kyle et al., 2001; Visser et al., 2003).

1.2 Bone quality and aging

Bone quality, may referred as bone mineral density, increased exponentially during the adolescent when the human muscle growth is peaked, and gradually starts to decrease as getting old. Not only the bone density starts to decrease at middle phase of human life span, age of 35, but also the rate of decline accelerates as the age increases. At age of 70, the remains of bone mineral is only 70% of the peak bone mineral once one acquired from adolescent. (Smith & Gilligan, 1996).

Although bone loss is natural phenomena during aging, the bone loss can be accelerated with metabolic problems accompanied by aging. (Xian et al., 2012; Xiao et al., 2010). Bone loss can induce weakening of bone and increase sensitivity to fractures through decrease of bone strength, and ultimately, it causes osteoporosis.

Osteoporosis, one of the most prevalent diseases for the elderly population, occurred all over the body evenly, and caused by low bone mineral density and destruction of micro structure of bone(Chan & Duque, 2002). With increase bone fracture rate caused by decrease of bone mineral density, physical activity of elderly people has been restricted greatly and endangered their health by causing metabolic syndromes(Xian et al., 2012; Xiao et al., 2010).

In case of United State, about 10 million people over 50 years old are suffering from osteoporosis, and 1.5 million of new prevalence are occurring every year (Post, Cremers, Kerbusch, & Danhof, 2010). Among the people suffering from osteoporosis, one out of two female and one out of five male have danger of experiencing osteoporotic fractures and danger has been increased exponentially

with increase of age (Khosla, Oursler, & Monroe, 2012; Oursler, Osdoby, Pyfferoen, Riggs, & Spelsberg, 1991). Also in Republic of Korea, in 2009, 10.6% of total population is aged over 65, and female population over 65 is growing every year. With this instance, increase of elderly population with osteoporosis and osteopenia became one of the urgent problem to solve for public health (통계청, 2007; 통계청, 2006)

2. Fibroblast growth factor 2 (FGF-2)

2.1 FGF-2 and muscle

FGF-2 (previously known as basic-FGF) is known to be one of the growth hormone that expressed in vascular smooth muscle and regulates vascular regeneration (Eliakim, Oh, & Cooper, 2000). However, not only FGF-2 regulate vascular regeneration, previous studies showed that FGF-2 is one of the most potent mitogens for myoblast, and it would appear to be critical for myogenesis in developing muscles(Grounds, 1998; Olwin, Hannon, & Kudla, 1994). The study which analyze correlation between in vivo FGF-2 protein level and immunohistochemical muscle regeneration demonstrated that high FGF2 protein level is in situations of good muscle regeneration (Anderson, Mitchell, McGeachie, & Grounds, 1995; Olwin et al., 1994). In addition, studies conducted by Clarke and his colleague also demonstrated that FGF-2 protein is expressed in skeletal muscle and promotes muscle regeneration (Clarke & Feeback 1996) via satellite cell proliferation (Clarke, Khakee & McNeil, 1993). FGF-2 induces proliferation of satellite cells and FGF-2 delays fusion of myocytes causing muscle hypertrophy and inducing muscle regeneration followed by injuries (Clarke et al., 1993). These data imply that the exogenous application of FGF-2 may enhance the muscle formation and may regulate the muscle maintenances (Grounds, 1998).

In addition, in the study conducted to investigate difference FGF-2 responsiveness in muscle in relation to the age difference, the satellite of 2 weeks and 9 month old rat did not showed any difference in the pattern of myoblast proliferation, yet delayed

on set of satellite cell cell-cycle (Johnson & Allen, 1995). Which indicates that, yet on set of satellite cell cell-cycle is different in relation to the age, FGF-2 induced satellite cell activation is important step of myoblast proliferation (Grounds, 1998).

2.2 FGF-2 and bone

Extracellular FGF-2 is important bone growth regulator (Noff, Pitaru, & Savion, 1989). Downstream activation caused by reaction between FGF-1 and FGF-2 receptor regulate various cell differentiation (Xiao et al., 2010). It is well demonstrated by cell culture experiment that level of FGF-2 can be used to measure bone mineral density (Noff et al., 1989). (Fig 2)

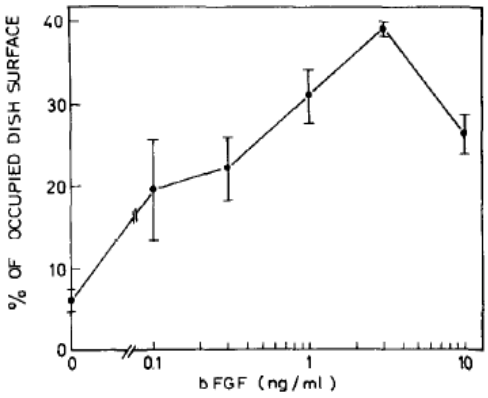

Figure 1. Cell culture of human bone like cell with FGF-2/bFGF. Demonstrating increase of applied FGF-2 level, % of occupied dis surface increase (Noff et al., 1989)

Current study demonstrated that increase of FGF-2 have positive effect on bone formation through cell culture experiments. It showed that human bone like cell culture with FGF-2 and Dexamethasone demonstrated significantly more cell count compared to Dexamethasone alone, and as time pass, DNA for bone differentiation has been increased significantly, and as result, the mineral contents showed significant difference (Pri-Chen et al., 1998). This indicated that downstream of FGF-2 and FGF-2 receptor activate transcription.

One study demonstrated direct effect of FGF-2 on bone mineral density through histomophometry. In this study, female rats has been overiectomized, and injected with FGF-2 systemically. Liang (Liang, Pun, & Wronski, 1999) reported that with FGF-2 injection, Osteoclast has been decreased significantly in proximal and distal bone, and Osteoblast and Osteoid, which has positive relationship, increased significantly. Liang showed dose dependent manners of FGF-2 effects on Osteoblast and Osteoclast as well (Liang et al., 1999). As FGF-2 injection increased, active Osteoblast increased and Osteoclast has been decreased. Also, he demonstrated that immediate decrease of Osteoblast after cessation of FGF-2 injection (Liang et al., 1999). With this instance, Liang reported that FGF-2 is potent regulator for bone formation (Liang et al., 1999).

