Dissertation of the Degree of Master of Sport Science

Effects of Treadmill Exercise on Regulation of Tight Junction Proteins in Aged Mice

트레드밀 운동이 노화 쥐에서 장 점막의 세포접합 단백질 조절에 미치는 영향

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Department of Physical Education

College of Education

Graduate School of Seoul National University

Hyungeun Shin

Abstract

Tight junction protein is representative regulator of gut permeability. Also, it has been noted for controlling inflammatory responses through tight junction. Therefore, in this study, we examined that whether tight junction protein is changed in aged mice, and to further, confirmed the effect of treadmill exercise on the tight junction protein.

In *in vitro* study, doxorubicin that induces cell senescence was treated to Caco2 cells (colon cell) to mimic aging effect. After that, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), exercise mimic chemical that stimulates AMPK level, was also administered to Caco2 cells. In animal study, 2 months and 21 months C57BL/6J mouse were treated with treadmill exercise for 4 weeks (YE=5, OE=5). Then, the tight junction protein expression level was examined by western blot. Also, serum lipopolysaccharide (LPS) and zonulin level were analyzed to identify gut permeability.

In vitro studies showed that doxorubicin downregulates tight junction protein expression levels in Caco2 cell, and also AICAR treatment upregulates tight junction protein expression levels. In animal study, 4 weeks treadmill exercise upregulated claudin-1 ($p<0.05$) and occludin ($p<0.01$) protein expression level in 21 months old mice. Also, zonula occluden-1 ($p<0.01$) protein expression level was increased in 2 months young mice after treadmill exercise. In addition, old mice group had higher level of serum LPS compared to young mice group, but the level was downregulated in both 2 months and 21 months mice group after treadmill exericse. Zonulin, which is known as degrading tight junction protein, is not significantly changed by both age and

exercise.

This study compared that tight junction protein expression level in old mice compared to its level in young mice, and also clarified that the effect of treadmill exercise on tight junction protein in both young and old mice.

Keyword : Aging, Exercise, Intestinal Tight Junction Proteins, Lipopolysaccharide, Zonulin,

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I. Introduction

1. Need for Research

In recent years, the population of the elderly has been increasing rapidly, resulting in decreased quality of life and increased mortality (Kennedy et al., 2014). Most chronic disease treatments currently focus on reducing mortality without considering health status. The life expectancy of the elderly is gradually increasing, but their healthspan is not keeping pace (Crimmins, 2015). Aging is a representative risk factor for various chronic diseases that limit healthspan. About 80% of elderly have chronic diseases such as obesity, high blood pressure, diabetes, and heart disease, and about 50% have at least two of these diseases (Jin, 2017). In other words, the importance of maintaining healthy aging without deteriorating physical function is increasing (Jin, 2017). Since aging is the accumulation of harmful changes at the molecular and cellular levels over time (Singh & Newman, 2011), various treatments to delay aging have been proposed (Morley, Anker, & von Haehling, 2014). Recently, gut microbiome have attracted attention as one of the strongest causes of physical frailty and cognitive frailty, and it is gradually revealed that there is a strong relationship between aging and gut microbiome (O'Toole & Jeffery, 2015). revious studies reported that gut microbiome regulate aging through mediators such as butyrate, bacterial metabolites, etc. Specifically, the concept of “Gut-Muscle Axis”was presented, suggesting a strong correlation between gut microbiome and muscle quality (Ticinesi et al., 2017).

Gut micrbiome induced by aging have been reported to increase gut permeability (Nicoletti, 2015). Representative regulator of gut

permeability is a tight junction protein (Visser, Rozing, Sapone, Lammers, & Fasano, 2009). Intestinal epithelial cells form epithelial junctional complex junctions composed of tight junctions, adherens junctions, and desmosomes (Zihni, Mills, Matter, & Balda, 2016). In particular, the tight junction protein, called the gate keeper, plays an important role in controlling gut permeability (M. Zuhl et al., 2014). Tight junction protein is known to maintain homeostasis in organs through paracellular diffusion of ions and solutes, forming a gut barrier (Zihni et al., 2016). When tight junction protein expression is decreased, gut permeability increased (Visser et al., 2009). Through increased gut permeability, Lipopolysaccharide (LPS) or other intestinal by-products (e.g. indoxyl sulfate), components of the Gram-negative bacteria outer membrane, are circulated into the blood (Grosicki, Fielding, & Lustgarten, 2018). Circulating serum LPS activates the inflammatory signaling pathway in each endocrine organ of the body (Oberholzer, Oberholzer, & Moldawer, 2001). Tight junction proteins consist of transmembrane proteins such as Occludin and Claudins, and cytosolic proteins such as Zonula Occludins (ZOs), etc. Occludin and Claudins are major tight junction proteins that regulate the integrity between epithelial cells, and ZOs interact with them to form cytoskeleton. Regulatory mechanisms of tight junction proteins have been found to be balanced by a variety of mechanisms (Zihni et al., 2016). Previous studies have reported that the expression levels of tight junction proteins (Claudin-1 and Occludin) are significantly reduced in aging models (Dun et al., 2018). As a result, aging models have been impaired gut barrier integrity, which increases gut permeability and makes harmful substances in the intestine pass through the barrier to promote inflammatory responses (Dun et al., 2018).

