Dissertation of the Degree of Master of Science

Effects of Exercise-Induced Apelin on Muscle Function and Cognitive Function in Aged Mice

운동에 의해 유도된 Apelin이 노화쥐의 근기능과 인지기능에 미치는 영향

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Abstract

Endogenous peptide, apelin, has been noted for improving muscle function. Furthermore, there is evidence that apelin not only improves muscle function in aging but also affects cognitive function. Therefore, in this study, we confirmed the correlation between muscle function and cognitive function in aging mice, and to further, examined the effect of exercise-induced apelin in muscles on cognitive function.

In *in vitro* study, the proliferation and cell viability of C212 cells (skeletal muscle cell), neuro2a cells (neuronal cell) and C6 cells (glial cell) were directly measured after apelin treatment. Forskolin (exercise mimetic chemical that upregulates cAMP) was also administered to C2C12 cells. C2C12 cells were treated with apelin and forskolin, after the treating, its secretomes were treated to C6 cells. In animal study, 8 months and 28 months C57BL/6J mouse were treated with treadmill (YE=5, OE=4) and ladder (YR=4, OR=3) exercise each. Then its muscle function and cognitive functions were measured by isometric force test, grip strength test, rotarod test and Y-maze test. Also, apelin expression levels of serum and skeletal muscles were analyzed, then its correlations with cognitive functions were analyzed.

In vitro studies have shown that the forskolin upregulates apelin expression levels in C2C12 cell, and apelin upregulated viability of C2C12, N2A and C6 cell. In addition, C2C12 skeletal secretomes which were treated with apelin upregulated C6 cell's viability. In animal study, both treadmill and ladder exercise upregulated grip strength ($p<0.05$), rotarod latency to fall ($p<0.05$) and isometric force

of twitch and tetanus in significant ($p<0.05$). Two types of exercise also upregulated cognitive functions (Y-maze test) in 28 months old mouse ($p<0.05$). Also, muscle function index (normalized skeletal muscles weight, grip strength and tetanic force) and cognitive functions showed significant positive correlation ($p<0.05$) and exercise-induced apelin expression in skeletal muscle and cognitive function (Y-maze test) also showed significant positive correlation in old mouse ($p<0.05$). In addition to it, serum apelin expression level upregulated by both exercise type in old mice and it showed positive relationship with cognitive function ($p<0.05$).

This study clarified the relationship between muscle function and cognitive functions. Also results suggest that apelin might be a molecule that intermediate the relationship between muscle function and cognitive function.

Keyword : Aging, Exercise, Apelin, Muscle function, Cognitive function

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I. Introduction

1. Need for Research

According to the report of Korea health promotion institute (KHPI) in 2018, Korea's life expectancy is rapidly increasing. However, the difference between life expectancy and healthy life expectancy is getting bigger, therefore healthy life expectancy is 8.9 years lower than life expectancy. Therefore, it is necessary to narrow the gap between life expectancy and healthy life expectancy. There's a lot of factors that affect healthy life expectancy, including mental problems such as Alzheimer's disease and cognitive function problem (Vos et al., 2012). In recent years, sarcopenia, the progressive decline of the skeletal muscle function and muscle mass, has been attracting attention as a representative geriatric disease, and cognitive frailty has been highlighted as a factors that reduces the health life (Kelaiditi et al., 2013). Recent studies have reported that sarcopenia affects cognitive function negatively (Tolea, Chrisphonte, & Galvin, 2018). This suggests that alleviating the sarcopenia might bring improvement of cognitive frailty too.

It is worth noting that exerkine apelin, recently introduced in *Nature medicine* in July 2018, can alleviate sarcopenia (Vinel et al., 2018). apelin has been shown by a various methods to demonstrate that apelin induces regeneration of skeletal muscles and that apelin increases in muscle through exercise (Vinel et al., 2018). However, this study was limited to only in skeletal muscle and endurance treadmill exercise.

Several studies have identified the role of apelin in the brain. In a previous study conducted in 2011, it was found that seizure induces

neuronal cell death, and loss of neuronal cells eventually leads to a status epilepticus, and epileptic seizure leads to a vicious cycle in which neuronal cell death. However, apelin acts on the endogenous ligand of apelin receptor (APJ), a receptor in the cell membrane of the brain, and mitigated cranial nerve damage by a protective regulation of the brain cells of the hippocampal and cerebral cortex (X. Zhang et al., 2011). Apelin has been shown that its role of protecting and regulating of neuronal cells (Cheng, Chen, Bai, & Xin, 2012). Other studies have reported that apelin alleviates neural cell death and neural dysfunction in the brain (Bao, Zhang, Han, & Dai, 2015). In other previous studies, apelin-13 was administered intracerebrally to an animal model that mimics Parkinson's disease by administering 6-OHDA to the rat model. Afterwards, Morris water maze and novel object recognition test were conducted to measure the cognitive function. As a result, apelin-13 significantly upregulated the cognitive function of Parkinson's disease model (Haghparast, Esmaeili-Mahani, Abbasnejad, & Sheibani, 2018). Based on these results, it can be concluded that apelin has a positive effect on cognitive function. However, these researches were limited to only in brain function.

As mentioned earlier, muscle function and cognitive function are positively correlated. Apelin also has a positive effect on muscle function and cognitive function, respectively. However, there has been little research into the mechanisms that explain crosstalk between muscle and cognitive function. Therefore, in this study, the correlation between muscle and cognitive function will be examined through apelin.

2. Purpose of Research

Although the apelin increased by endurance treadmill exercise (Vinel et al., 2018), but there's no research about comparing endurance treadmill exercise and resistance ladder exercise. Therefore, firstly apelin expression level will be defined by exercise type. After then, relationship between muscle function and cognitive function will be defined. Lastly, via *in vitro* and *in vivo* studies the role of exerkine apelin in skeletal muscle on cognitive function will be confirmed.

3. Hypothesis of Research

In order to clarify the purpose of this study, the following research hypotheses were set up.

First, the amount of apelin expression in mouse treated with endurance treadmill exercise and resistance ladder exercise will vary from group to group

Second, muscle function and cognitive function will have a positive correlation.

Third, the amount of apelin expression in skeletal muscle and serum will have a positive correlation with cognitive function

.

II. Study Background

1. Treadmill Exercise and Ladder Exercise

After 30 years old, as people older, muscle function and muscle mass decreasement occur (Jung et al., 2015). Ladder climbing exercise is resistance exercise for mice model, and it could reverse the muscle function loss and muscle mass loss in aged rats, and its effects were somewhat related to myokine expression (Jung et al., 2015). Treadmill exercise is kind of endurance exercise and it can prevent aging of skeletal muscle via proliferation of muscle capillaries, and increasement of oxidative enzyme activity, also it upregulate muscle strength and brings hypertrophy of skeletal muscle (Kemi, Loennechen, Wisloff, & Ellingsen, 2002; Rogers & Evans, 1993).

2. Apelin

Apelin was first discovered by Masahiko group in 1998, actually apelin was discovered as ligand for G protein-coupled receptor APJ (Tatemoto et al., 1998). Apelin is composed of 77 amino acids and its sequence is encoded in C-terminus (Tatemoto et al., 1998). First discovered role of apelin was balancing electrolyte of blood and its pressure (Tatemoto et al., 2001). Apelin act as a peptide that cleaved in endoplasmic reticulum, and its C-terminus maintained in apelin-36, apelin-17 and apelin-13 (Mesmin, Dubois, Becher, Fenaille, & Ezan, 2010). Until these days its role has been discovered and it effects on a lot of physical metabolism, such as muscle regeneration, antioxidant, protection of neurons, and amelioration of stress (Shen, Yue, Fu, Tian, & You, 2019; Vinel et al., 2018; J. Zhang, Lin, Xu, & Tang, 2019).

3. Y-maze Test

Y-maze test is simple test that measures mice cognitive function related to spatial working and learning memory, a lot of researches used this tool for measuring cognitive function (Leinenga & Gotz, 2015; Park et al., 2019). Therefore, we used Y-maze test to measure cognitive function of mice.

4. Neuronal Cell and Glial Cell

Neurogenesis in the adult hippocampus is functionally effects on cognitive function. Especially it contributes to learning and memory functions (Oomen, Bekinschtein, Kent, Saksida, & Bussey, 2014). Therefore, confirming proliferation and viability of neuronal cell is somewhat related to cognitive function (Park et al., 2019).

Glial cells can contribute to maintaining the integrity of neuronal cell function in brain, furthermore it can contribute to the recovery of brain function from the damages (Morita, Lee, & Her, 2009). Also glial cell maintains homeostasis and support and protection of neuronal cell (Jessen & Mirsky, 1980). Furthermore, neurogenesis have critical role in plasticity of structural and maintaining network on Alzheimer's disease which is cognitive impairments occur in brain (Mu & Gage, 2011).