More current study confirmed that increase of bone matrix FGF-2 level is one responsive to the increase of Bone Morphogenic protein – 2 (BMP-2) which is important factor causing accumulation of Runx 2 to nuclear (Agas et al., 2013).

3. Aging and FGF-2

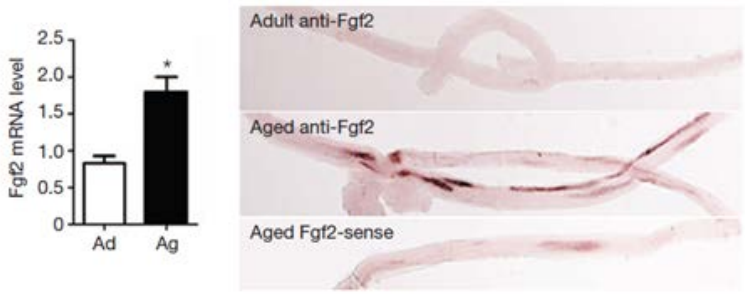

Figure 2. FGF-2 mRNA level change in aged muscle fiber and histology of FGF-2 in single muscle fiber of aged, and adult muscle fiber (Chakkalakal, et al., 2012)

Current study demonstrated the FGF-2 in muscle fiber in aging status. In aging status, to maintain the homeostasis of satellite cell, muscle fiber FGF-2 is increased (Chakkalakal, Jones, Basson, & Brack, 2012). Normally, Satellite cell is in quiescence to maintain the stem cell pool (Boyle, Wong, Rocha, & Jones, 2007; Morrison & Spradling, 2008). However, FGF-2 the well know growth factor which have potential to satellite cell mitogenic activity, increased greatly in the aged fibers (Chakkalakal et al., 2012). With increased FGF-2 in the muscle fiber, the cell cycle of satellite cell has been increased and it cause the cell depletion, causing the acceleration of aging(Chakkalakal et al., 2012). At the same time, by inhibition FGF-2 signal by treating spry 1, cell cycle of satellite cell has been decreased (Chakkalakal et al., 2012). It was suggested that FGF-2 induction in aged muscle fiber is one of the cell's self-protect mechanism attempting to repair the aged muscle fiber (Chakkalakal et al., 2012; Floss, Arnold, & Braun, 1997; Lefaucheur & Sebille, 1995). Strategies to prevent chronic FGF-2 production from the aged muscle fiber may maintain stem cell number to prevent aging of the muscle cell (Chakkalakal et al., 2012).

4. Exercise

Exercise has been recommended to improve bone mineral density in the young adolescents during their growth period by giving mechanical load (Hamrick, 2011). Traditionally, Mechanical loading has been stimulates the bone growth by providing increased strain or increase the frequency of low strain (Hamrick, 2011). Current reports demonstrated that increase in rate of BMD decrease is caused by malfunction of bone endocrine ability, and significant decrease of bone mineral would cause decrease of structural rigidity, osteoporosis and ultimately, it restrict the physical activity by increased the fracture rate (Xian et al., 2012; Xiao et al., 2010).

Also, various studies implied that effectiveness of exercise on maintaining muscle mass and function as well. One of the skeletal muscle's impressive and important characteristic is plasticity (Siparsky, Kirkendall, & Garrett, 2014). As muscle's ability to adapt environments through life span, trainability of skeletal muscle and muscle gain has been demonstrated on elderly population (Siparsky et al., 2014). With appropriate exercise, adults between ages of 60 to 80 experienced 20% to 30% of aerobic fitness improvements, as well as peripheral muscle adaptations (Heath, Hagberg, Ehsani, & Holloszy, 1981; Seals, Hagberg, Hurley, Ehsani, & Holloszy, 1984). In addition, men over 66 years of age demonstrated 5% increase of strength per day with weight lifting 80% of their 1 RM for 12 weeks (Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988). As result, strength improvement with cross-sectional areal improvement of the thigh muscle all leading to improvement in functional mobility (Fiatarone et al., 1990).

Moreover, muscle produce and/or secrete various growth factors and hormones during contraction. Among these factors and hormones, muscle induced IGF-1 and FGF-2 are important growth factor for muscle hypertrophy and maintenance, and increased with muscle contraction (Eliakim et al., 2000). Muscle contraction is one of the stress that causes physiological sarcolemma injury, and it induced FGF-2 production and secretion from cytoplasm of muscle fibers (Clarke et al., 1993). Also Clarke (Clarke & Feeback, 1996) demonstrated that elevated FGF-2 level in muscle with chronic mechanical stress. Moreover, single acute wrist exercise was able to decrease serum FGF-2 level and recovered slowly to its' normal range after few hours (Eliakim et al., 2000). Eliakim (Eliakim et al., 2000) reported that exercise site may require FGF-2 to recover membrane disruptions and circulating FGF-2 was 'captured' by muscle in exercise site, indicating altering FGF-2 level may work as regulator of muscle regeneration.

III. METHOD

1. Animal

Nineteen months old male wild-type C57BL/6 mice (Biomedical mouse resource center, Korea) was used for this experiment. All mice will housed in a controlled environment with a 12:12-h light-dark cycle with room temperature maintained at 22℃. All mice were provided with water and food ad libitum. The animals will be cared for in accordance with the Guide for the Care and Use of Laboratory Animals issued by Institute of Laboratory Animal Resources, USA, 1996, and a protocol will be approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. All of the experiments will be conducted to minimize the number of animals utilized and the suffering caused by the procedures of the present study

2. Experimental design

Twenty nineteen months old male C57BL/6 mice will be randomly assigned to two groups, Resistance Training group (ORT; n=7); Old Control group (OCON; n=7); Endurance Training group (OET; n=6). All mice was provided a chow containing 12.5% of calories provided from fat, 24.5% from protein, and 63% from carbohydrates (Purina rodent chow 5057, Purina Korea) and food intake was measured weekly. ORT group was forced to climb vertical ladder (1-m ladder with 1.5-cm grid and inclined at 85°) 3 times a week for 12 weeks and OET group was force to run treadmill 3 times a week for 12 weeks. 48 hours after last exercise training, animal sacrifice was performed to minimize the effect of the last bout of exercise. Following sacrifice, left and right tibialis anterior and soleus muscle was surgically removed and weighted. Also, left and right femur was surgically removed. After the extractions of muscle and bone, left tibialis anterior, soleus and femur was frozen in liquid nitrogen and stored at -80°C for protein analysis.