Another previous study reported that AMP-activated protein kinase (AMPK) promotes CDX-2 expression, enhancing intestinal barrier function and intestinal epithelial cell differentiation (Sun, Yang, Rogers, Du, & Zhu, 2017). When 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator, was treated in Caco-2 cells, ZO-1, stimulated by calcium, improved reassembly ability and increase the expression level of villin, E-cadherin, and DPPIV, resulting in decreased gut permeability (Sun et al., 2017). In addition, Patients with type 2 diabetes have increased gut permeability, however continuous exercise intervention makes them have improved gut permeability, decreasing inflammatory responses (Pasini et al., 2019). According to Teerapornpuntakit et al.(2009), mRNA level of ZO-1 and CLDN2 in the ileum increased 1.5 fold and mRNA level increased 2-3 fold in the duodenum after 60 minutes of moderate aerobic exercise (Teerapornpuntakit, Dorkkam, Wongdee, Krishnamra, & Charoenphandhu, 2009). This suggests that moderate intensity exercise can increase intestinal tight junction proteins, enhancing the gut barrier and inhibiting the inflammatory response. On the other hand, another previous studies reported that continuous of high intensity exercise intervention can damage gut barrier, increasing gut permeability (M. N. Zuhl et al., 2014). Specifically, 10 days of aerobic exercise significantly decreased the expression of CLDN1 in the ileum, and Zonulin expression level was upregulated (Holland et al., 2015). These findings indicate that the effects of exercise on gastrointestinal system(GI system) and its mechanisms are not clear, suggesting the need to study the frequency, intensity and time of exercise (Holland et al., 2015).

2. Purpose of Research

The purpose of this study is to compare the expression levels of tight junction proteins (Claudin-1, Occludin, Zonula occludin-1) in gut mucosa of aged mice and young mice and identify the effect of treadmill exercise on tight junction protein.

3. Hypothesis of Research

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

Hypothesis Ⅰ. Aged mice will have weaker gut barrier function compared to young mice.

Hypothesis Ⅱ. Gut barrier function weakened by aging will be enhanced by 4 weeks of endurance treadmill exercise.

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II. Study Background

1. Treadmill Exercise

Treadmill exercise is a kind of endurance exercise that can effectively affect both humans and animals. Only 2 weeks treadmill exercise can alter mRNA levels in gut (Holland et al., 2015). Although high intensity endurance exercise reduces gut barrier function through increasing the core temperature (Bogerd et al., 2018), moderate intensity endurance exercise functions to preserve intestinal lymphocyte cytokines and apoptosis proteins (Packer & Hoffman-Goetz, 2012). Also, previous studies reported that aerobic exercise can improve gut barrier function in diabetic patients (Pasini et al., 2019).

2. Tight junction protein

Tight junctions (TJs) are multifunctional complexes that bind tightly between adjacent epithelial cells. Through tightly binding the spaces between the cells, TJs can prevent the diffusion of microorganisms and other antigens (Farquhar & Palade, 1963). TJ consists of more than 50 proteins which are transmembrane proteins such as Occludin, Claudins, Zonula Occludins and cytosolic proteins (Ulluwishewa et al., 2011). Claudin is mainly responsible for TJs strand formation, and that Occludin is an accessory protein that regulates the diffusion between cells (Furuse, Sasaki, Fujimoto, & Tsukita, 1998). ZOs interact with them to firm cytoskeleton (Zihni et al., 2016). Specifically, intestinal TJ protects the host from external stimuli and modulates immune responses through the formation of cytoskeleton between cells (Zihni et al., 2016).

Human intestinal epithelial tissue is composed of thin epithelial cells that separate the lamina propria from the intestinal lumen. Tight junction protein is a key factor in regulating gut barrier by tightly binding the space between epithelial cells (Ulluwishewa et al., 2011). This TJ is not a fixed form but a dynamic structure that is constantly reconstructed through interactions with microorganisms, other antigens, and other external stimuli (Chelakkot, Ghim, & Ryu, 2018). In other words, the imbalance of intestinal TJ induced by external stimuli weakens the gut barrier and causes pathological conditions (Fasano, 2001). About 35~40% of elderly suffer from gastrointestinal discomfort, and one of the factors causing this gastrointestinal problem is the imbalance of the gut barrier (Hall, Proctor, Fisher, & Rose, 2005). Previous study reported that the expression levels of Claudin-1, Occludin, ZO-1, and JAM-A

decreased in aged mice, and the permeability was increased due to the imbalance of the gut barrier (Dun et al., 2018; Tran, Greenwood-Van Meerveld, & Sciences, 2013). Also, although gut barrier was weakened in patients with type 2 diabetes, continuous exercise intervention make it enhanced through regulating of inflammatory response (Pasini et al., 2019). In the same context, ZO-1 and CLDN2 mRNA levels in the small intestine increased after doing moderate endurance exercise (Teerapornpuntakit et al., 2009). However, another previous study reported that continuous aerobic exercise can damage the gut barrier (M. Zuhl et al., 2014), and cause inflammatory response (M. N. Zuhl et al., 2014). These previous studies show that the effects of exercise on gut barrier are still controversial, and it is necessary to study the effect of various exercise type and intensity. In addition, in vitro study, when caco2 cell is treated with 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator, differentiation is promoted and decreased gut permeability. In this regard, moderate endurance exercise will enhance the TJPs and thus play a protective role in inhibiting the host's inflammatory response.

3. Lipopolysaccharide

Endotoxins of Gram-negative microbes are components of the outer membrane and perform essential functions for bacterial viability (Rietschel et al., 1994). When they set free, Lipopolysaccharide (LPS) is released to induce pathophysiological effects on hosts (Rietschel et al., 1994). LPS in the outer membrane of Gram-negative microbes is composed of lipid A, core polysaccharide, and O-antigen (Raetz & Whitfield, 2002). Lipid A, the hydrophobic region of LPS, is a phospholipid that constitutes a monolayer of Gram-negative bacteria outer membrane. Most lipid A is recognized by Toll-like receptor 4 (TLR4), an endogenous immune response system present in macrophages or animal cells (Aderem & Ulevitch, 2000). TLR4, activated by lipid A in macrophages, promotes biosynthesis of inflammatory regulators such as TNF-α, IL-1β, etc (Hoshino et al., 1999).

III. Methods and Materials

1. Animal Care

Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of SNU, SNU-17122924. All mice groups were housed in controlled environment in 22℃, humidity 60% 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 effects of 4 weeks of treadmill exercise on tight junction protein and gut barrier in different age and exercise. The mice were divided according to its age and exercise intervention, young group without exercise (YC, n=6), young group with exercise (YE, n=5), old group without exercise(OC, n=5), old group with exercise (OE, n=5). All the young group aged 2 months old and old group aged 21 months old C57BL6/J mice (Fig. 1).