III. Methods and Materials

1. Animal Care

Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of DGIST, IACUC-18050202. All mice groups were housed in controlled environment in 22°C and 12:12 hour light and dark cycle. Mice were fed with water and food in *ad libitum*.

2. Research Design

This study aims to clarify the expression pattern of apelin induced by exercise in different age and exercise type. This study also aims to analyze the correlation between exercise-induced apelin and cognitive function. The mice were divided according to its age and exercise type, young group without exercise (YC, n=4), young group with endurance treadmill exercise (YEE, n=5), young group with resistance ladder exercise (YRE, n=5), Old group without exercise (OC, n=3), Old group with endurance treadmill exercise (OEE, n=4) and Old group with resistance ladder exercise (ORE, n=3). All the young group aged 8 months old and old group's age were 28 months old C57BL6/J mice (Fig. 1).

In *in vitro* study, Neuro 2A cell, C2C12 cell and C6 cells were used for confirming apelin's direct effect on neuronal cell and skeletal muscle cell (Fig. 2).

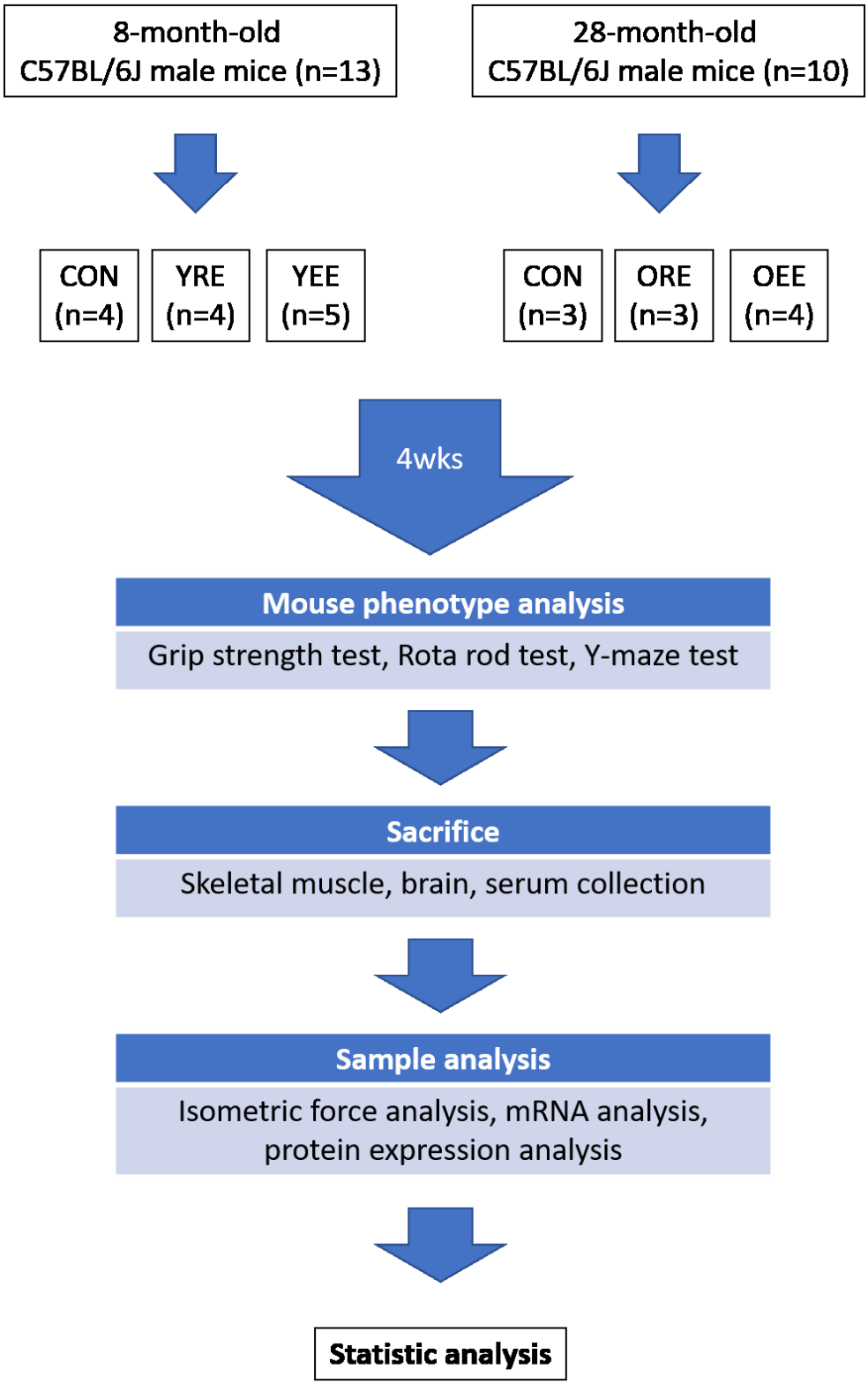

Figure 1. Animal study design

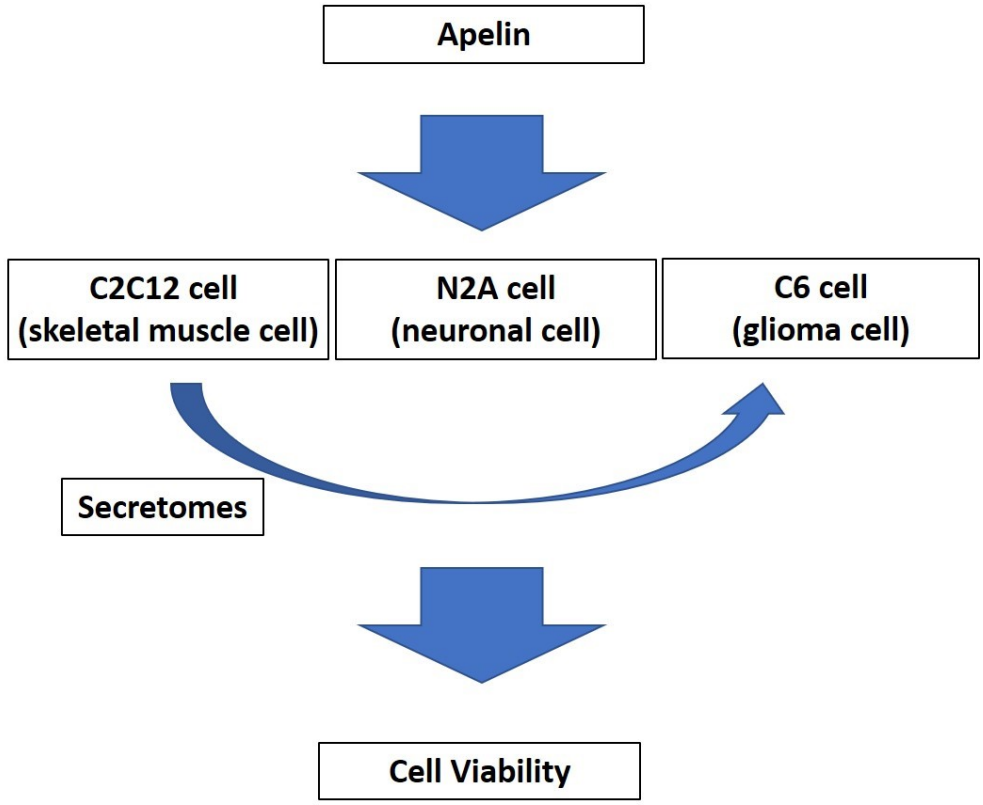

Figure 2. *in vitro* study design

3. Exercise Protocol

In treadmill exercise (Columbus, Exer 3/6), YEE mice allocated to perform treadmill running and subject to 10 degrees incline, 10 m/min for 30 minutes at 1-2 week, 2 m/min were increased every 2 weeks. OEE mice ran on 2 m/min lower speed then YEE mice.

In ladder exercise, YRE and ORE group performed 3d/wk for 4 weeks. Ladder was set at 85 degrees and a 1-m ladder with 1.5 interval grid. YRE group started with half of its body weight and ORE group started with 10% of its body weight. YRE and ORE group did 10 times per one session in ladder exercise. Exercise intensities were increased after four successful trials, and its intensity is shown below (Table 1).

