The Impact of Frailty Syndrome on Skeletal Muscle Histology and the Preventive Effects of Exercise

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Abstract

Background: Aging-induced frailty syndrome leads to significant functional decline in skeletal muscle. The specific histological changes in skeletal muscle due to frailty syndrome are not well-defined. While the positive effects of exercise on skeletal muscle histology are documented, its preventive impact on frailty syndrome requires further exploration. This study investigates the impact of frailty syndrome on skeletal muscle histology and evaluates the preventive effects of exercise.

Method: Mice were divided into five groups: young, adult, old, frailty syndrome, and exercise. The exercise group underwent endurance exercise for 60 minutes, three times a week, for 16 weeks. Body weight and lean mass ratio were measured. Muscle fiber cross-sectional area (CSA), myofiber number, extracellular matrix (ECM) content,and myosin heavy chain (MHC) isoforms of the plantaris, tibialis anterior, and quadriceps muscles were evaluated.

Results: Compared to the old group, the frailty syndrome group showed increased body weight and ECM content, with reductions in CSA and fiber number ($P < 0.05$). The proportion of MHC isoforms in skeletal muscles remained unchanged. Exercise significantly reduced body weight and ECM content, while increasing CSA, fiber number, and lean mass ratio ($P < 0.05$).

Conclusion: Frailty syndrome induces histological changes in skeletal muscle, contributing to functional decline. Exercise can mitigate these changes, highlighting its potential in preventing skeletal muscle muscle dysfunction associated with frailty syndrome. These findings provide valuable insights for the prevention, diagnosis, and treatment of frailty syndrome.

Keywords: Aging; Frailty; Histology; Exercise; Prevent

1. Introduction

Thrust of the paragraph: Frailty Syndrome Induced by Aging

Aging is an inevitable natural phenomenon in the life process, and the aged population is significantly increasing worldwide (Rochon & Smith, 1996). During the aging process, various diseases often occur, leading to a decline in the quality of life for elderly individuals and an increased social burden. Among the diseases associated with aging is frailty syndrome. For "healthy aging," it is essential to conduct comprehensive research on frailty syndrome.

Thrust of the paragraph: Identification of Frailty Syndrome

Frailty syndrome is a common multifactorial condition in the elderly, characterized by a reduced ability to resist external stressors (Chen et al., 2014; Soong et al., 2015). With the global aging population significantly increasing, the number of elderly individuals suffering from frailty syndrome is also rising annually (Wilson et al., 2012). Frailty syndrome is theoretically defined as a clinically recognizable state of increased vulnerability resulting from aging-associated decline in reserve and function across multiple physiological systems, such that the ability to cope with everyday or acute stressors is compromised (Xue, 2011). Specifically, the three states of frailty syndrome include: pre-frailty, frailty, and frailty complications (Ahmed et al., 2007; Lang et al., 2009). Furthermore, once a person is pre-frailty, they are is more likely to progress to frailty and frailty complications, thus emphasizing the downward spiral effect of this syndrome (Ahmed et al., 2007; Gill et al., 2006). Frailty syndrome is characterized by unexplained weight loss, fatigue, muscle weakness, slow gait, and reduced physical activity (Fried et al., 2001). Frailty syndrome increases the risk of falls, hospitalization, disability, and death, posing a significant challenge to "healthy aging" (Chen et al., 2014). The occurrence of frailty syndrome is associated with the decline of multiple physiological systems, including the skeletal muscle system.

Thrust of the paragraph: Frailty Syndrome Mice Model and Muscle Dysfunction

Studying diseases through animal models can avoid many ethical issues faced by human studies and allows for controlled experimental processes. Currently, the most popular animal model for researching frailty syndrome is the one developed by Thompson et al., which was reverse-translated from the human frailty phenotype (Baumann et al., 2018, 2019; Fried et al., 2001; Kwak et al., 2020). Thompson et al. found that skeletal muscle function was significantly reduced in the frailty syndrome mouse model compared to healthy aging mice (Baumann et al., 2018, 2019; Kwak et al., 2020). Unfortunately, the specific reasons for the reduced skeletal muscle function in frailty syndrome mice were not further explained.