All the mice were acclimated to cage and treadmill exercise for 3 days prior to exercise intervention. After 3 days of adaptation, exercise group is allocated to perform treadmill exercise and subjected to gradually increase for 4 weeks. Mice were sacrificed 24 hours after the exercise intervention to avoid effect of last bout of exercise.

Figure 1. Research design

3. Exercise Protocol

All the mice were acclimated to cage and treadmill exercise for 3 days prior to exercise intervention. After 3 days of adaptation, exercise group is allocated to perform treadmill exercise for 4 weeks. 1 session consists of warm up, exercise, and cool down. Exercise intensity is 12 m/min for 30 minutes at 1−2 week, 2 m/min were increased every week. 1 session was repeated twice, with a break of 1 hour between exercise.

The exercise protocol of this study is as follows.

Table 1. Exercise Protocol

<table>
<tr><th colspan="7">Exercise protocol</th></tr>
<tr><td></td><td colspan="4">Warm up</td><td>Exercise</td><td>Cool down</td><td rowspan="3">1hr break time</td></tr>
<tr><td>Ex</td><td>0m/min, 2min</td><td>5m/min, 1min</td><td>8m/min, 1min</td><td>10m/min, 1min</td><td>1st week: 12/min + 2m/min per week (Total 30min)</td><td>5m/min, 2min</td></tr>
<tr><td></td><td colspan="5">1 session * 2 times</td></tr>
<tr><td colspan="7">With a break of 1 hour between exercise</td></tr>
</table>

4. Rotarod Test

Rotarod test was performed before and after 4 weeks of treadmill exercise intervention in order 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).

5. Tissue Collecting

Mouse tissues were collected 24 h after the exercise intervention to avoid any effects of the last bout of exercise. Mice were anesthetized with isoflurane and whole blood was removed from the heart. The entire colon was separated and rinsed with PBS solution to remove feces, and limb skeletal muscle was collected. All samples were frozen immediately in liquid nitrogen and stored at -80 ° C for further analysis.

6. 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 PVDF membranes with mini trans blot, following the membrane was blocked in 5% skim milk for 30 minute at room temperature and washed in TBS-Tween 20 Buffer. Primary antibodies were used: anti-Claudin1 (Santa Cruz Biotechnology #sc166338), : anti-Occludin (Abcam, #ab168986, 1:500 dilution), anti-ZO1(Invitrogen, #40-2200, 1:1000 dilution), anti-phospho-Akt (Ser473, Cell signaling technology, #9271S, 1:1000 dilution), anti-Akt (Cell signaling technology, #9272S, 1:1000 dilution), anti-phospho-AMPKα(Thr172, Cell signaling technology, #1:1000 dilution), anti-AMPKα (D5A2, Cell signaling technology, #5831T, 1:1000 dilution), anti-GAPDH (Cell signaling technology, #2118, 1:5000 dilution). After 3 washes of 10 min each, the PVDF membranes were then incubated with a peroxidase-conjugated secondary anti-rabbit or anti-mouse and specific antibody signals were detected by Immobilion western chemiluminescent HRP substrate (Millipore, #WBKLS0500).

7. Real-time PCR

RNA was isolated by using TRIzol reagent (Invitrogen, #15596026) according to the manufacturer's instructions. Following RNA isolation, RNA was reverse transcribed into cDNA using CycleScript RT premix (Bioneer, #K-2044-CFG). Real-time PCR was performed by utilizing CFX 96 PCR system (Biorad, Germany) and SYBR Green with low ROX (Enzynomics, #RT500M). Each primer's forward and reverse sequences were Claudin-1 forward primer (5-GCACATACCTTCATGTGGCTCAG-3), Claudin-1 reverse primer (5-TGGAACAGAGCACAAACATGTCA-3), Occludin forward primer (5-TCCTATAAATCCACGCCGGTTC-3), Occludin reverse primer (5-CTCAAAGTTACCACCGCTGCTG-3), Zonula occluden-1 forward primer (5-CGGGACTGTTGGTATTGGCTAGA-3), Zonula occluden-1 reverse primer (5-GGCCAGGGCCATAGTAAAGTTTG-3), Beta-actin forward primer (5-TGGCACCCAGCACAATGAA-3), Beta-actin reverse primer (5-CTAAGTCATAGTCCGCCTAGAAGCA-3). Thermal cycling proceeded with 40 cycles with denaturation 95℃ for 10s, annealing 60℃ for 15s, and elongation 72℃ for 25s.

8. Enzyme-Linked Immunosorbent Assay (ELISA)

The measurement of serum lipopolysaccharide(LPS) and zonulin expression level was performed by enzyme-linked immunosorbent assay (ELISA). Mouse Lipopolysaccharides (LPS) ELISA Kit(CUSABIO, Cat#CSB-E13066m) and Mouse zonulin ELISA Kit (Mybiosource, Cat#MBS748504) were used for analyzing. Serum was diluted in dilution solution by 10 times and 4 times respectively.

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9. *In vitro* study

Caco2 intestinal epithelial cells (KCLB, #30037.1, Korean cell line bank) were incubated in Eagle's Minimum Essential Medium (EMEM; Lonza, USA) supplemented with a 20% fetal bovine serum (FBS) at 37℃ with 5% CO2. Caco2 cells were treated with doxorubicin (Glentham Life Science, #GA4969) to induce cell senescence. After incubation with 10uM doxorubicin for 3hr pre-treatment, AICAR (Abcam, #ab120358) was treated to lead exercise mimic effect in Caco2 cells for 24 hr. Cells were seeded in 96well plates to quantify cell viability using cell counting kit-8 (CCK-8; Dojin Laboratories,#CK04). Also, Cells were seeded in 12 well plates at a density of 1.5 * 10^6cells for mRNA analysis.