Table 1. Ladder exercise protocol

		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
YRE(g)	1	18	18	18	20	23	23	23	26	26	26	30	30
	2	21	21	21	23	26	26	26	29	29	29	33	33
	3	24	24	24	26	29	29	29	32	32	32	36	36
	4	27	27	27	29	32	32	32	35	35	35	39	39
	5	27	27	27	32	35	35	35	38	38	38	42	42
	6	27	27	27	36	38	38	38	41	41	41	45	45
	7	27	27	27	39	41	41	41	44	44	44	48	48
	8	30	30	30	42	45	45	45	47	47	47	51	51
	9	30	30	35	45	45	45	45	50	50	50	54	54
	10	30	30	40	48	48	48	48	53	53	53	57	57
ORE(g)	1	3	3	7	8	10	10	10	13	13	13	15	15
	2	3	3	7	8	10	10	10	13	13	13	15	15
	3	3	3	7	8	10	10	10	13	13	13	15	15
	4	3	3	7	8	10	10	10	13	13	13	15	15
	5	6	6	9	10	12	12	12	15	15	15	17	17
	6	6	6	9	10	12	12	12	15	15	15	17	17
	7	6	6	9	10	12	12	12	15	15	15	17	17
	8	6	6	9	10	12	12	12	15	15	15	17	17
	9	8	8	11	13	15	15	15	17	17	17	19	19
	10	8	8	11	13	15	15	15	17	17	17	19	19

4. Grip Strength Measurement

The grip strength was measured using Grip Strength Meter (Bioseb, France). After the 4 weeks of exercise, the test was performed in mice's upper limbs by allowing the animals to grasp a grid plate attached to the force gauge, followed by pulling the animals away from the gauge gently, and 3 times of trial was performed. Its average grip strengths were used (Song, Cho, Lee, Lee, & Song, 2018).

5. Rotarod Test

Rotarod test was performed to test mice balancing ability on rotating rod. After the 4 weeks of exercise, Mice were gently placed on a rotarod (B.S. Technolab), and adaptation to rotarod was performed with 4 rpm of rotating rod during 3~5 min. After the 10 minutes or over resting, maximum five min trials on an accelerating rotarod 1rpm/8sec was performed until its latency to fall was measured. This protocol modified from Chen's group (L. Chen et al., 2005).

6. Tissue Collecting

The mice tissues were collected 48 hours after the exercise intervention to avoid effect of last bout of exercise. 20% Urethane solution were injected intraperitoneally. After the paralyzation, blood was collected from mice eye. Then, its limb skeletal muscles were collected. After the perfusion with PBS, then its whole brains were collected. All samples were directly put it into 4% paraformaldehyde or aqueous nitrogen.

7. Western Blot

The total proteins were extracted using RIPA buffer (ThermoFisher Scientific, #89900), containing phosphatase inhibitor (Sigma-Aldrich, #4906845001) and protease inhibitor (Roche, #4693159001), separated by 10~16% tris-glycine SDS-PAGE, transferred to NC membranes using Iblot 2 NC mini stacks (Invitrogen, #IB23002), following primary antibodies were used: anti-Apelin (Covalab, #pab0222), anti-GAPDH (Cell signaling technology, #2118), the antibodies were diluted 1:5,000 and 1:500 with TBST(Biosesang, #HT2007) containing 5 % skim milk each. The NC membranes were then incubated with a peroxidase-conjugated secondary anti-rabbit and specific antibody signals were detected by Immobilion western chemiluminescent HRP substrate (Millipore, #WBKLS0500).

8. Isometric Force Test

Isometric force test was performed to measure mice muscle function in *ex vivo*. After the dissecting of soleus (SOL) and extensor digitorum longus (EDL), its tendons connected to isometric force measuring machine (Grass tech, S48 stimulator). Then twitch force was measured with short electric stimulation, and tetanic force was measured with 300 ms long electric stimulation. After the twitch and tetanic force measurement, the fatigue recovery rate was measured. Every 2 second interval stimulation was induced in muscle 15 times. After the fatigue inducing, its isometric force was measured every 30 second.

9. Enzyme-Linked Immunosorbent Assay (ELISA)

The measurement of serum and skeletal muscle apelin expression level was performed by enzyme-linked immunosorbent assay (ELISA). Apelin C-terminus ELISA kit (Raybiotech, EIAM-APC) was used for analyzing. Serum was diluted in dilution solution by 4 times.

10. Y-maze Test

Cognitive function of mice was measured by Y-maze test. Y-maze have same length of 3 arms (60 cm long, 11.5 cm wide and 25 cm high) and its angle between arms was 120 degrees. After 4 weeks of exercise, mice were gently put in the Y-maze and its movements across the arms were counted during 8 min, 3 arms were labled as 1,2 and 3. If the mouse did not pass the same number during the three movements, they gave 1 point. Then its alteration percentage was calculated by total movements and its score.

11. *In vitro* Study

In *in vitro* study, C2C12 cell (ATCC, CRL-1772), C6 cell (KCLB, #10107), and neuro-2a (ATCC, CCL-131) were used. Apelin-13 (Bachem, #4029109.001) was directly treated to cells in dose dependently. In C2C12, neuro-2a cell, these were treated with 1uM doxorubicin to mimic aging of cells (Bielak-Zmijewska et al., 2014). To collect Apelin and Exercise mimetic molecule (forskolin) treated C2C12 cell's secretoms, we treated apelin and forskolin (sigma-aldrich, #F6886) to differentiated C2C12 cell for 24 hours, after that, C2C12 cells were washed with 1X PBS (Lonza, #17-516F), 2 times and 1X PBS was used for collect secretomes for 4 hours. After then, syringe filter (Satorius, #16534) was used for filter cell debris in secretomes collected 1X PBS, and these secretomes were concentrated with centrifugal filter (Millipore, UFC800324). Then concentrated secretomes were treated to C6 cell in 96 well plates to confirm its cell viability with cell counting kit (Dojindo, #CK04) (Harris et al., 2004).

IV. Results

1. The Effects of 4 weeks of Treadmill and Ladder Exercise on Muscle Function Index

After the 4 weeks of treadmill and ladder exercise, normalized wet weight of soleus (SOL), extensor digitorum longus (EDL), gastrocnemius (GAS) and tibialis anterior (TA) muscle did not changed in young mice group (8 months), however OC groups showed lower normalized muscle weight, treadmill exercise group (OEE) showed upregulated muscle weight in all muscle type, and SOL was the only muscle type that upregulated in ORE group (Fig. 3A-D). Rotarod latency to fall time didn't changed by any exercise type in young mice, aged mice showed shorter time of latency to fall, however treadmill and resistance exercise upregulated latency to fall time (Fig. 3E). Normalized grip strength didn't changed by any exercise type in young mice, when it comes to aging, mice had tendency to show weakness in grip strength test, however only treadmill exercise upregulated grip strength in aged mice (Fig. 3F).