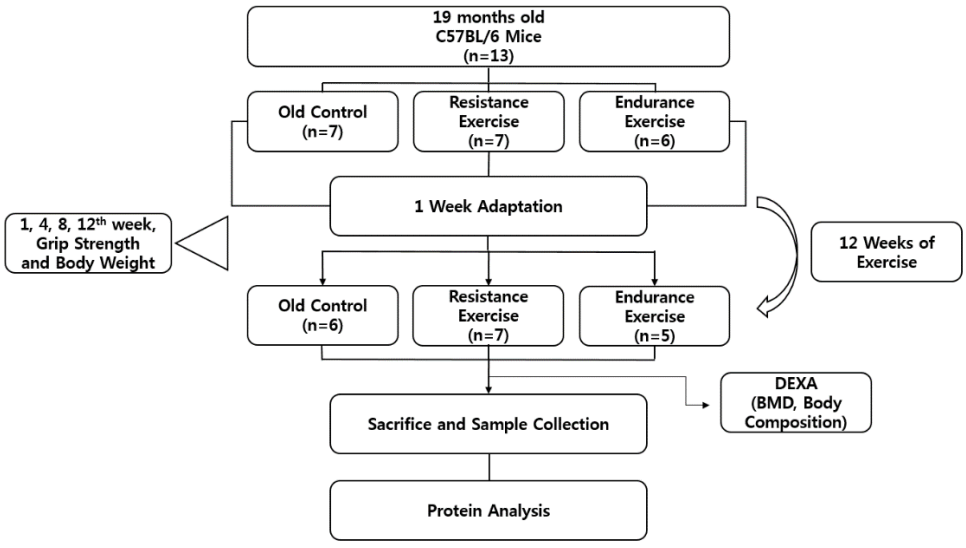

Figure 3. Experimental design

3. Exercise protocol

The C57BL/6 mice in the ORT group was force to climb vertical ladder 3 days per week for 12 weeks. Resistance ladder climbing exercise was conducted by using 1-m ladder with 1.5-cm grid and ladder was set to attain 85 degreed with ground. The adaptation for ORT group mice was conducted by letting mice to climb up the ladder without any resistance for one week. To reduce the stress, outsource stimulus such as food reward, electrical stimulation and forced air was not given to the mice during ladder climbing exercise.

The resistance was given to the mice by adding weights on the tail, and the load was increase gradually as the exercise proceeds. The mice was positioned at the very bottom of 1-m ladder and mice was motivated to climb up the 1-m ladder with weight on the tail. When mice reaches the very top of the ladder, 2 minute rest was given before next trial of ladder climbing. Each exercise trial was consisted with 8 consecutive ladder climbing trials.

After one week of adaptation period, during the first actual exercise session, four ladder climb trail was conducted without weight and 5 grams of weight will be added to their tail. To progressively increase resistance on their tail, first 4 trails of each exercise session was start with the 2 grams of additional weight that was added during last 4 trails of previous exercise session. Then, if the mice successfully conduct the first 4 trail, the resistance was increased by 2 grams for last 4 trails. However, because ORT group is consisted with the aged mice, after 8^{th} week of exercise, increase rate of weight was decreased compare to first 8 weeks. Instead of

adding 2 gram from last 4 trials of previous exercise session, after 8th week, the first 4 trials was conducted with the weight from last 4 trials of previous session, and if they carry out the exercise successfully, 2 grams of additional weight was added for next 4 trials. However, because the animals were very old and fragile, very careful attention and adjustment of exercise intensity were given.

For the OET group, force treadmill running was conducted 3 days per week for 12 weeks. Treadmill running was conducted on specialized treadmill for the mice that was built by our laboratory. To reduce the stress, the outsource stimulation was not provided to mice to motivate to run the treadmill.

The treadmill training protocol was followed. Before the actual exercise program was conducted the OET group was conducted adaptation of a week. For the exercise program, OET group was force to run from 30 min to 1 hour per session with not including 3 min warm down. The each exercise session was segmented by intensity and time, and Intensity and time were gradually increased by weeks. The exercise protocol for ORT and OET are presented in figure 4.

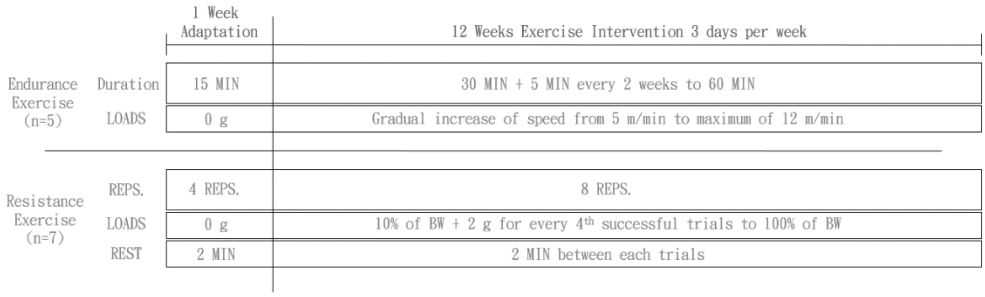

Figure 4. Exercise protocol

4. Measurements

Table 1. Measurements

Measurements	Measurement/ Method	Model/Antibody	Company
Body Composition	DEXA	Discovery W	Hologic, USA
Bone Mineral Density	DEXA	Discover W	Hologic, USA
Grip Strength	Grip Strength Meter	Grip Strength Meter	Bioseb, France
Muscle/Bone FGF-2	ELISA	FGF basic mouse ELISA kit	abcam, USA

4.1 Body composition

Figure 5. DEXA (Dual Energy X-Ray Absorptiometry)

According to experiment design, DEXA analysis was performed right before the sacrifice and sampling to measure body composition and bone mineral density by using QDR Discovery W (Hologic, USA). Mice was anesthetize by 20% Urethane after overnight fasting, then was placed supine position on the DEXA. Because detection range for small animal have to be greater than 150 grams, adult Sprague dawley rat was placed on the DEXA together in order to fulfill the detection range of DEXA itself. Data collected from DEXA was analyzed by Standard Software (QDR for Windows XP Operating System, USA). This analyze software allow us to select the sub-region to measure and analyze composition and bone mineral density. By using this feature of standard software, front limb and hinder limb region was selected carefully and analyzed.