IV. Results

1. The effects of AICAR and Doxorubicin on Intestinal Tight Junctions in Caco2 cells

To clarify the effects of treadmill exercise on the intestinal tight junctions in aging process, we designed exercise-aging mimetics at the cellular level. AMP-analog AICAR was used to mimic aerobic exercise effect, and doxorubicin was used to induce cell senescence. Pre-incubation with 10uM doxorubicin for 3hr was performed before AICAR treatment.

Cell death is major characteristics of aging process. We identified the effects of various doxorubicin dosages through cell counting kit-8 (CCK-8) assay to know optimal cell survival. The results of the cell viability CCK-8 assay demonstrated that the concentration at which the cell viability was 80% was 10uM dosage of doxorubicin (Fig. 2A). Therefore, we used 10uM doxorubicin dosage to cause cellular senescence and 1Mm AICAR to activate AMP-activated protein kinase in Caco2 cells (Sopjani et al., 2010). We found that 10uM doxorubicin significantly downregulated the claudin-1, occluding, zonula-occluden1 mRNA expression level. However, 1mM AICAR treatment rescued the expression level (Fig. 2B-D).

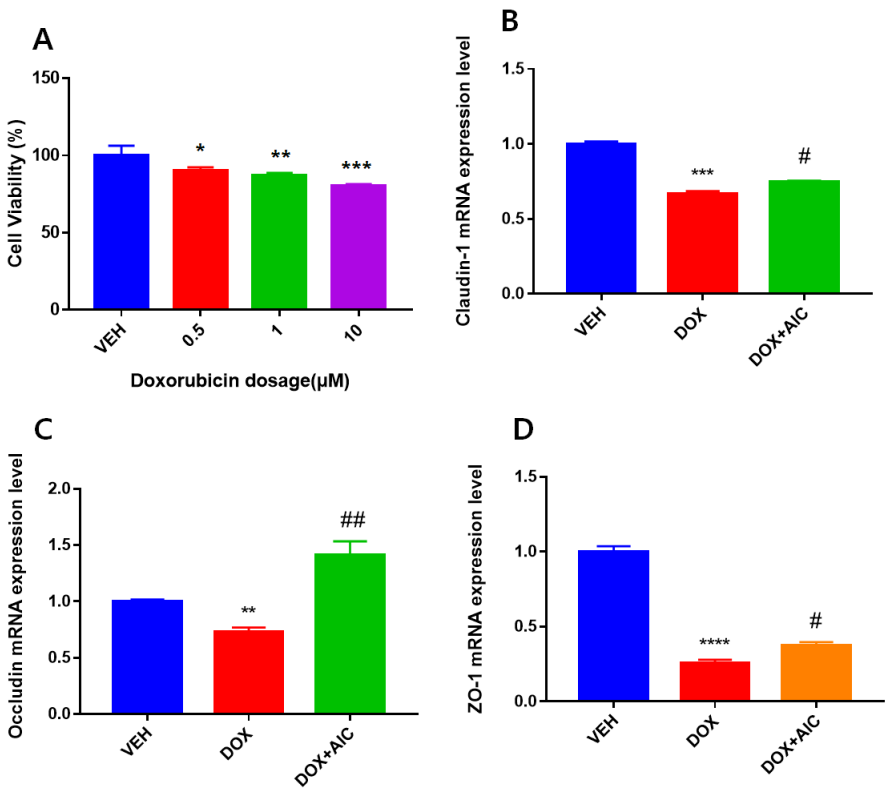

Figure 2. The Effects of AICAR and Doxorubicin on Tight Junctions in Caco2 cells. (A) Doxorubicin effects on Caco2 cell viability (B) Claudin-1 mRNA expression level, (B) Occludin mRNA expression level, (C) Zonula occluden-1 (ZO-1) mRNA expression level in colon. **p<0.01 vs. YC, #p<0.05, ##p<0.01 vs. OC. Stastical analysis was performed using two-tailed Student's t-test.

2. The Effects of 4 weeks of Treadmill Exercise on Body Composition and Skeletal Muscle Wet Weight.

Old mice group showed upregulated body fat compared to young mice group, but it didn't changed by treadmill exercise in both young and old mice (Fig. 3A). When it comes to lean mass, old mice group had tendency to decrease ($p=0.0837$). However, only treadmill exercise improved lean mass in young mice group, but not significantly (Fig. 3B). Old mice group showed lower normalized muscle weight of soleus (SOL) and gastrocnemius (GAS) compared to young mice group. However, both exercise groups did not upregulate weight of SOL and GAS muscle after the 4 weeks of treadmill exercise (Fig. 3C-D).