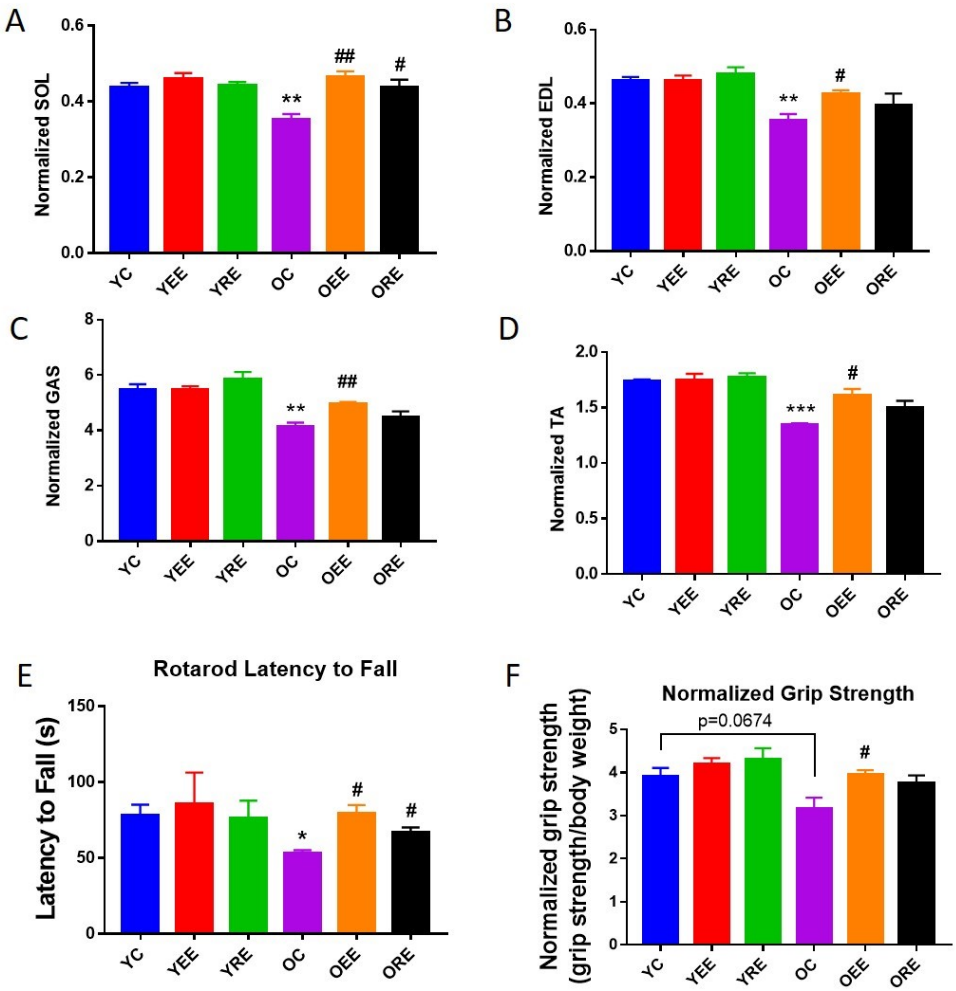

Figure 3. The Effects of 4 weeks of Treadmill and Ladder Exercise on Muscle Function Index. (A, B, C and D) This indicates soleus (SOL), extensor digitorum longus (EDL), gastrocnemius (GAS), tibialis anterior (TA) normalized wet weight, (E) Rotarod latency to fall time, (F) Normalized grip strength test result after 4 weeks of exercise. *p<0.05, **p<0.01 vs. YC, #p<0.05, ##p<0.01 vs. OC. Statistical analysis was performed using two-tailed Student's *t*-test.

2. The Effects of 4 weeks of Treadmill and Ladder Exercise on Skeletal Muscle Isometric Force

We also performed isometric force test in *ex vivo* skeletal muscle to measure muscle function in all mice groups. Twitch force of both SOL and EDL muscle didn't showed downregulation effect in aged mice, and both treadmill and ladder exercise didn't upregulated twitch force of SOL and EDL in young mice group, however treadmill and ladder exercise upregulated twitch force of SOL and EDL in aged mice group (Fig. 4A and 4C). Tetanus force of SOL and EDL showed lower level in aged mice compared to young mice group, however both treadmill and resistance exercise upregulated tetanus force of SOL and EDL in aged mice group (Fig. 4B and 4D). Fatigue recovery rate of EDL muscle downregulated by aging and upregulated by only ladder exercise in aged mice group (Fig. 4E).

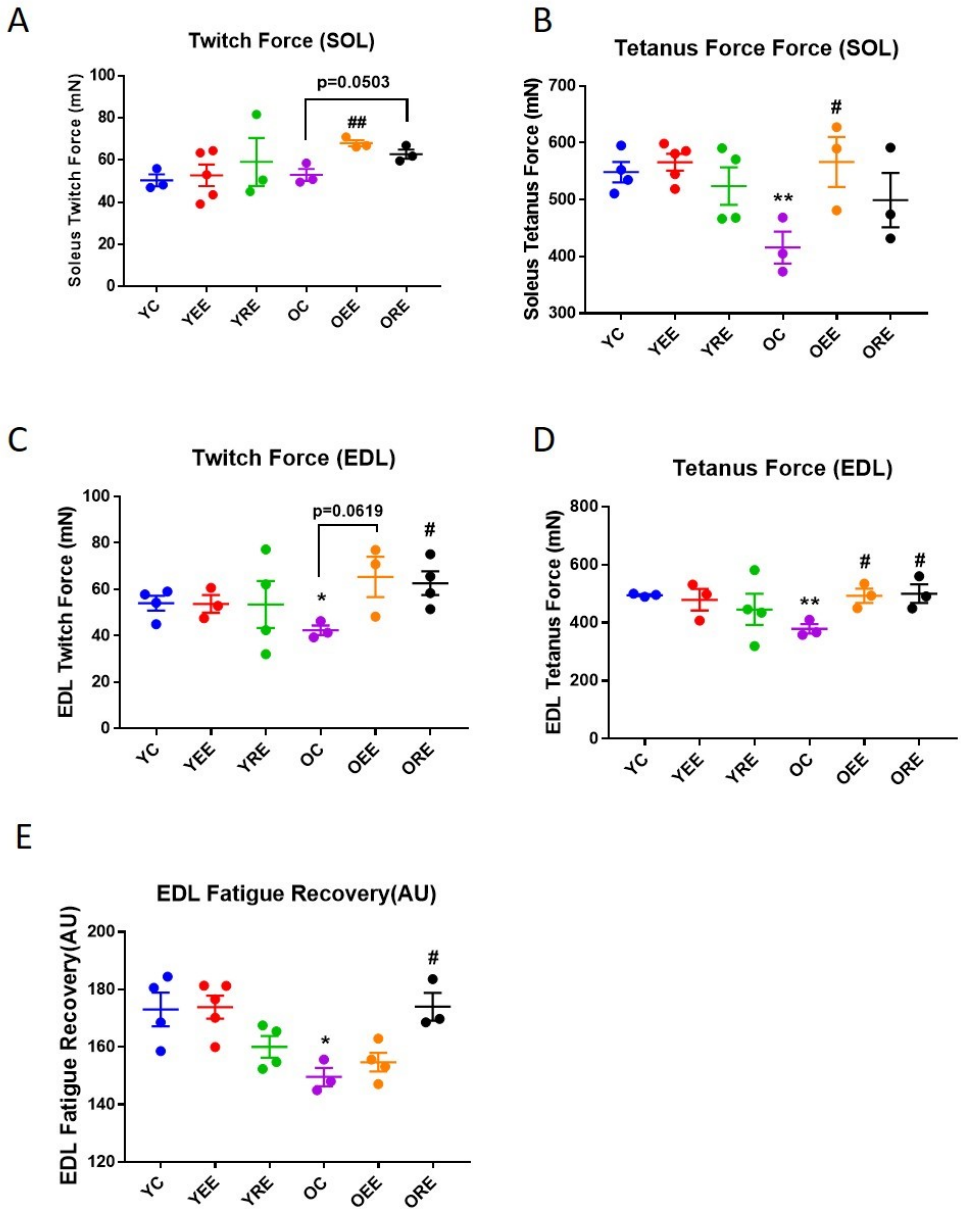

Figure 4. The Effects of 4 weeks of Treadmill and Ladder Exercise on Skeletal Muscle Isometric Force. (A and C) Twitch force of SOL and EDL, (B and D) Tetanus force of SOL and EDL. (E) Fatigue recovery rate of EDL. *p<0.05, **p<0.01 vs. YC, #p<0.05, ##p<0.01 vs. OC. Statistical analysis was performed using two-tailed Student's *t*-test.

3. The Effects of 4 weeks of Treadmill and Ladder Exercise on Cognitive Function (Y-maze test)

Y-maze test can be thought as a cognitive function index in mice. Its alteration (%) didn't changed by both exercise type in young mice group, but OC showed downregulated alteration (%) and both treadmill, ladder exercise upregulated its alteration (%) in aged mice group (Fig. 5A). As you can see, both treadmill exercise and ladder exercise upregulated muscle function index and cognitive function index in aged mice group.

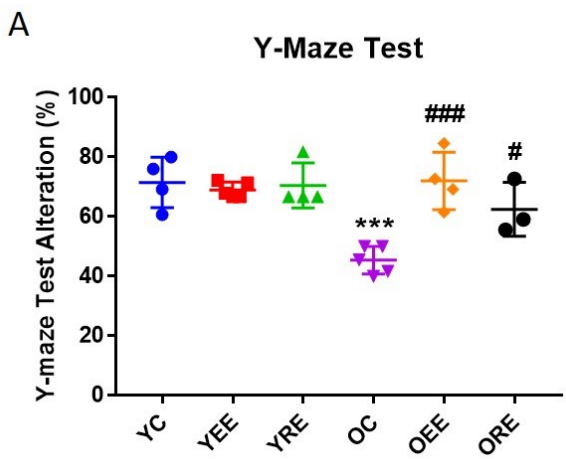

Figure 5. The Effects of 4 weeks of Treadmill and Ladder Exercise on Cognitive Function (Y-maze test). ***p<0.001 vs. YC, #p<0.05, ###p<0.001 vs. OC. Statistical analysis was performed using two-tailed Student's *t*-test.

4. The Correlation between Muscle function Index and Cognitive Function (Young, Old, n=23)

We confirmed that treadmill and ladder exercise improve muscle function and cognitive functions in aged mice, However, we could not be sure that this result indicates that the muscle function is related to cognitive function. Therefore, we analyzed the correlation between muscle function index and cognitive function (Y-maze test) in all mice group. Normalized wet weight of all muscle type showed significant positive correlation with Y-maze test alteration (%) (Fig. 6A-D). Also normalized grip strength showed positive correlation with Y-maze test alteration (%) (Fig. 6E).