Thrust of the paragraph: Histololgy Can Reflect Muscle Dysfunction

The reduced skeletal muscle function in frailty syndrome can be explained by histological changes. First, it helps reveal the cellular and molecular mechanisms involved in the process of muscle frailty syndrome. Second, skeletal muscle histology research can identify pathological changes associated with muscle-related diseases such as frailty syndrome. Third, the findings can aid in developing new therapeutic approaches, including gene therapy, stem cell therapy, and pharmacological interventions, to improve muscle function. Finally, understanding the structural and functional changes in skeletal muscle can provide key insights into frailty syndrome research, helping to delay muscle degeneration and improve the quality of life for the elderly (Evans et al.,

2010). However, the histological changes in skeletal muscle caused by frailty syndrome have not been reported yet.

Thrust of the paragraph: Muscle Histology Can Improve by Exercise

The positive effects of endurance exercise on skeletal muscle histology have been widely reported. Studies have shown that endurance exercise promotes muscle strength and power by increasing the number and cross-sectional area (CSA) of muscle fibers (Bell et al., 2000; Green et al., 1999; Sanchez et al., 2005). The extracellular matrix (ECM) is crucial for the transmission of muscle force and structural support (Hay, 1981; Mecham, 2011). Endurance exercise can remodel the ECM through regulated signaling pathways. For example, endurance exercise remodels the ECM by regulating the matrix metalloproteinase (MMP) family, thereby improving the extracellular environment of muscle fibers and promoting muscle health (Das et al., 2008; Gibala et al., 2009). Additionally, myosin heavy chain (MHC) isoforms are essential for muscle function. Endurance exercise can improve muscle function by adjusting the proportion of MHC isoforms (Wilson et al., 2012). Therefore, endurance exercise can promote muscle health by improving skeletal muscle histology. However, it remains unclear whether improving histology through endurance exercise before the onset of frailty syndrome can prevent aging-induced frailty syndrome, thereby maintaining skeletal muscle health.

Thrust of the paragraph: Research Purpose

This study aims to investigate the histological characteristics of frailty syndrome and examine the preventive effects of endurance exercise on them. This study can provide a diagnostic basis for future research on frailty-related diseases in humans and animals. Specifically, histological changes are crucial in diagnosing frailty-related diseases, helping physicians identify and classify diseases and assess pathological changes. These features are also essential for predicting disease prognosis and selecting treatments, guiding the development of personalized treatment plans. Furthermore, the positive effects of endurance exercise on the prevention of frailty-related diseases provide scientific support for the formulation of future exercise prescriptions. Lastly, histological research is valuable for the development of new drugs and the understanding of disease mechanisms.

2. Methods

2.1 Animals and Experimental Design

The experimental animals used were male C57BL/6N mice, divided into five groups: young (2 months, YM, n=10), adult (13 months, AM, n=10), old (22 months, OM, n=33), frailty syndrome (22 months, FS, n=13), and endurance exercise (22 months, EOM, n=10) (Figure 1). The room temperature (22 ± 2 °C), humidity (50–60%), and light (12/12 h light/dark cycle) were controlled. The mice had ad libitum access to food, water, and activity. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University (HYU 2017-0265A; 2019-0017A). The methods followed relevant guidelines and regulations and were reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

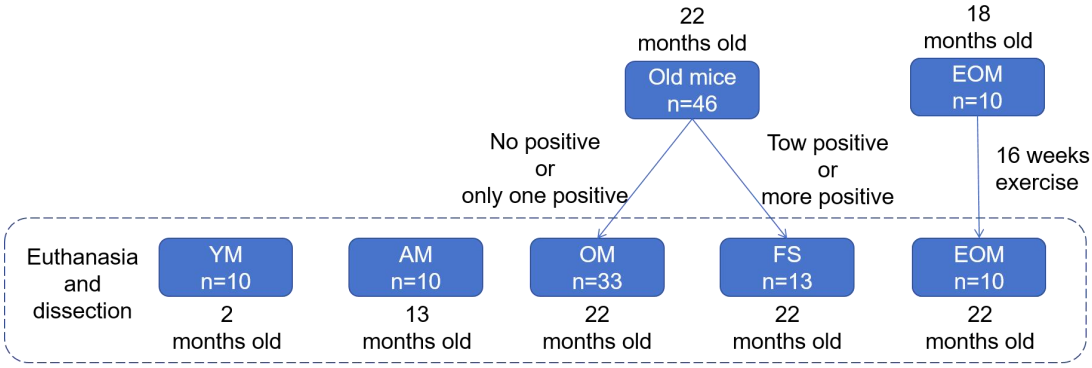


Figure 1. Animals and experimental design.