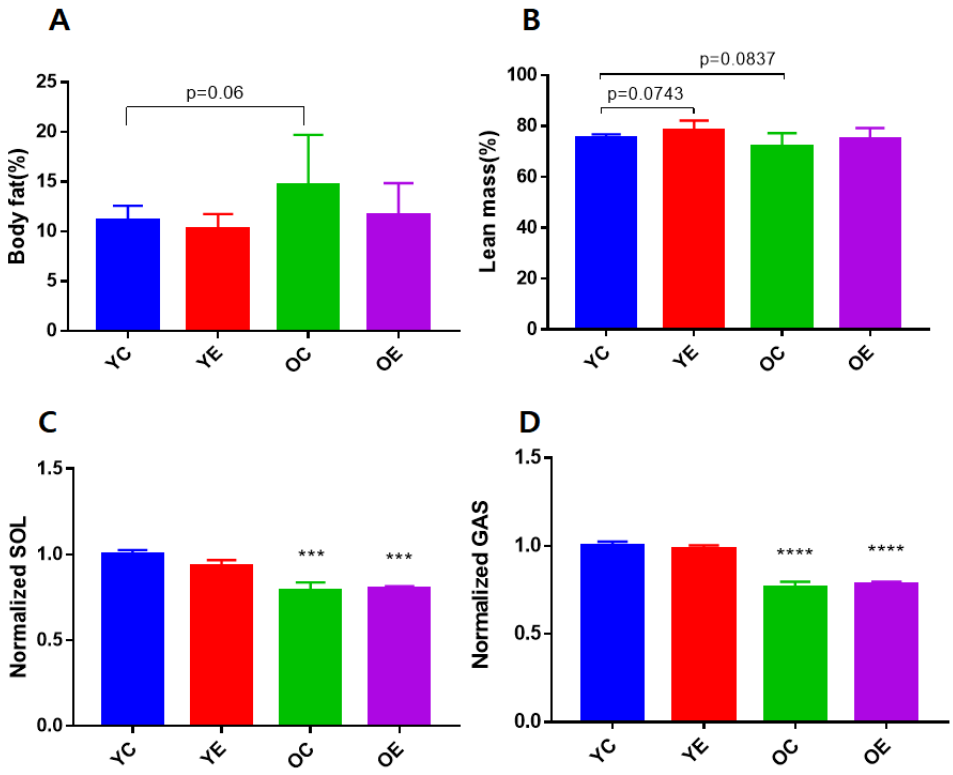

Figure 3. The Effects of 4 weeks of Treadmill Exercise on Body Composition and Muscle Wet Weight. (A) Body fat (%), (B) Lean mass (%), (C and D) Soleus wet weight/BW (mg/g), Gastrocnemius wet weight/BW (mg/g) of young sedentary (YC), young exercise (YE), old sedentary (OC), old exercise (OE). ***p<0.001 vs. YC. Stastical analysis was performed using two-tailed Student's t-test.

3. The Effects of 4 weeks of Treadmill Exercise on Motor Function and p21 expression level

Rotarod test can measure motor coordination and muscle function. There was significant difference in Rotarod latency to fall between YC and OC group, however OE group had tendency to improve motor coordination compared to OC group (Fig 4A). In addition, OC group had higher level of p21 that is representative aging marker than YC group. After 4 weeks of treadmill exercise, upregulated p21 protein expression level was significantly recovered to level of young mice group (Fig. 4B).

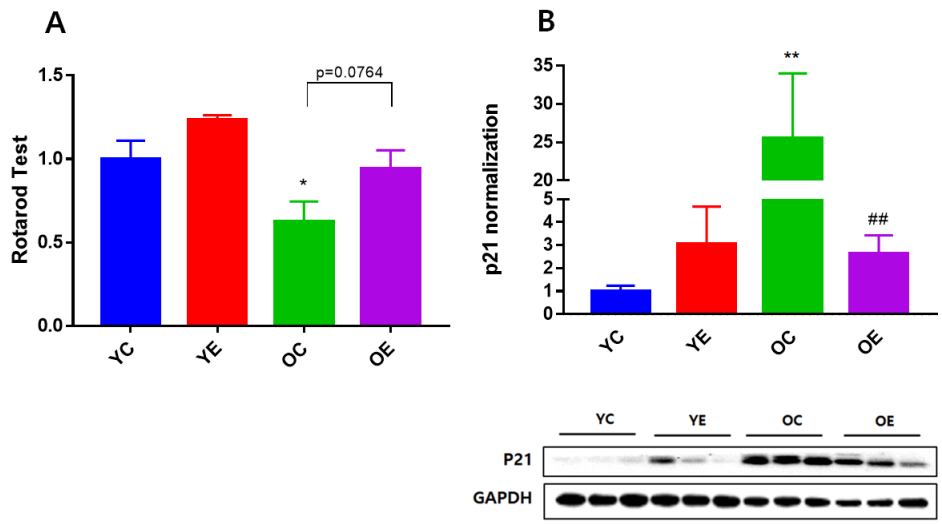

Figure 4. The Effects of four weeks of Treadmill Exercise on Motor function and p21 expression level. (A) Motor function test Rota rod latency to fall time, (B) p21 protein expression level of Gastrocnemius (GAS). *p<0.05, **p<0.01 vs. YC. #p<0.05, ##p<0.01 vs. OC. Statistical analysis was performed using two-tailed Student's t-test. All the results were presented as mean ± SEM.

4. The Effects of 4 weeks of Treadmill Exercise on Total Intestine Length

Total intestine length was normalized by body weight. It's length was not changed by both age and treadmill exercise (Fig. 5A).

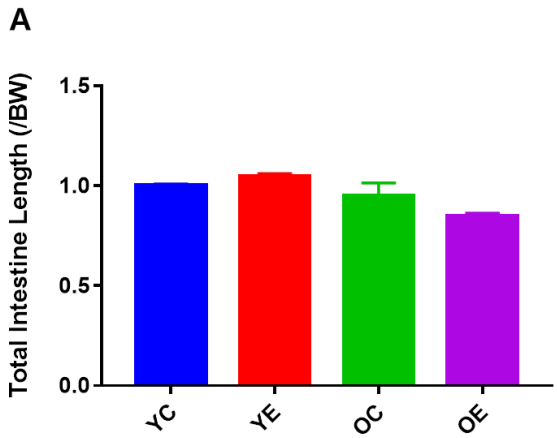

Figure 5. The Effects of 4 weeks of Treadmill Exercise on Total Intestine Length. Stastical analysis was performed using two-tailed Student's t-test.

5. The Effects of 4 weeks of Treadmill Exercise on Intestinal Tight Junction Proteins expression level

Claudin-1 protein expression level was not significantly changed by age. The expression level was upregulated in old exercise group compared to old control group. However, it was limited to old mice groups. Young exercise group was not changed by 4 weeks of treadmill exercise (Fig. 6A). Also, occludin protein expression level was not significantly changed by age, and there was no significant difference between young control group and young exercise group. Only treadmill exercise upregulated the protein level in old exercise group (Fig. 6B). Zonula occluden-1 protein expression level was not changed by both age and treadmill exercise (Fig. 6C).