4.2 Grip-strength

Figure 6. Grip strength meter

The grip strength was measured with Grip Strength Meter (Bioseb, France), and method which was used in previous report was modified for measuring (Meyer, Tilson, Byrd, & Riley, 1979). Grip strength was measured by allowing the animals to grasp a still wired grill attached to the force gauge. This procedure was followed by pulling the animal away from the gauge until when the mice releases the grill while pulling mice back on the tail gently. It provide the maximal force for grip strength, and the value of force was recorded in unit of grams. Measuring the maximal grip strength was performed for five times, and highest measurement was used for analysis. The grip strength was measured during pre-test, fourth week, eighth week and 12 week of exercise period. For analysis the median value instead of mean value was taken because the animal was old and easily fragile, the measurement may consisted the error.

4.3 Protein extraction

After the DEXA measurement, anesthetized mice's left and right tibialis anterior and soleus muscle was rapidly removed and stored at -80 Celsius degree until the protein analysis. To extract the protein from both muscle and bone will be homogenized in 500 ul of extraction buffer (RIPA). The extracts was then centrifuge at 12,000 rpm at 4 Celsius degree for 15 minutes to differentiate insoluble materials. Insoluble materials was discarded and determination of protein concentrations in the supernatants quantification of protein assay will be performed using the Pierce BCA protein assay kit (Thermo Scientific, USA) and Bradford assay kit(Bio-rad, USA). BCA protein assay kit was used to determine protein concentration in femur because although Bradford assay kit give much more sensitive reaction, Bradford assay was interfered with minerals in the body extracted solution.

4.4 Protein analysis

Quantification and measurement of FGF-2 on protein quantified femur, soleus and tibialis anterior muscle sample with BCA and Bradford assay was performed by using FGF basic mouse ELISA kit (ab100670) (abcam, USA). The ELISA was conducted by following the procedure provided with ELISA kit.

5. Data Analysis

Statistical analysis was performed using the SPSS 20.0 software (SPSS Inc.). Results will be expressed as mean±SE. One way ANOVA was performed to exam the difference between groups in body weight, lean mass, fat mass, muscle wet weight, BMC, BMD and FGF-2 level. Dun-Sidak 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 FGF-2 protein level.The level of significance was set at $p<0.05$.

IV. RESULT

1. Whole body composition and Body Weight

Table 2. Whole body scan of DEXA and body weight

	OCON	ORT	OET	*p* value
BMC (g)	0.692±0.044	0.747±0.071	0.650±0.056	0.542
BMD (g/cm^2)	0.115±0.002	0.116±0.005	0.116±0.003	0.974
Fat mass (g)	4.983±0.379	5.4±0.347	5.160±0.555	0.763
Lean mass (g)	27.583±0.776	27.186±0.689	26.020±1.157	0.459
Percent fat	15.250±0.764	16.543±0.989	16.620±1.705	0.644
Total mass (g)	35.35±1.208	35.586±0.798	33.640±1.160	0.408

Whole body DEXA was scanned 48 hours after the last exercise session for both resistance and aerobic exercise, right before the dissection of mice with 16 hours of fasting. DEXA result showed no significant improvement in BMC, BMD, Fat Mass, Lean mass, Percent fat and Mass in both ORT, OET group compared to OCON group. Also, the body weight was measured right before dissection, and body weight as well showed no significant difference.

2. Grip strength

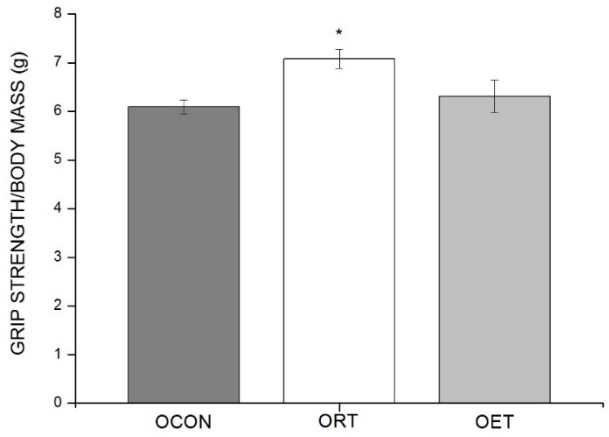

Figure 7. Relative grip strength of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). *; vs. OCON

For grip strength, median values of Grip strengths measured at 2nd, 4th, 6th, 8th, 10th, and 12th weeks of each group were adjusted with body weights. The result showed significant improvement in grip strength in resistance group. (OCON vs. ORT, 6.092±0.145 vs. 7.083±0.194, $p<0.05$) However, there was no significant difference between OCON group and OET group, and OCON group and OET.

3. Appendicular lean mass

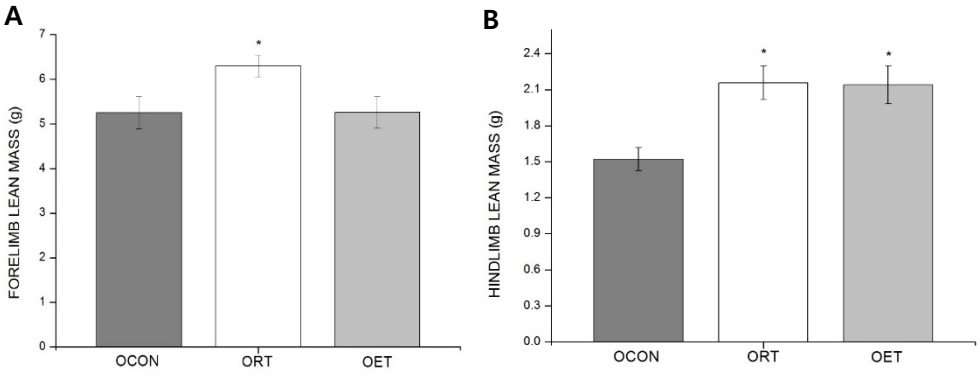

Figure 8. Appendicular lean mass of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb lean mass, B; hindlimb lean mass. *; vs. OCON

For each DEXA result, forelimbs and hindlimbs were carefully analyzed by using computer program QDR for Windows XP Operating System, USA, provided by Hologic Inc. The result showed significant difference in forelimb lean mass in ORT group compare to the OCON group. (OCON vs. ORT, 5.25±0.360 vs 6.3±0.239, $p<0.05$) However, there was no significant difference between OCON group and OET group in lean mass of forelimb. Also, lean mass of hindlimbs of ORT group was significantly heavier compare to OCON group(OCON vs. ORT, 1.52±0.01 vs. 2.15±0.141, $p<0.05$), and OET group showed significantly heavier lean mass compare to OCON group as well (OCON vs OET, 1.52±0.01 vs. 2.14±0.167, $p<0.05$).