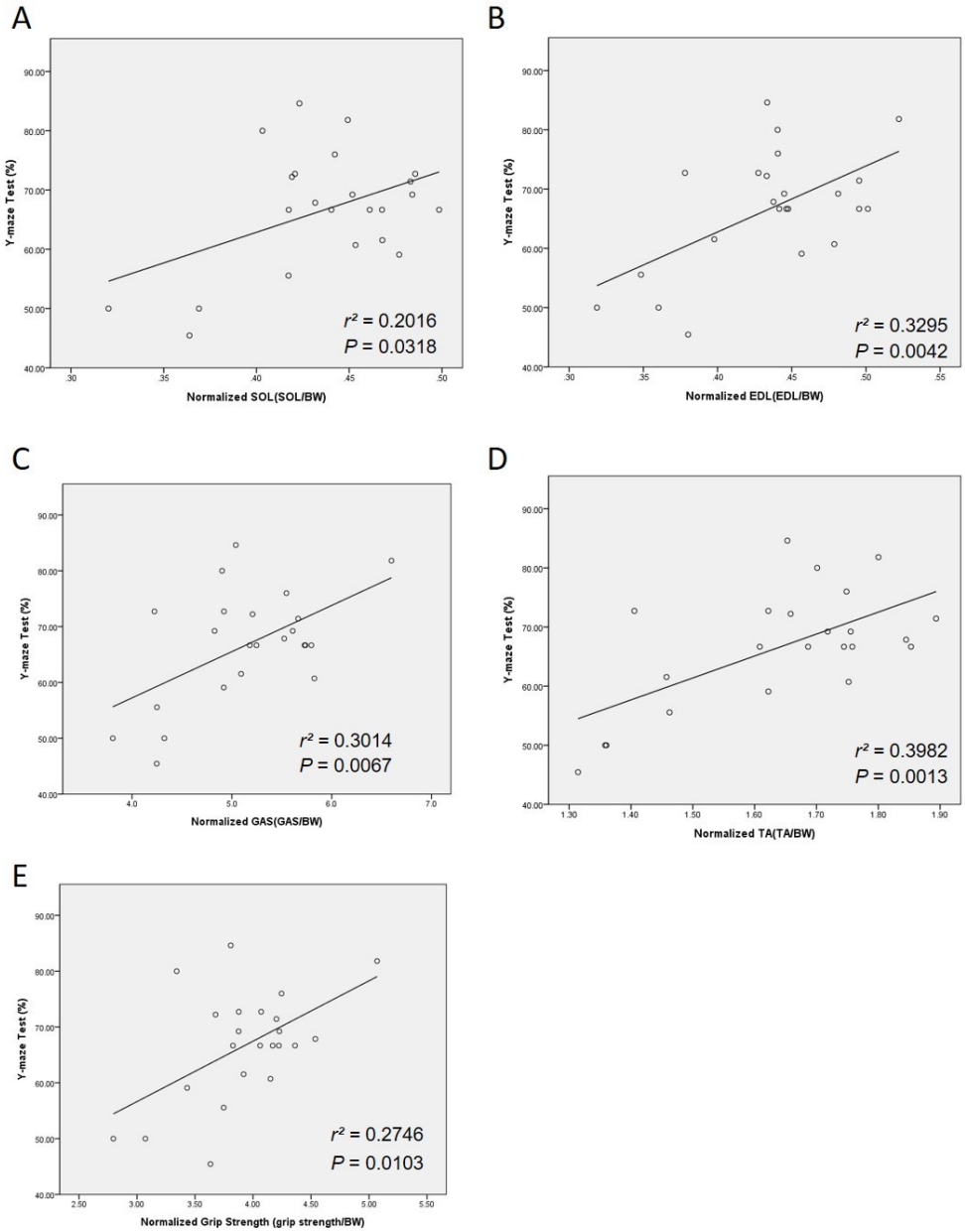

Figure 6. The Correlation between Muscle Function Index and Cognitive Function (Young, Old N=23). (A, B, C and D) Correlation between the normalized muscle wet weight of SOL, EDL, GAS and TA and Y-maze test. Correlation between the normalized grip strength and Y-maze test. The lines indicate linear regression, and the Pearson's correlation coefficient was used.

5. The Correlation between Isometric Force and Cognitive Function (Young, Old, n=23)

Isometric force test can measure twitch force and tetanic force of each muscle. Twitch force of SOL didn't showed correlation with Y-maze test (Fig. 7A). However tetanic force of SOL, twitch force of EDL and tetanic force of EDL showed tendency to positive correlation with Y-maze test (Fig. 7B-D)

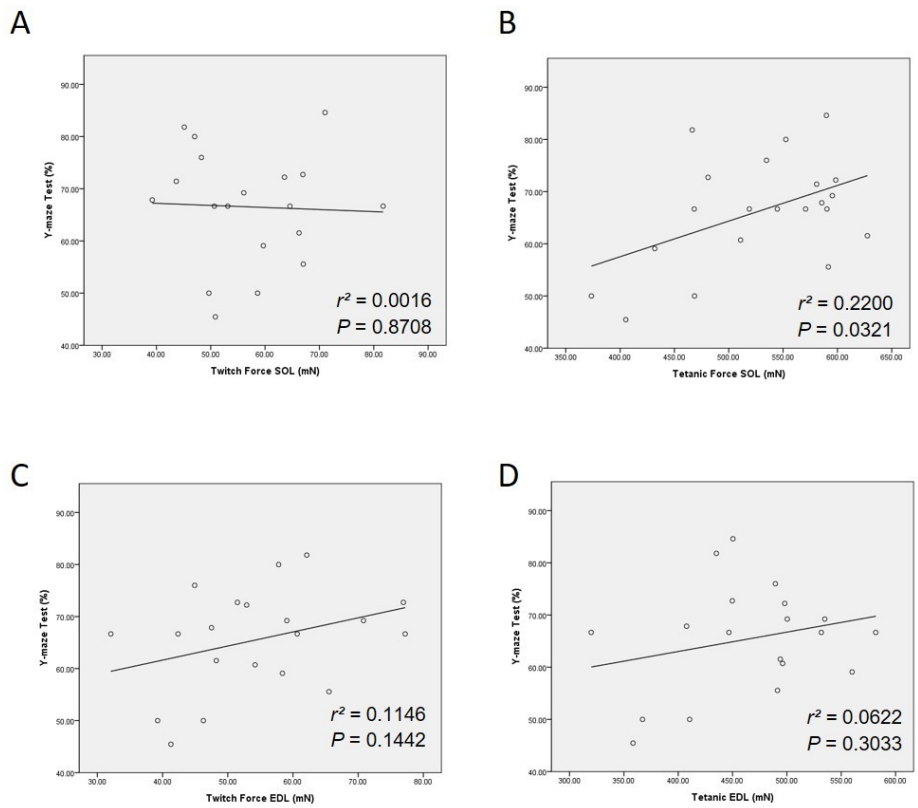

Figure 7. The Correlation between Isomertric Force and Cognitive Function (Young, Old N=23). (A and C) Correlation between the twitch force of SOL, EDL and Y-maze test. (B and D) Correlation between the tetanus force of SOL, EDL and Y-maze test. The lines indicate linear regression, and the Pearson's correlation coefficient was used.

6. The Correlation between Muscle Function Index and Cognitive Function in Aged Mice

Isometric force test results and Y-maze test showed just tendency of positive correlation in all mice group (Fig. 7A-D). Therefore, we confirmed again normalized grip strength, rotarod latency to fall and twitch force correlation in only aged mice group. Normalized grip strength and rotarod latency to fall showed more powerful correlation with Y-maze test alteration (%) in only aged mice than all mice group (Fig. 8A and 8B). In addition, twitch force of SOL and EDL showed significant positive correlation with Y-maze test alteration (%) in only aged mice group (Fig. 8C and 8D).