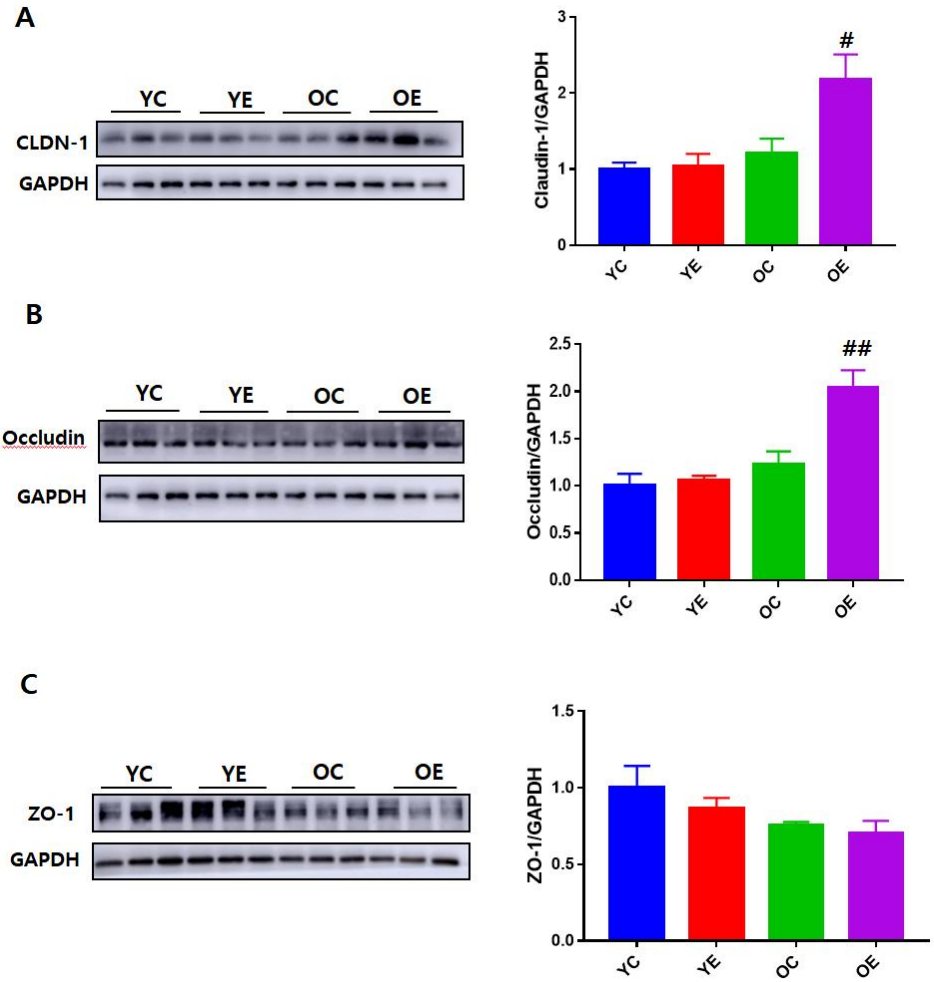

Figure 6. The Effects of 4 weeks of Treadmill Exercise on Intestinal Tight Junction Proteins expression level. (A) Claudin-1 protein expression level, (B) Occludin protein expression level, (C) zonula occludens-1 (ZO-1) expression level in colon. **p<0.01 vs. YC, #p<0.05, ##p<0.01 vs. OC. Stastical analysis was performed using two-tailed Student's t-test.

6. The Effects of 4 weeks of Treadmill Exercise on AMPK, AKT, ACC phosphorylation

To elucidate the role of AMPK on upregulated intestinal tight junction proteins, AMPK, AKT, and ACC phosphorylation was identified. In the study, p-AMPK/AMPK, p-AKT/AKT, and p-ACC/ACC ratio was not significantly different between groups (Fig 7A-C).