4. Muscle wet weight

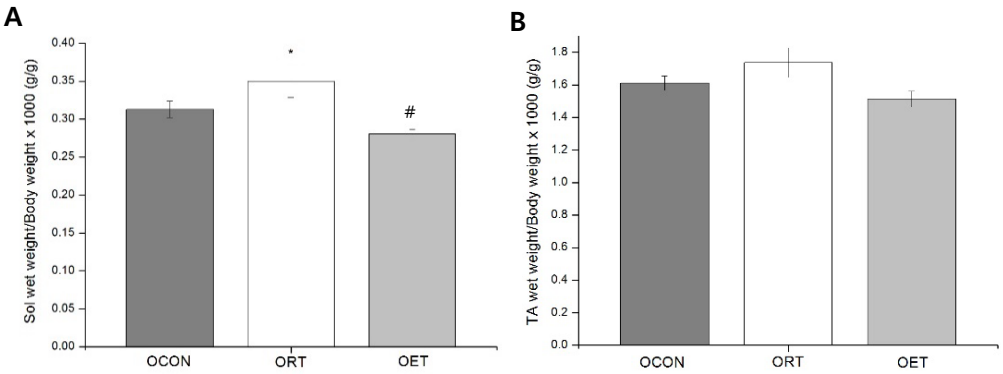

Figure 9. Relative muscle wet weight of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; relative muscle mass of soleus muscle, B; relative muscle mass of tibialis anterior muscle. *; vs. OCON, #; vs. ORT

Right after the dissection of muscle, average of both side of soleus and Tibialis Anterior muscle wet weights were measured. By adjusting with body weight, there was a significant difference in Soleus muscle wet weight only between ORT and OCON. (OCON vs. ORT, 0.313 ± 0.011 vs. 0.35 ± 0.0215, $p<0.05$). However, there was no significant difference in Tibialis Anterior muscle wet weights between OCON group and OET group.

5. Appendicular BMC

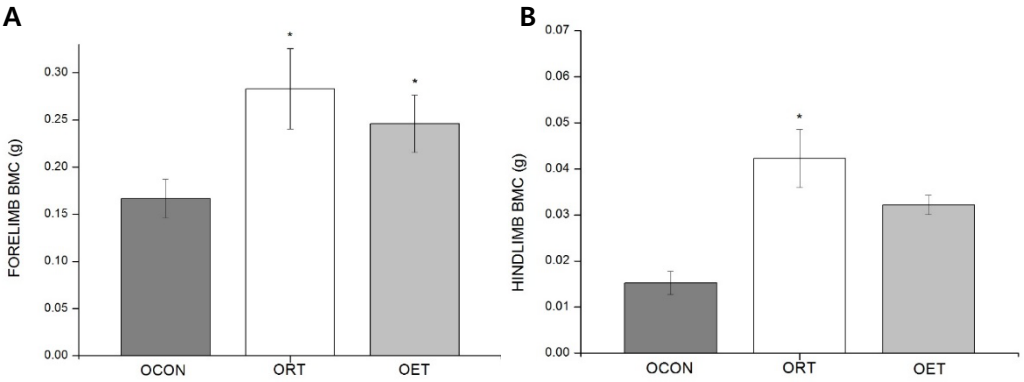

Figure 10. Appendicular BMC of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb BMC, B; hindlimb BMC. *; vs. OCON

By careful analyzing DEXA result by using computer program, forelimb and hindlimb BMC was measured. There were significantly increased in forelimb BMC in ORT group compared to OCON group (OCON vs. ORT, 0.166±0.00204 vs. 0.283±0.0425, $p<0.05$) and OET group compared to OCON group as well (OCON vs. OET, 0.166±0.00204 vs. 0.246±0.0303, $p<0.05$). Also, the result showed that significant increase in hindlimb BMC in only ORT compared to OCON (OCON vs. ORT, 0.0152±0.002 vs. 0.423±0.0063, $p<0.05$), however there was no significant difference in OCON and OET.

6. Appendicular BMD

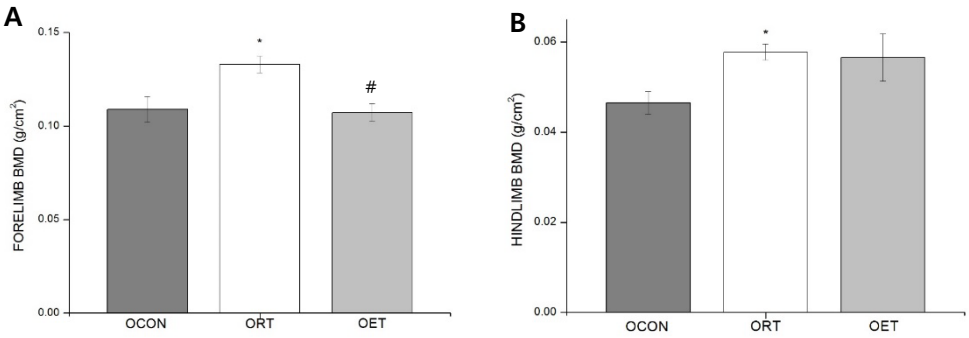

Figure 11. Appendicular BMD of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; forelimb BMD, B; hindlimb BMD. *; vs. OCON, #; vs. ORT

For forelimb and hindlimb BMD, DEXA results were carefully analyzed by using same program. The result showed that increase in BMD in ORT group compared to OCON group, (OCON vs ORT, 0.1089±0.007 vs. 0.1329±0.0045, $p<0.05$) however, there was no significant difference between OCON and OET groups. Also, the result showed that increase in BMD in ORT group compared to OCON group (OCON vs. ORT, 0.0465 ± 0.0025 vs. 0.05778 ± 0.00176, $p<0.05$), however, there was no significant difference between OCON and OET groups.