2.2 Screening of Frailty Syndrome Mouse Model

The screening criteria for the frailty syndrome mouse model used in this study were previously described in detail (Baumann et al., 2018, 2019; Fried et al., 2001; Kwak et al., 2020). Briefly, before euthanasia, mice that fell in the bottom 20% for slowness (rota-rod test), weakness (grip strength test), poor endurance (treadmill test), or low activity (voluntary wheel test), or in the top 20% for body weight were considered positive marker for frailty syndrome. These criteria were used to identify cut-off values for frailty syndrome at 22 months of age (Table 1).

Table 1. Frailty Syndrome Criteria and Cut-off Values

Fried Phenotype (Fried et al., 2001)	Mouse Frailty Syndrome Phenotype (Baumann et al., 2018, 2019)	Cut-off Values
Low activity	Voluntary wheel test	Lower 20% (138.60m/day)
Poor endurance	Treadmill test	Lower 20% (2.87 min)
Weakness	Grip strength test	Lower 20% (29 sec)
Slowness	Rotarod test	Lower 20% (55.67 sec)
Lower body weight	Higher body weight	Upper 20% (48.8g)

Mice with two or more positive frailty syndrome markers were identified as having frailty syndrome, while mice with one or no positive frailty syndrome marker were considered non-frailty syndrome (Baumann et al., 2018, 2019; Kwak et al., 2020) (Figure 2).

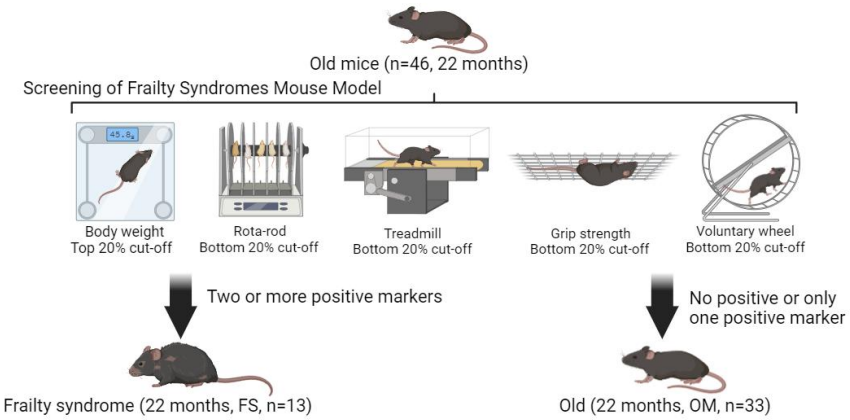


Figure 2. The frailty syndrome mouse model identification process.

Body Weight

Body and skeletal muscle raw weights were obtained on an electronic scale (CS200; OHAUS, Parsippany, NJ).

Walking Speed

Walking speed was evaluated using the Rota-Rod test (Leem et al., 2022). An adaptation phase was conducted over the first three days, including a pre-test exercise at 5 rpm for 1 minute, performed once daily. The formal test used an acceleration mode, with the speed progressively increasing from 5 to 50 rpm over a span of 5 minutes (Model 76-0770, Harvard Apparatus Inc., Holliston, MA, USA). The latency to fall from the apparatus was measured. Each mouse underwent three trials with a ten-minute interval between tests, and the best performance among the three trials was considered the final outcome measure.