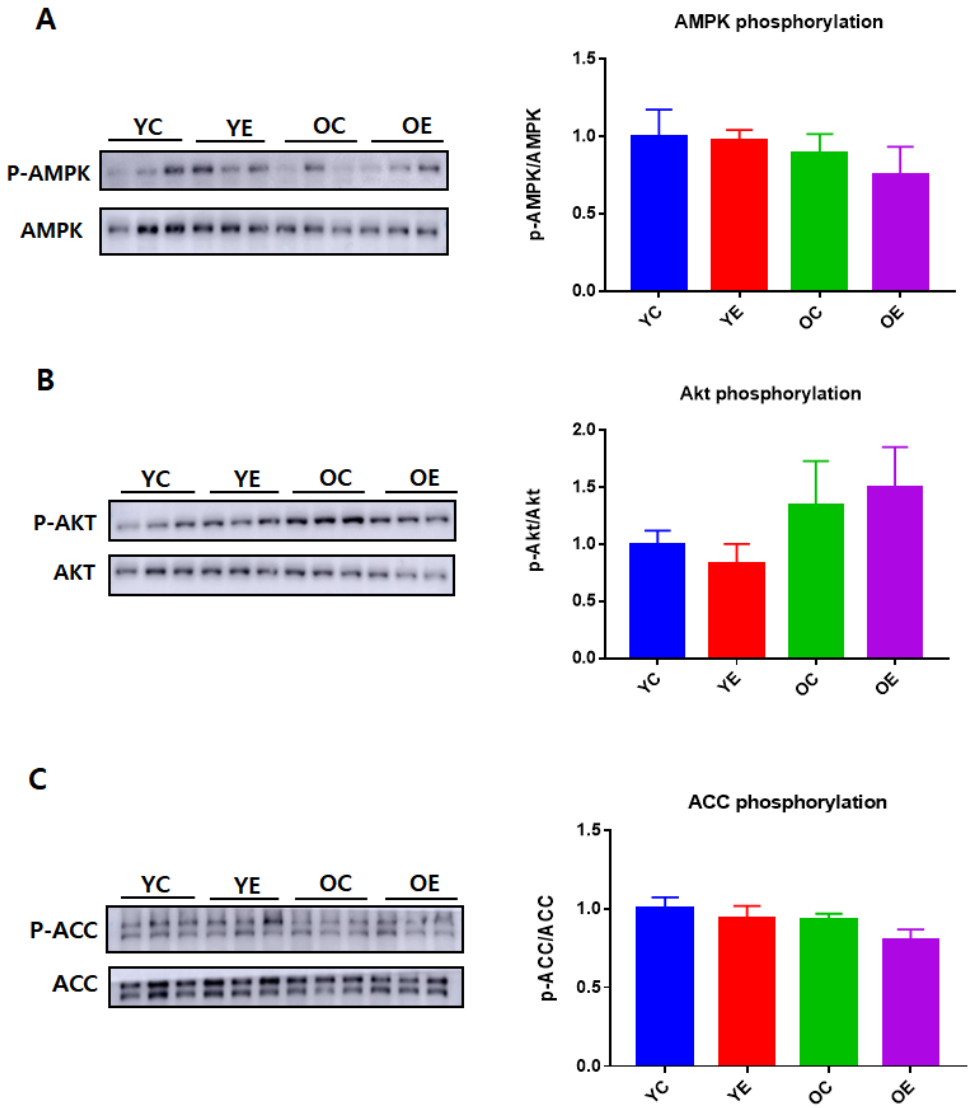

Figure 7. The Effects of 4 weeks of Treadmill Exercise on AMPK, AKT, ACC phosphorylation. (A) p-AMPK/AMPK ratio, (B) p-AKT/AKT ratio, (C) p-ACC/ACC ratio in colon. Stastical analysis was performed using two-tailed Student's t-test.

7. The Effects of 4 weeks of Treadmill Exercise on Serum Lipopolysaccharide and Zonulin Level

OC group had higher level of serum LPS than YC group. However 4 weeks of treadmill exercise significantly downregulated serum LPS level in both young and old mice groups (Fig. 7A). OC group had tendency to show increase in serum zonulin level compared to YC group, but zonulin level had no significant difference after 4 weeks of treadmill exercise (Fig. 7B).

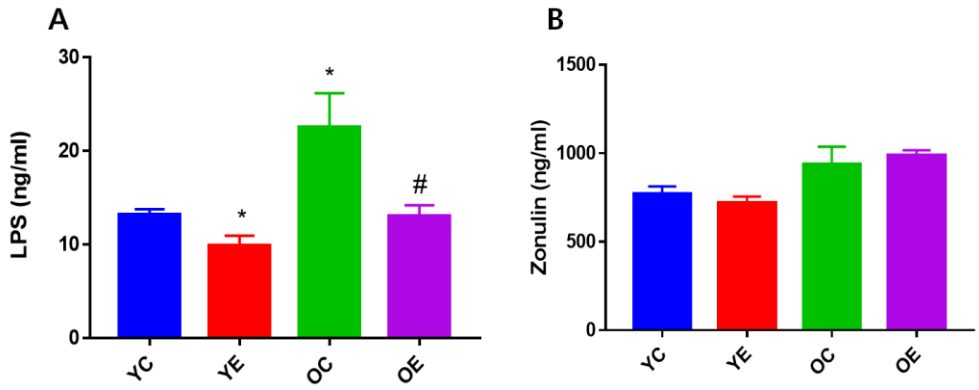

Figure 8. The Effects of 4 weeks of Treadmill Exercise on Serum Lipopolysaccharide and Zonulin Level. (A) serum LPS (ng/ml)level (YC, n=6, YE, n=4, OC, n=5, OE, n=5), (B) serum Zonulin level (YC, n=6, YE, n=5, OC, n=5, OE, n=5). *p<0.05 vs. YC. #p<0.05 vs. OC. Statistical analysis was performed using two-tailed Student's *t*-test.

V. Discussion

In this study, 4 weeks of treadmill exercise was performed on 2 month-old and 21 month-old C57BL/6J mice. The purpose of this study was to investigate the changes in the expression levels of intestinal tight junction proteins by age and exercise intervention, and the effects of these changes on skeletal muscles. Most previous studies examined the effects of aging and exercise intervention on intestinal tight junction protein respectively, but this study examined the effects of exercise on aged mice. In addition, since the effect of exercise on intestinal tight junction protein expression was controversial according to exercise intensity and type, this study tried to confirm the appropriate intensity of exercise on intestinal tight junction protein.

Previous study reported that normalized skeletal muscle weight was decreased in aged model, and treadmill exercise upregulated skeletal muscle weight (Pasini et al., 2012). In this study, the decrease in normalized skeletal muscle weight in old mice group is consistent with preceding study. However, skeletal muscle weight was not changed in both young and old mice group by 4 weeks of treadmill exercise. The previous study performed 8 weeks of treadmill exercise (Pasini et al., 2012), but our study conducted 4 weeks of treadmill exercise. Thus, exercise duration was not enough to increase muscle mass.

Most of previous studies demonstrated that treadmill exercise upregulate muscle function such as rotarod latency to fall (Y. H. Chen et al., 2018; Zelikovich, Quattrocelli, Salamone, Kuntz, & McNally, 2019). In this study, Rota rod test was performed to

measure agility and coordination ability. Rota rod latency to fall times was measured before and after exercise intervention in young and old mice group. Similar to skeletal muscle weight changes in this study, old mice group showed downregulation of muscle function compared to young mice group. After 4 weeks of treadmill exercise, rotarod latency to fall times was improved, but not significantly. Previous study performed the exercise intervention for 8 weeks (Zelikovich et al., 2019), but since this study performed 4 weeks of treadmill exercise, it was not enough exercise duration to increase muscle function.

In this research, gastrocnemius muscle was used for analysis since it is kind of mixed fiber type muscle (Sher & Cardasis, 1976). p21, cyclin-dependent kinase inhibitor, has been known to be lead to aging (Choudhury et al., 2007). Lifelong exercise training recovered age-related downregulation of p21 mRNA level in skeletal muscle (Dethlefsen et al., 2018). In this study, OC group had higher level of p21 protein expression level in gastrocnemius than YC group. Also, in OE group, p21 protein expression level was reduced by 4 weeks of treadmill exercise, which is consistent with previous study. Thus, the result of rotarod latency to fall and aging marker p21 biochemical analysis showed that the mice used in this study delays aging through treadmill exercise.