7. FGF-2 protein level in muscle

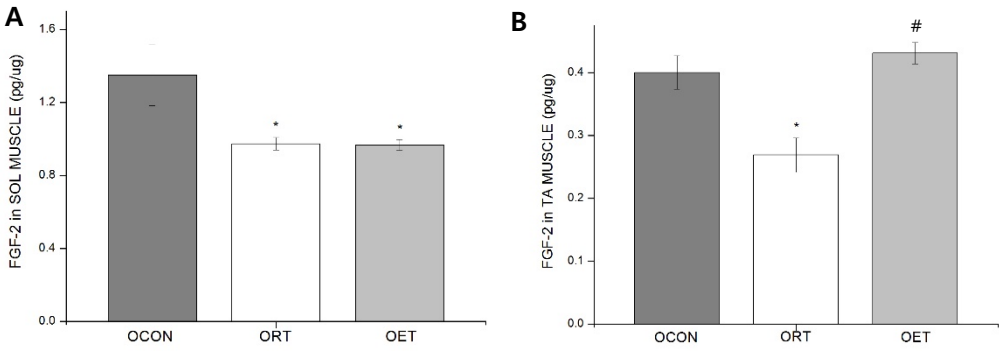

Figure 12. FGF-2 protein level in muscle of OCON (old control group), ORT (old resistance exercise group) and OET (old endurance exercise group). A; FGF-2 level in soleus muscle, B; FGF-2 level in tibialis anterior muscle. *; vs. OCON, #; vs. ORT

By conducing ELISA, FGF-2 protein level of Soleus and tibialis Anterior muscle were measured. The result showed significant decrease in FGF-2 protein level of ORT group and OET group compare to the OCON group in Soleus muscle (OCON vs. ORT, 1.35 ± 0.1667 vs. 0.9434 ± 0.035, $p<0.05$; OCON vs. OET, 1.35 ± 0.1667 vs. 0.967 ± 0.030, $p<0.05$). In tibialis Anterior muscle, only ORT group showed significant decrease compared to OCON group in FGF-2 protein level (OCON vs. ORT, 0.400 ± 0.027 vs. 0.268 ± 0.027, $p<0.05$).

8. FGF-2 protein level in bone

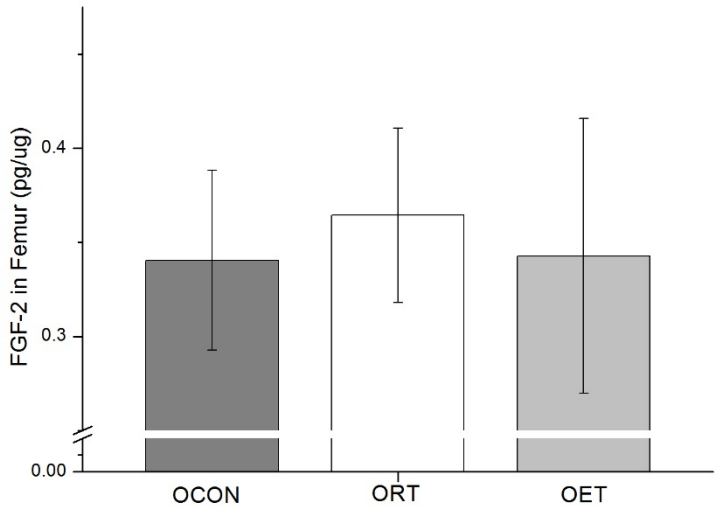

Figure 1311. FGF-2 protein level in femur

By conducting ELISA, level of FGF-2 in femur was measured. The result of ELISA showed there was no significant difference in femur FGF-2 Level, however, there was increased tendency in FGF-2 level of femur.

9. Correlation between muscle FGF-2 and hindlimb lean mass

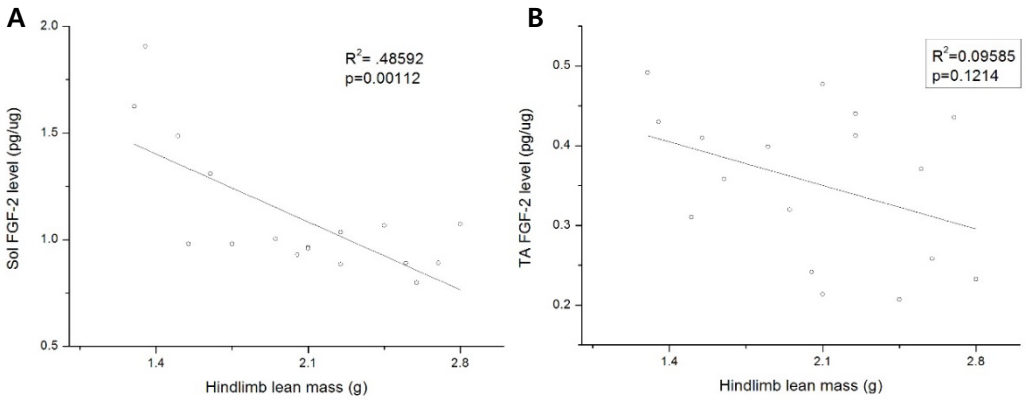

Figure 1412. Correlation between muscle FGF-2 protein level and hindlimb lean mass. A; correlation between hindlimb lean mass and soleus muscle FGF-2 protein level, B; correlation between hindlimb lean mass and tibailis anterior muscle FGF-2 protein level

For correlation analysis, FGF-2 protein level in muscles and hindlimb lean mass from DEXA was taken. The result showed negative correlation only between FGF-2 level of Soleus muscle and hinder limb lean mass. (r^2=0.48592, *p*=0.0012) however, there was no relationship between tibialis anterior muscle FGF-2 protein level and hindlimb lean mass.