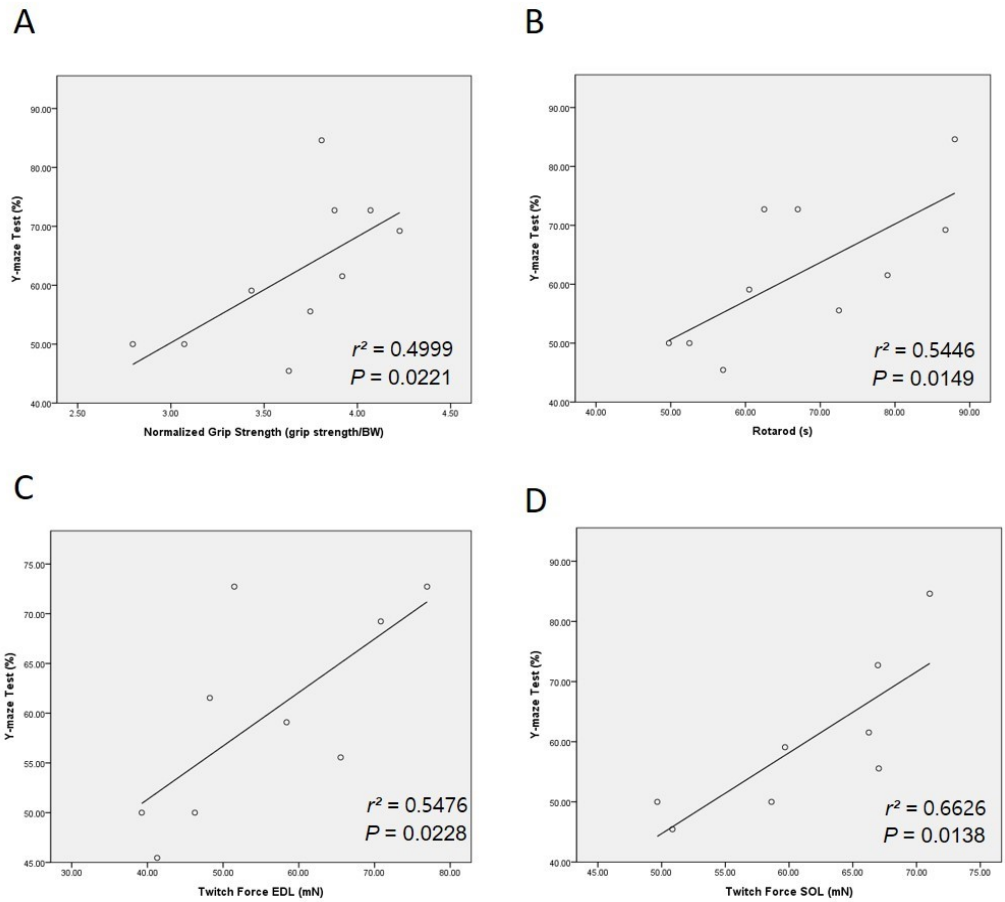

Figure 8. The Correlation between Muscle Function Index and Cognitive Function in Aged Mice (N=10). (A and B) Correlation between the normalized grip strength, rotarod and Y-maze test. The lines indicate linear regression, and the Spearman's correlation coefficient was used.

7. The Effects of Apelin on *in vitro* Cells and Its Secretomes.

Animal study results showed exercise effects on muscle function index and cognitive function (Y-maze test). Also, it showed positive correlation between muscle function index and cognitive function. However this results didn't mean that muscle function improvement improves cognitive function. Therefore, we hypothesized that muscle function improvements and cognitive function improvements are mediated by exercise-induced apelin. Actually, doxorubicin is treated to induce aging in C2C12, Neuro 2A cell. Apelin treatment in C2C12 cell and neuro 2A cell upregulated cell viability of both cells. Forskolin is exercise mimetic chemical that upregulates cAMP in cell that upregulates MAPK signal pathway. C6 cell also showed upregulated cell viability by an apelin treatment as exercise mimetic (Fig. 9C). To confirm C2C12 cell secretome effect on C6 cell, firstly we confirmed that C2C12 cell apelin expression level is upregulated by exercise mimic. As a result, exercise mimetic upregulated apelin expression level in C2C12 cell (Fig. 9D). After treating forskolin and apelin on C2C12 cell, its secretomes were collected and treated to C6 cell, as a result, apelin and forskolin treated C2C12 cell's secretome upregulated C6 cell's viability (Fig. 9E).

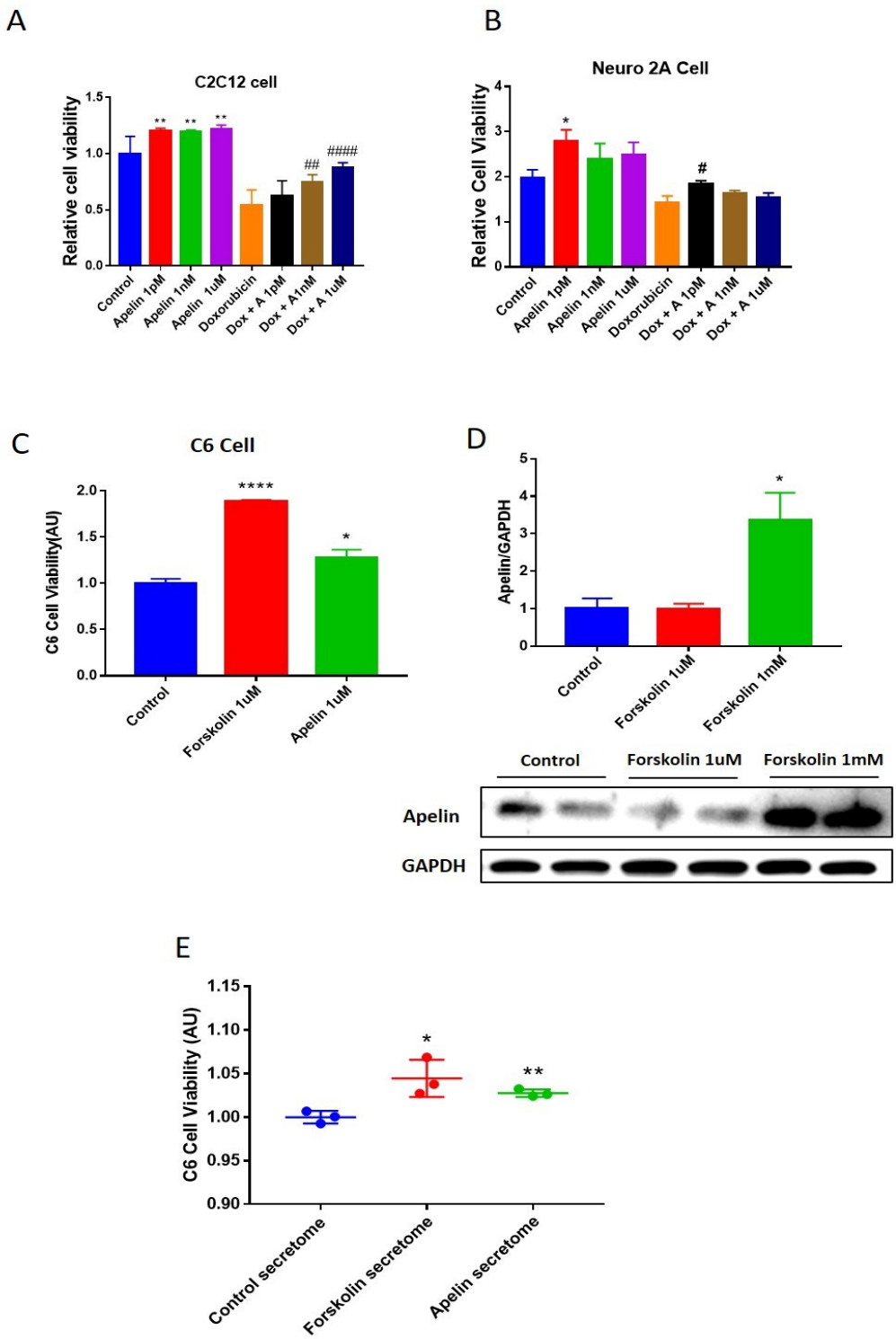

Figure 9. The Effects of Apelin on *in vitro* Cells and Its Secretomes. (A, B and C) Direct apelin effects on C2C12, N2A and C6 cell viability (n=3 each group). (D) Apelin expression level in forskolin

treated C2C12 cell (n=4 each group). (E) C2C12 cell secretome treated C6 cell viability (n=3 each group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. Control. #p<0.05 vs. Doxorubicin. Statistical analysis was performed using one-tailed and two-tailed Student's *t*-test.

8. The Effects of 4 Weeks of Treadmill and Ladder Exercise on Apelin Expression Levels and Its Correlation with Cognitive Function

In vitro study suggests that apelin as a muscle function and cognitive function mediator. However, it was limited to *in vitro* study, so we confirmed exercise effects on apelin expression level in skeletal muscle and serum. Also, we confirmed the correlation between apelin expression level and Y-maze test alteration (%) in mice group. Aging and both exercise type didn't effect on skeletal muscle apelin expression level in mice (Fig. 10A). However, apelin expression levels in serum showed significant downregulation in OC group and both treadmill and ladder exercise upregulated serum apelin expression levels in aged mice group, but exercise type differences were not existing (Fig. 10B). Interestingly, skeletal muscle apelin expression level didn't showed correlation with Y-maze test, but serum apelin expression level showed tendency to positive correlation with Y-maze test in all aged mice group (n=23) (Fig. 10C and 10D). In addition, skeletal muscle and serum apelin expression level showed positive correlation with Y-maze test in only aged mice group (n=10) (Fig. 10E and 10F).