The shortening of the large intestine is called "colon shortening". Most previous studies displayed that colon shortening is one of the biological markers to evaluate the inflammation of intestine. In this study, the length of the large intestine showed a tendency to increase with age, but there was no significant change by treadmill exercise.

Tight junction proteins play an important role in binding the

spaces between the epithelial cells, preventing the diffusion of microorganisms and other antigens (Farquhar & Palade, 1963). According to previous studies, high-intensity exercise has been reported to reduce the expression level of tight junction protein (M. N. Zuhl et al., 2014) and moderate intensity exercise increases the expression level (Pasini et al., 2019). Thus, the effect of exercise has not been clarified yet and the need for study of exercise intensity has been suggested. This study displayed that there was no difference in claudin-1 and occludin expression level between young mice group and old mice group. However, claudin-1 and occludin expression level was significantly upregulated in old exercise group compared to old control group. Combined with the results of roatrod latency to fall times and aging marker p21 protein expression level in the only OE group compared to OC group, the protocol of this study was demonstrated to be sufficient exercise intensity in aged mice only. Also, the intensity of exercise used in our study was inevitably high intensity in order to match total amount of exercise between aged mice and young mice, but there was a 30 minutes break between sessions to offset high intensity-induced side effects such as heat stress.

In addition, previous study demonstrated that intestinal tight junction protein is mediated via activation of AMP-activated protein kinase in Caco-2 cell monolayers (Sun et al., 2017). In our research, *in vitro* study showed that claudin-1, occludin and zonula occluden-1 was upregulated by AICAR treatment. However, *in vivo* study demonstrated that p-AMPK/AMPK, p-AKT/AKT ratio is not significantly changed, inconsistent with previous studies. Caco2 cells, which represent intestinal cells, were tested in a fully controlled state (Peng, Li, Green, Holzman, & Lin, 2009), but it is

thought that other mechanisms may have worked because mice may have various adaptation mechanisms against exercise intervention.

In the previous study, elderly had lower level of serum LPS level compared to young participants (Ghosh et al., 2015), and serum LPS level was reduced by physical exercise in diet-induced obesity model (Oliveira et al., 2011). Interestingly, our study was the first to confirm that decreased LPS level in aged mice was increased by exercise. This study showed that serum LPS level was decreased in aged mice, and reduced LPS level was upregulated by treadmill exercise.

Zonulin, also known as pre-haptoglobin 2, is a primary biological marker that regulate intestinal barrier. It can cause reversible disruption of tight junction structure, increasing gut permeability. Previous study reported that exercise can increase zonulin mRNA expression level in rats (Holland et al., 2015). Also, exercise can improve leaky gut by decreasing zonulin level in type 2 diabetes (Pasini et al., 2019). However, contrary to previous studies, the study showed that zonulin level was not changed by 4 weeks of treadmill exercise. The meaning of this result is difficult to interpret, but one study showed that there were no changes in zonulin level after high-intensity interval cycling (Karhu et al., 2017).

VI. Conclusion

In conclusion, we examined that exercise or AICAR-induced exercise mimic condition could increase intestinal tight junction proteins in aging model. We suggest that treadmill exercise could be a good treatment for maintaining gut health in the elderly. However, there was a limitation that the age of the old mice model used in this study was lower than that of other previous studies. Further studies to validate exercise effects on intestinal tight junction proteins using much older mice will be of great interest.

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Abstract (Korean)

장 점막의 세포접합 단백질은 인접한 상피 세포의 사이를 단단하게 결합시키는 다기능 복합체로서, 장의 투과성을 조절하는 대표적인 단백질이다. 또한, 세포와 세포 사이의 밀접한 접합을 통해 염증 반응을 제어하는 것으로 알려져있다. 따라서, 본 연구에서는 노화가 일어났을 경우 장 점막의 세포 접합 단백질이 어ㄸ□ㅎ게 변화하는지 확인하고 4주간의 트레드밀 운동이 이에 미친 영향을 미치는지 확인하였다.

세포 수준의 연구에서는, 세포 노화를 유도하는 Doxorubicin을 Caco2 세포 (장 세포)에 처리하였다. 이후, AMPK를 활성화시키는 운동 모방 화학물질인 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)를 처리하여 장 점막의 세포 접합 단백질의 발현량 변화를 확인하였다. 동물연구에서는 2개월과 21개월령의 C57BL/6J 마우스를 이용하여 트레드밀 운동을 4주동안 처치하였다. 이후, 장 점막의 세포접합 단백질의 발현량을 확인하였으며, 장내 침투성을 확인하기 위하여 serum Lipopolysaccharide와 serum Zonulin의 발현량을 확인하였다.

세포 실험 결과, Doxorubicin은 Caco2 cell에서 장 점막의 세포접합 단백질의 발현량을 유의하게 감소시켰으며, AICAR 처리는 감소된 발현량을 유의하게 증가시켰다. 동물 연구 결과, 4주간의 트레드밀 운동은 노화 마우스에서 Claudin-1과 Occludin의 단백질 발현량을 유의하게 증가시켰다. 그러나, ZO-1의 발현량은 유의한 차이가 나타나지 않았다. 장 점막의 세포접합 단백질을 분해하는 것으로 알려진 serum Zonulin은 연령과 운동에 의해 모두 유의한 차이를 나타내지 않았다. 이 외에도 노화 마우스는 젊은 마우스에 비해 serum LPS의 발현 수준은 높았으나, 운동을 통하여 유의하게 감소한 것을 확인할 수 있었다.

본 연구는 노화 마우스와 젊은 마우스에서 장 점막의 세포접합 단백질의 발현량을 비교하였으며, 4주간의 트레드밀 운동이 이에 미치는 영향

을 확인하였다.