V. DISSCUSION

As far as we know, it was the first study that investigate changes of FGF-2 protein level of muscle and bone in response to exercise in aged status. Not only 12 weeks of resistance exercise group increased appendicular lean mass, soleus and tibialis anterior muscle mass, relative grip strength, 12 weeks of resistance exercise also increase BMC and BMD compared to control group. On the other hand, hindlimb lean mass and BMC were increased in endurance exercise group compared to control group. In addition, soleus muscle and tibialis anterior muscle FGF-2 protein level were reduced in resistance exercise group compared to control group, whereas only soleus muscle FGF-2 protein level was reduced in endurance exercise group compared to control group, and endurance exercise group only reduce FGF-2 protein level in soleus muscle. However, in femur, FGF-2 protein level did not show any difference among three groups. At last, soleus muscle FGF-2 protein level showed a negative correlation with hindlimb lean mass.

Resistance exercise increased appendicular lean mass, muscle wet weight and relative grip strength.

It is known that resistance exercise increases lean mass, and decreases fat mass in aged status (Heath, Hagberg, Ehsani, & Holloszy, 1981; Seals, Hagberg, Hurley, Ehsani, & Holloszy, 1984). However, in this study, DEXA result showed no significant changes in whole body composition between old control group and exercise groups. To eliminate the effects of nutrition consumption difference, rough

food intake per day were recorded, and did not show a significant difference among three groups throughout 12 weeks of intervention period. However, body organelles and skeletal system muscle cannot be differentiated by DEXA, whole body composition results might have affected by body organ. However, when DEXA results were analyzed by appendicular body parts, the body composition differences were shown to be significant in lean mass, BMC, and BMD between OCON and ORT, OET groups.

In this study, ORT group showed a significant increase in forelimb and hindlimb compared to OCON group, and OET group showed a significant increase only in hindlimb lean mass compared to OCON group. These results not only indicate the both types of exercise can increase lean mass in the appendicular parts of body, resistance exercise is more effective on increasing lean mass of the affected parts of body(Egan & Zierath, 2013). In support to the lean mass, relative muscle wet weight of soleus muscle in ORT group showed a significant increase compared to OCON, however, there was a no difference found between OCON group and OET group.

The strength of mice over 12 weeks was assessed by measuring grip strength. Over 12 weeks of exercise intervention period, grip strength was measured at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} weeks. Due to their age, daily conditions of individual mice affected either their motivation or actual strength, variation of grip strength between each trial were considerable. To reduce variations, the median values among 12 weeks were taken for analysis. In spite of this limitation, the relative grip strength of ORT increased compared to OCON group. Which indicated that the resistance exercise conducted for 12 weeks had sufficient intensity to observe exercise induced changes in muscle. However, the absolute grip strength nor relative grip strength showed a

significant difference in OET compared to OCN group. These results were consistence with previous studies (Egan & Zierath, 2013), which demonstrated the effectiveness of resistance exercise in increasing strength compared to the endurance exercise.

Resistance exercise reduced muscle FGF-2 protein level in soleus and tibialis anterior muscle.

One of the purpose of this study was to investigate the effect of two types of exercise on muscle FGF-2 protein level in aged mice. From previous study, muscle FGF-2 level was found to be important for satellite cell proliferation, and it promotes muscle regeneration (Clarke & Feeback, 1996; Clake, Khakee, & McNeil, 1993). However, in aged mice, single muscle fiber FGF-2 protein level was dramatically increased compared to that of adult mice and it induced satellite cell depletion (Chakkalakal, Jones, Basson, & Brack, 2012). In our lab's unpublished data, similar trends with pervious study in soleus muscle FGF-2 protein level were observed between young mice and old mice. In this study, with 12 weeks of exercise intervention, 19 months old mice showed a significant difference on muscle FGF-2 protein level in muscle. However, FGF-2 protein level responded differently to different types of exercise intervention. In soleus muscle, FGF-2 muscle protein level was decreased in both ORT and OET groups compared to OCON group, and only tiabialis anterior muscle FGF-2 protein level of ORT was decreased compared to OCON group. The result was consistent with the previous study that demonstrated a difference in exercise responsiveness between soleus and tibialis anterior muscle

(Drzymala-Celichowska, Karolczak, Redowicz, & Bukowska, 2012). Also, in another previous study, Leiter and his colleague demonstrated satellite activation on four different muscle (gastrocnemius muscle, extensor digitorum muscle, tibialis anterior muscle and quadriceps muscle) after 3 weeks of voluntary wheel running exercise and the result showed that only quadriceps were responsive to voluntary wheel running and voluntary wheel running did not induce any change in satellite cell activation (Leiter, Peeler, & Anderson, 2011). However, in this study, muscle type differences were not considered; for the future study, the investigation of the effect of two different types of exercise on type 1 and type 2 muscle in relation to changes of muscle FGF-2 protein level to be conducted in order to elucidate the unanswered question from this study.

Resistance exercise increased appendicular bone quality.

In this study, the BMC and BMD of whole body did not show any difference among three groups, however, the appendicular part BMC and BMD showed a significant increase in exercise groups compared to OCON group. In forelimb, both ORT group and OET group showed a significant increase compared to OCON in BMC, and only ORT group showed a significant increase compared to OCON group in BMD. However, in hindlimb, only ORT group showed significant increase compared to OCON group in both BMC and BMD, and OET showed an increasing trend in BMC. In the previous studies, 6 weeks of resistance ladder climbing exercise demonstrated a significant increase in BMD (Ahles et al., 2013) and 6 weeks of voluntary wheel running only showed an increase in BMC (Newhall, Rodnick, van

der Meulen, Carter, & Marcus, 1991). This study showed a consistence result with the previous studies. Although BMD and BMC are qualified measurements in determining bone quality, bone strength, fracture force, and volume of the bone should be also taken into consideration as of more precise measurements of bone quality in constructing a more structured study in the future.

Exercise did not change FGF-2 protein level in bone.