Grip Strength

Grip strength was evaluated using the inverted-cling grip test (Graber et al., 2013). An adaptation phase was conducted prior to the official test, with mice undergoing the adaptive test once daily for three days. During the official test, each mouse was placed at the center of a wire mesh screen, and a stopwatch was started. The screen was then inverted over the course of 2 seconds, with the mouse's head descending first, and held 40-50 cm above a padded surface. The time until the mouse fell was recorded. This measurement was repeated three times with ten-minute intervals between tests, and the longest duration was recorded as the final measurement.

Endurance

Endurance capacity was assessed using a treadmill test (Alghannam et al., 2016). An adaptation period was conducted three days prior to the official test, during which mice ran at 5 cm/s for 5 minutes daily on a 0-degree incline. During the official test, the treadmill speed increased by 1 cm/s every 20 seconds, starting from 5 cm/s, with a 0-degree incline. The trial concluded when the mouse touched the shock pad (set at 0.6 mA) three times.

Physical Activity

Physical activity was quantified using the voluntary wheel test (Manzanares et al., 2018). Running distance was monitored using a voluntary wheel (MAN86130, Lafayette Instrument Company, Lafayette, IN, USA), with each rotation equating to a distance of 0.4 meters. The average running distance over a 5-day period was recorded for each mouse.

2.3 Exercise Protocol

The EOM group (18 months old) underwent 16 weeks of endurance exercise. At the end of the endurance exercise period (22 months old), they were euthanized. The EOM group was subjected to endurance exercise on a treadmill. One week before the formal commencement of the endurance exercise, all old mice underwent three sessions of adaptive training at an exercise intensity of 5 m/min for 20 minutes each. After the initiation of the formal experiment, each exercise session was performed for 60 minutes, three times a week, for 16 weeks. Exercise intensity was gradually increased from 4 to 16 m/min per week and subsequently maintained, and

the treadmill incline was kept at 0°. Instead of electrical stimulation, the mice were gently touched with a wooden stick to encourage them to run (Figure 3).

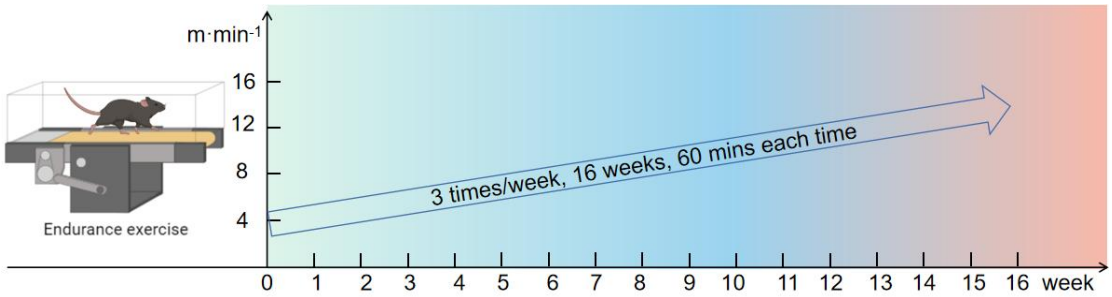


Figure 3. Endurance exercise protocol for EOM

2.4 Preservation and Preparation of Skeletal Muscle

After the mice were euthanized, the tibialis anterior (TA), quadriceps (QD), and plantaris (PL) muscles were carefully removed. The left leg muscles were fixed in 10% formalin for 24 hours, then dehydrated, and embedded in paraffin. The sections were prepared for future hematoxylin and eosin (H&E) and Masson's trichrome staining. The right leg muscles were weighed, frozen in liquid nitrogen, and subsequently stored at –80 °C for subsequent MHC isoform analyses.

2.5 Masson's Trichrome Staining and Analysis

Paraffin-embedded tissues were sectioned at 5 μm. After deparaffinization and rehydration, the tissue sections were stained using Masson's trichrome staining kit (G1340, Beijing Solarbio Science & Technology Co., Ltd.). The sections were subsequently placed in xylene and alcohol for dehydration and transparency, and finally sealed with neutral resin. Images were acquired using a slide scanner (Axio Scan. Z1, Zeiss) at 200x magnification. For each mouse, a single section of each skeletal muscle was randomly photographed to obtain 5 images, each covering an area of 0.271 square millimeters. The average of these five images was used to represent the ECM value of that skeletal muscle for the mouse. ImageJ software was used for analysis. A threshold was established to remove the blank background, the ECM signal was isolated using color deconvolution and thresholding, and the area of the ECM signal was subsequently compared with the net area of the muscle section using ImageJ software.