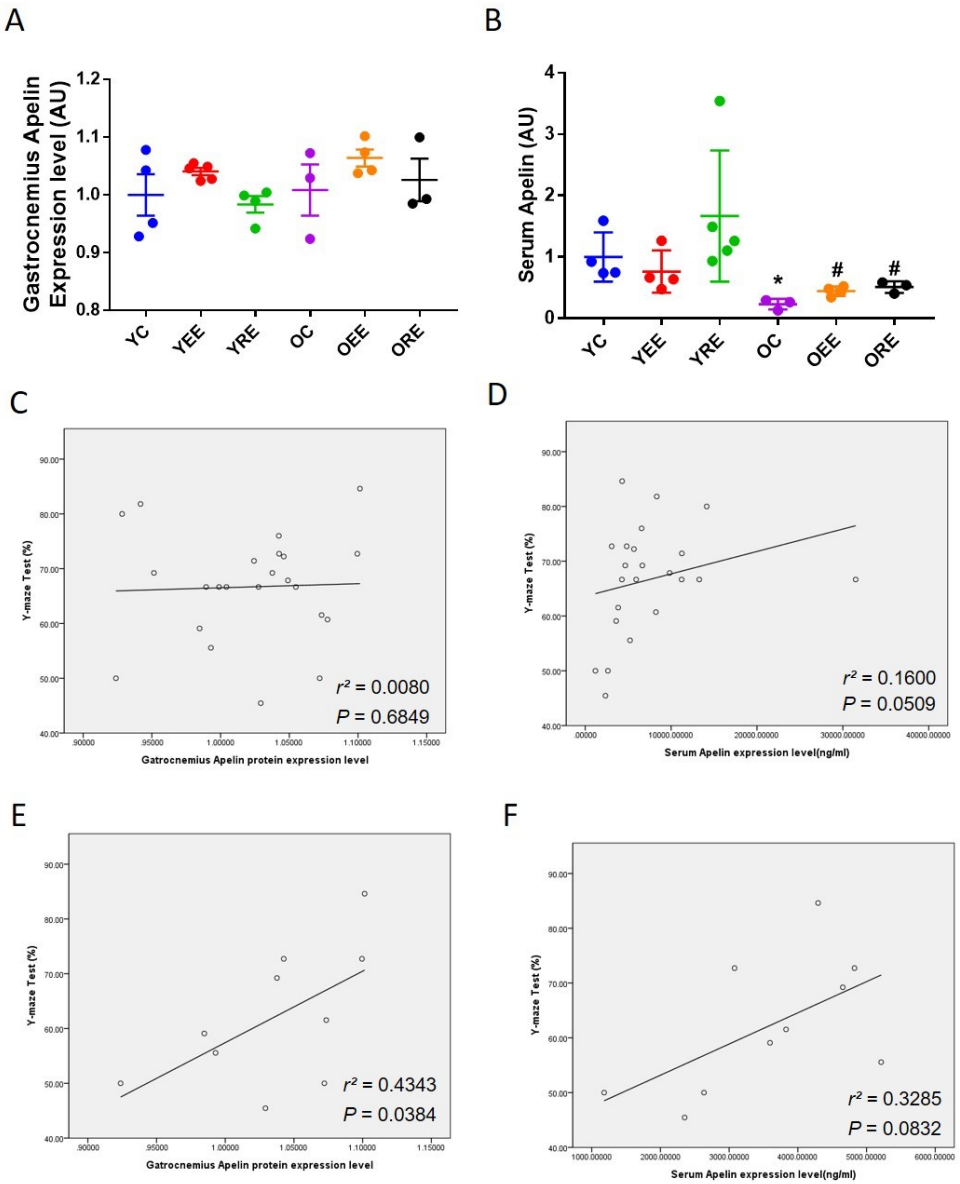

Figure 10. The Effects of 4 Weeks of Treadmill and Ladder Exercise on Apelin Expression Levels and Its Correlation with Cognitive Function. (A and B) GAS and serum apelin expression levels in animal models. (C and D) The correlation between GAS, serum apelin expression levels and Y-maze test score in young and aged mice group (n=23). (E and F) The correlation between GAS, serum apelin expression levels and Y-maze test score in aged mice group (n=10). *p<0.05 vs. YC, #p<0.05 vs. OC. Statistical analysis was performed using two-tailed Student's *t*-test. The lines indicate linear regression, and the Pearson's correlation coefficient was used.

VI. Discussion

This research reported exercise-induced apelin's roles for muscle function and cognitive function. 4 weeks of treadmill and ladder exercise upregulated its muscle weight significantly, but it seems to be only limited to old mice group. Actually, young group mice didn't showed upregulation of muscle weight in skeletal muscle by an exercise (Fig. 3A, 3B, 3C and 3D). In previous study, young mice under 12 months showed normalized skeletal muscle weight upregulation by treadmill exercise and ladder (H. Fu, Desvergne, Ferrari, & Bonnet, 2014; Stotzer et al., 2018). However, this research showed upregulation of skeletal muscle weight in aged mice group only. On the other hand, in terms of aging in skeletal muscle, it showed similar results as previous studies, aged one showed less weighed skeletal muscle weight than young group (Cartee, Hepple, Bamman, & Zierath, 2016; O'Leary, Vainshtein, Iqbal, Ostojic, & Hood, 2013). Most of previous study showed that treadmill and ladder exercise upregulate muscle function such as grip strength and rotarod latency to fall (Y. H. Chen et al., 2018; J. E. Kim et al., 2013; J. S. Kim, Yoon, Kim, Choi, & Song, 2016; Son et al., 2016), but in this study, only aged group showed upregulation of muscle function index by treadmill exercise and ladder exercise (Fig. 3E and 3F). Similar to skeletal muscle weight changes in previous studies, aged mice group showed downregulated muscle function compared to young mice group (J. S. Kim et al., 2016). Results showed that exercise were not that effective to young mice group's muscle weight and muscle function, but in molecular level, young mice group showed upregulation of p-AMPK/AMPK ratio in YEE group, therefore

it can be said that exercise was effective to both young and aged mice group. In terms of muscle isometric force test, previous study also showed that exercise upregulates muscle twitch force and tetanic force in both young and aged mice group (Gonzalez, Messi, & Delbono, 2000; Hayes & Williams, 1996). In addition, aged mice group showed lower muscle twitch and tetanic force than young mice group (Gonzalez et al., 2000; Hayes & Williams, 1996). In my research, only aged group showed upregulation of isometric force by exercise, but young mice group doesn't showed upregulation of isometric force by exercise (Fig. 4A, 4B, 4C and 4D), nevertheless, exercise definitely affected on aged mice muscle function.

In the study, we performed both endurance exercise and resistance exercise to young and old mice group. In case of endurance exercise, there's almost same exercise protocol was performed, but in case of resistance exercise, different exercise protocol was performed to mice depend on their age. Because OC, ORE, OEE mice were too old to perform same weight as young mice group, so we followed previous study's resistance ladder exercise protocol (J. S. Kim et al., 2016).

The Y-maze test has been used for testing cognitive function of mice in previous researches (Y. Fu et al., 2017; Leinenga & Gotz, 2015; Park et al., 2019). Therefore, this research used Y-maze test for measuring cognitive function of mice (Fig. 5A). Previous researches already showed cognition decreasment of aged mice and aged human, and also effects of exercise on cognitive function upregulation (Pedersen, 2019). The results showed that upregulation effects of exercise on Y-maze alteration (%), and downregulation effects of aging on Y-maze alteration (%) (Fig. 5A).

In previous studies, both human and animal research results showed

that muscle function and cognitive function are positively related each other (Tolea et al., 2018; Yoon, Lee, & Song, 2018). However, previous studies were limited to only few indexes of muscle function such as grip strengths or muscle weights. Therefore, in this study, we used isometric force analyzer that even more accurate tool for muscle function measuring. As a result, similar to previous studies, most of skeletal muscle function index were positively related to cognitive function index (Fig. 6 and 7). Interestingly, old group's muscle function index showed more significant positive correlation with cognitive function index (Fig. 8A, 8B, 8C and 8D). According to the results, it can be said that muscle function and cognitive function are more strongly correlated in aged mice.