In the previous studies, it was demonstrated that IGF-1 and FGF-2 induce osteoblast proliferation and differentiation. However, these two growth factors trigger different pathways to increase osteoblast proliferation and differentiation (Robubi et al., 2014), resulting increase in bone quality. Especially, FGF-2 induces an increase of BMP-2 and increase in osteoblast differentiation, thus resulting an increase in the mineralization of bone (Agas, Sabbieti, Marchetti, Xiao, & Hurley, 2013). Furthermore, Hausdof and his colleague (2010) demonstrated an increase of FGF-2 level in osteoblast with external strain (Hausdorf et al., 2011). Hence, in this study, it was expected that bone FGF-2 protein level would be increased in respond to exercise, however, there was no difference in FGF-2 protein level among three groups. Yet, the reason for the unresponsiveness of FGF-2 protein to exercise in bone is unexplainable in this study; only a couple of speculation can be made: (1) the strain that was applied to bone by exercises was not enough to change osteoblast FGF-2 protein level in aged status, or (2) it might be accounted to the decreased level of muscle FGF-2 from exercise in aged status, since muscle and bone interact via FGF-2 (Hamrick, McNeil, & Patterson, 2010). To answer this question, further studies

should be designed while thoroughly considering the increment of intensity and the measurement of the potent osteogenic factors such as IGF-1, BMP-2 and RUNX 2 in order to distinguish the IGF-1 induced osteoblast proliferation and FGF-2 induced osteoblast differentiation (Agas et al., 2013; Xian et al., 2012).

Muscle FGF-2 protein level and hindlimb lean mass showed a negative correlation.

The correlation analysis was conducted between muscle FGF-2 protein level and hindlimb protein level. The correlation analysis showed a negative correlation between soleus muscle FGF-2 protein level and hindlimb lean mass, however, no correlation was found between tibialis anterior muscle FGF-2 protein level and hindlimb lean mass. However, as it was mentioned before, tibialis anterior muscle was not responsive to endurance exercise from previous study (Leiter et al., 2011), tibialis anterior muscle FGF-2 protein level also showed negative correlation between hindlimb lean mass as well by excluding endurance exercise group (data not shown).

Conclusion

This study was first started with a notion that there might be a relationship between muscle quality and bone quality. Numerous studies indicated that muscle and bone interact via FGF-2 in paracrine and endocrine fashion in normal status (Hamrick, 2011; Hamrick et al., 2010). However, in aged status, although physiological statuses

are very different compared to normal status, very few studies were conducted to investigate FGF-2 and other growth hormones. As far as we know, this was first study that was conducted to investigate the change in muscle and bone FGF-2 protein level in relation to exercise. However, this study was only able to deduct descriptive explanation on FGF-2 protein level and the physiologic changes of muscle and bone. Further studies are required to investigate difference in muscle and bone FGF-2 protein level from the effect of exercise on different types of muscle (type 1 and type 2 muscle) and different parts of bone(cortical bone and trabecular bone) in aged status.

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국문 초록

12주의 저항성운동과 유산소운동이 노화 쥐 골격근과 뼈의 FGF-2에 미치는 영향

김진수

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서론: 최근, 근육과 뼈의 내분비기관으로서 역할이 밝혀져, 연구가 활발히 이루어 지고 있다. 특히 근육에서 분비되는 Fibroblast growth factor-2는 근육의 성장과 재생에 긍정적인 영향을 미치며, 뼈에서도 조골세포의 분화 과정에 긍정적인 역할을 미친다고 알려져 있다. 따라서 본 연구에서는 노화 상태에서 운동에 근육과 뼈의 FGF-2 단백질에 어떠한 영향을 미치는 지 알아 보고자 한다.

방법: 19 개월령 노화 쥐(C57BL/6 mice) 20 마리를 OCON (old control group, n=7), ORT (old resistance exercise group, n=7), OET (old endurance exercise group, n=6, 3 그룹으로 무선 할당하여 12주동안 저항성 사다리 운동과 유산소 트레드밀 운동을 주 3회 실시 하였다. 식이 섭취를 매주 측정하였으며, 소형 동물 악력계를 이용하여 악력을 매 2주 측정하였다. 12주의 운동 중제 후, 체성분은 이중에너지 X선 흡수 계측법 (DEXA; Dual Energy X-Ray Absorptimetry)을 사용하여 측정하였고, 근육은 장지신근과 전경골근을 적출하여 무게를 측정하였으며, 뼈는 대퇴골을 적출하여, 단백질 분석을 위해 -70° C로 보관 되었다. 단백질 분석으로 항원 항체 반응을 이용한 효소결합 면역흡착 분석법(ELISA)을 이용하여 분석하였다. 통계 기법은 One-way ANOVA를 사용하여 분석하였으며, 사후 검증을 통하여 그룹간의 차이를 분석하였다. 또한 pearson correlation 분석이 뒷다리의 제지방과 근육 FGF-2 단백질의 상관관계 분석에 사용 되었다.

결과: 상대적 악력에서는 ORT그룹에 서 OCON그룹 보다 상대적으로 유의한 증가를 보였으며, 사지의 제지방량에서 앞다리 뒷다리 모두 유의한 증가를 보였으나, OET그룹에서는 뒷다리에서 OCON그룹에 비하여 유의한 증가를 보였다. 장지신근의 무게는 ORT그룹에서 OCON그룹에 비하여 유의한 증가를 보였다. 또한 사지의 BMC와 BMD모두 ORT그룹에서 OCON그룹보다 유의한 증가를 보였으나, OET그룹에서는 앞다리의 BMC만 OCON그룹보다 유의한 증가를 보였다. FGF-2의 단백질 양은 장지신근에서만 ORT그룹, OET그룹 모두에서 유의한 감소를 보였으나, 전경골근에서는 ORT그룹에서만 OCON그룹 보다 유의한 감소를 보였다. 그러나 대퇴골에서의 FGF-2 단백질 양은 세 그룹 모두에서 유의한 차이를 보이지 않았다. 마지막으로 뒷다리 제지방량과 근육의 FGF-2 단백질 양의 상관관계 분석에서는 장지신근의 FGF-2 단백질 양과 뒷다리의 제지방량에서만 유의한 음의 상관관계를 보였다.

결론: 12주의 저항성 사다리 운동은 노화된 C57BL/6 쥐의 사지의 제지방량, BMC 그리고 BMD서 유의한 증가를 보였고, 장지신근과 전경골근의 근육 무게를 증가 시켰다. 또한, 12주의 저항성 사다리운동은 노화된 C57BL/6 쥐의 장지신근과 전경골근의 FGF-2 단백질 양을 감소 시켰으나, 대퇴골의 FGF-2 단백질에서는 변화를 보이지 않았다. 마지막으로, 장지신근의 FGF-2 단백질 양과 뒷다리의 제지방과 유의한 음의 상관 관계를 나타내었다.