2.6 H&E Staining and Analysis

H&E staining was performed on paraffin-embedded tissue sections after deparaffinization and rehydration, as previously described by Robert D. Cardiff (Almond & Enser, 1984), with slight modifications. Briefly, rehydrated tissue sections were stained using ClearView™ hematoxylin (MA0101010, StatLab) for 3 minutes, rinsed in tap water for 30 seconds, differentiated in 1% hydrochloric acid alcohol for 5 seconds, washed in tap water for 3 minutes, and stained in ClearView™ eosin (MA0101015, StatLab) for 1 minute. They were subsequently dehydrated, rendered transparent, and sealed with neutral resin. Images were acquired using a slide scanner (Axio Scan. Z1, Zeiss) at 200x magnification. For each mouse, a single section of each skeletal muscle was randomly photographed to obtain 5 images, each covering an area of 0.271 square millimeters. The average of these five images was used to represent the cross-sectional area (CSA) and fiber number of that skeletal muscle for the mouse. The CSA and fiber number were

automatically analyzed using Cellpose (Stringer et al., 2021; Waisman et al., 2021).

2.7 Electrophoretic Separation of Myosin Heavy Chain Isoforms

The MHC isoform composition of each fiber was determined using SDS–PAGE and silver staining. Skeletal muscle was extracted for 60 min on ice in 50 vol buffer (0.1 M sodium phosphate, pH 7.3), to which a 1:100 protease + phosphatase inhibitor cocktail (WSE-7420, ATTO) was added. The total protein concentration was subsequently determined using the Pierce™ BCA Protein Assay Kit (23227, Thermo). Samples were diluted in 5× sample buffer (25% β-MEtOH [β-ME], 11.5% SDS, 50% glycerol, 312.5 mM Tris, pH 6.8, and 0.05% bromophenol) and dH_2O to achieve a final protein concentration of 0.266 μg/μL. The samples were subsequently boiled for 10 min, and 6 μg (30 μL) of protein was loaded onto a mini gel system (Mini Protean III: 8.3 × 7.3 cm). MHC isoforms were separated using 8% SDS–PAGE with 30% glycerol (Talmadge & Roy, 1993). The lower running buffer comprised 0.05 M Tris (base), 75 mM glycine, and 0.05% w/v SDS. The upper running buffer was 6× the concentration of the lower running buffer, and β-ME was added (final concentration: 0.2% v/v) (Kohn & Myburgh, 2006). After sample loading, electrophoresis was performed at a constant voltage of 140 V for 28 h. The temperature of the buffer was maintained at 4 °C during electrophoresis. After electrophoresis, the gels were stained with a silver staining kit (24612, Thermo), and the bands were quantified via densitometry using Image J software.

2.8 Statistical analysis

Analyses were performed using GraphPad Prism (version 9) software. One-way analysis of variance and Tukey's post-hoc test were used to compare the mean difference among groups. When the conditions of normality or homogeneity were not met in comparisons of three or more groups, the Kruskal–Wallis test along with Dunn's post-hoc comparison was employed. All results are expressed as the mean ± standard error of the mean. Statistical significance was set at $p < 0.05$. Asterisks indicate the following: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. Results

3.1 Endurance Exercise Prevents Frailty Syndrome by Reducing Body

Aging leads to an increase in body weight in mice, and frailty syndrome further exacerbates this increase ($P < 0.05$) (Figure 4A). Aging also reduces the lean mass ratio (ratio of skeletal muscle to body weight) ($P < 0.05$), but frailty syndrome does not cause a further decrease in this ratio ($P > 0.05$) (Figure 4B-D). After 16 weeks of endurance exercise, there was a significant reduction in body weight ($P < 0.05$) (Figure 4A), and the lean mass ratio also significantly increased ($P < 0.05$) (Figure 4B-D). These data indicate that aging leads to weight gain and a reduction in lean mass ratio. Furthermore, frailty syndrome exacerbates weight gain. Importantly, 16 weeks of endurance exercise can prevent frailty syndrome by reducing weight.