After the confirming of relation between muscle function and cognitive function, to find out what's in the mechanism of it. We used skeletal muscle cells, neuronal cells and glial cells in *in vitro* study. Doxorubicin was used for mimicking aging on the skeletal muscle cell and neuronal cell, doxorubicin is molecule that induce oxidative stress and act as anti-neoplastic agent in cells, therefore it can be used as an aging inducing molecules in skeletal muscle cell and neuronal cell (De Falco et al., 2016; Moruno-Manchon et al., 2018). In previous study, direct apelin treatment on primary donor skeletal muscle cell upregulated exercise signaling pathways and downregulated autophagy and inflammation markers expression in protein level (Vinel et al., 2018). Other previous studies showed that treatment of apelin increased BDNF expression level in hippocampus of rat (Shen et al., 2019). However, these previous studies were limited to only markers of muscle function and cognitive function, therefore, in this study, we used cell counting kit to measure cell viability and proliferation level in apelin treated skeletal muscle cell

and neuronal cell. The results showed cell viability and proliferation upregulation effects of apelin (Fig. 9A and 9B). In addition to it, we compared forskolin, which are cAMP upregulating molecule that mimics exercise (Sapio et al., 2017; Vinel et al., 2018), effects on glial cell and apelin treatment on glial cell, as a result, apelin upregulated cell viability and proliferation of glial cell as forskolin does (Fig. 9C). To confirm that exercise really upregulates apelin expression level in skeletal muscle, we treated forskolin on skeletal muscle cell. As a result, forskolin treating on skeletal muscle cell upregulated apelin expression level in skeletal muscle cell (Fig. 9D). This result is consistent with previous study (Vinel et al., 2018). Therefore we confirmed that exercise induces apelin expression in skeletal muscle cell, so we treated apelin enriched secretomes of skeletal muscle cells and exercise mimicked skeletal muscle cell's secretomes on glioma cell, as a results, glial cell upregulated its cell viability and proliferation by a both exercise mimicked secretomes and apelin enriched secretomes (Fig. 9E). No study has confirmed effects of apelin enriched skeletal muscle's secretomes on glial cells, so it can be said that the result is novel finding.

After the *in vitro* study to find out effects of apelin enriched secretomes of skeletal muscle cell, we confirmed exercise effects on young and aged mice group's skeletal muscle and serum. Unlike the previous studies, apelin expression level was not significantly changed by exercise and aging in skeletal muscle (Fig. 10A) (Vinel et al., 2018), In case of serum apelin expression level, aged mice showed downregulation of apelin expression level and it was upregulated by both exercise type, but young mice group showed no difference expression level of apelin in serum (Fig. 10B). In previous study, serum apelin level was upregulated by exercise in young mice

group only, but in my research, upregulation of serum apelin expression level by exercise was found in aged mice group only (Fig. 10B). It is somewhat confusing, but my study might indicate that apelin upregulation by exercise is easier in aged mice than young mice.

In this research, we only used GAS muscle, it is kind of mixed fiber type muscle. However, it contains more type 2 fiber than type 1 fiber (Sher & Cardasis, 1976). We confirmed that treadmill endurance exercise and resistance ladder exercise upregulate apelin expression level of GAS, therefore it can be suggested that exercise upregulate apelin expression on type2 fiber muscle (Fig. 10A). Besides to this study, we also confirmed apelin expression levels of tibialis anterior muscle. As a result, we confirmed that exercise upregulates apelin expression level on tibialis anterior muscle. Tibialis anterior muscle also mixed type fiber muscle and it contains more type 2 fiber than type 1 fiber, therefore, it can be suggested that exercise upregulates apelin expression levels on type 2 fiber dominant muscle. In addition to it, in our laboratory, previously we confirmed apelin expression levels on soleus and extensor digitorum longus after 8 weeks of treadmill exercise. As a result, contrast to GAS and tibialis anterior muscle, exercise downregulated apelin expression levels of soleus. In case of extensor digitorum longus muscle, there's no significant changes in apelin expression level. Soleus muscle is type 1 fiber dominant muscle and extensor digitorum longus muscle is type 2 fiber dominant muscle. Therefore it can be suggested that some reason, type 1 dominant muscle apelin expression level is downregulated by exercise (Son et al., 2016). However, in serum level, exercise still upregulated apelin expression level in serum. However, this previous study was performed through rat model,

therefore, it is necessary to examine exercise effect on skeletal muscle apelin expression level via muscle fiber type.

Furthermore, we confirmed correlation between apelin expression levels and cognitive function index (Y-maze test) to find out that exercise induced-apelin can be intermediator of muscle function and cognitive function interrelationship. It was novel finding that apelin expression level in serum was positively correlated to cognitive function index (Fig. 10D). In addition to this, in aged mice group, they showed even stronger positive relationship in skeletal muscle, serum apelin expression level and cognitive function index (Fig. 10E and 10F). Our findings can implicate potential roles of exercise-induced apelin on cognitive function, however mechanism finding of this study is limited to *in vitro* study only. Therefore, research about skeletal muscle specific knock out and overexpression of apelin will reinforce the results that exercise-induced apelin affects on skeletal muscle function and cognitive function relationship.

V. Conclusion

In conclusion, both treadmill and ladder exercise upregulated muscle function and cognitive function. Muscle function and cognitive function showed positive correlation in all mice group and especially in aged mice group. In *in vitro* study, apelin upregulated skeletal muscle cell, neuronal cell, glioma cell's viability, also skeletal muscle cell secretomes treated with exercise mimicking chemical forskolin and apelin upregulated C6 cell viability. Both type of treadmill and ladder exercise upregulated apelin expression in aged mice group and its apelin expression level showed positive correlation with cognitive function. Therefore, we can carefully assume that muscle function and cognitive function improvement by exercise is mediated by exercise-induced apelin expression in skeletal muscle and serum.

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Abstract (Korean)

내인성 펩타이드인 아펠린(apelin)은 근기능을 향상시키는 것으로 알려져 있다. 또한, 아펠린은 노화시에 근기능을 향상시킬 뿐만 아니라, 인지기능에도 영향을 미친다는 선행 연구가 진행되었다. 이를 토대로, 본 연구에서는 노령쥐의 근육기능과 인지기능의 상관 관계를 확인하고 근육에서 유발된 아펠린이 인지기능에 어떠한 영향을 미치는지 확인하였다.

세포 수준의 연구에서는, C2C12 세포(골격근 세포), N2A(신경 세포) 및 C6 세포(신경아 교세포)의 세포 증식률을 아펠린 처리 직후 측정하여 각각의 세포에 아펠린이 미치는 영향을 확인하였다. Forskolin (cAMP를 상향 조절하는 운동 모방 화학 물질)을 C2C12 세포에 하여 운동에 의한 근세포에서의 아펠린 발현양 변화를 확인하였으며, C2C12 세포를 아펠린과 forskolin으로 처리한 후, 그에 따른 secretome을 C6 세포에 처리하여 근육에서 분비된 아펠린이 신경교세포에 미치는 영향을 확인하였다. 동물 연구에서는 8 개월과 28 개월령의 C57BL/6J 쥐를 이용하여 각각 러닝 머신(YE = 5, OE = 4)과 저항성 사다리(YR = 4, OR = 3) 운동을 처치하였다. 그리고 근육 기능과 인지기능을 등척성 힘 측정, 악력 측정, 로타로드 측정 및 Y-maze 측정을 통해 확인하였다. 또한, 혈청 및 골격근의 아펠린 발현 수준을 분석한 후, 인지기능과의 상관 관계를 분석하였다.

세포 수준 연구는 forskolin이 C2C12 세포에서 아펠린 발현 수준을 증가시키고 아펠린이 C2C12, N2A 및 C6 세포에서 세포 증식률을 증가시킨다는 것을 보여 주었다. 또한, C2C12 골격근 세포에서 분비된 아펠린이 C6 세포의 세포 증식률을 증가시켰다. 동물 실험에서, 러닝 머신과 사다리 운동 모두에 의해 악력($p < 0.05$), 로타로드($p < 0.05$), 등축력($p < 0.05$)이 증가되는 경향성을 보였다. 28 개월 된 쥐에서도 두 가지 유형의 운동이 수행되는데, 골격근에서의 아펠린 발현양과 인지기능(Y-maze test) 수준이 유의한 상관 관계를 보였다($p < 0.05$). 이 외에도 혈청 아펠

린 발현 수준은 두 가지 운동 유형에 의해 증가하였고, 그 발현양은 인지기능과 정적인 상관 관계를 나타내었다($p < 0.05$).

이 연구는 근기능과 인지기능 사이의 관계를 더욱 명확하게 확인하였으며, 또한 연구의 결과를 토대로 아펠린이 근기능과 인지기능 사이의 관계를 중개하는 분자 일 수 있음을 확인하였다.