Body weight Plantaris Tibialis anterior Quadriceps

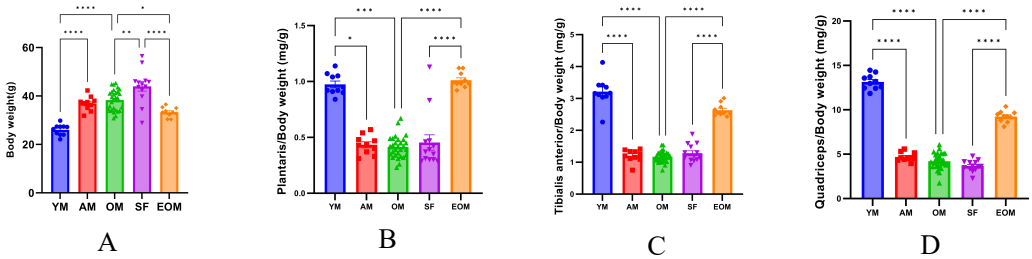
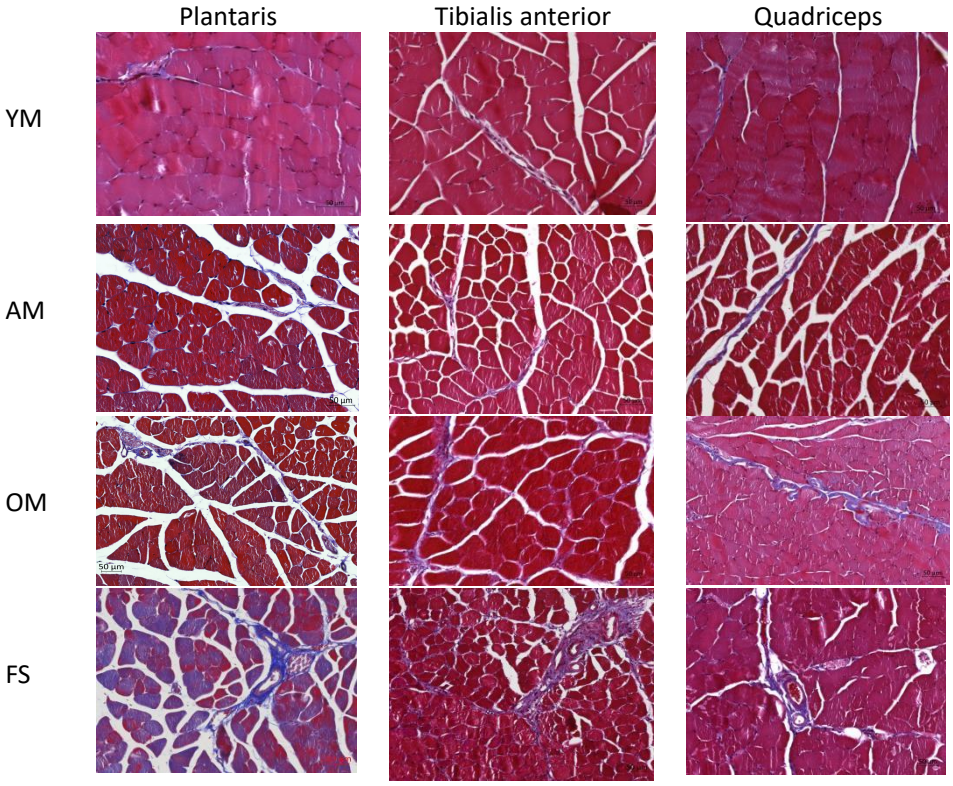


*Figure 4. Body weight and lean muscle ratio. (A) Body weights of the different groups of mice. (B, C, and D) Plantaris, tibialis anterior, and quadriceps to body weight ratios. YM = 2 months, n = 10; AM = 13 months, n = 10; OM = 22 months, n = 33; FS= 22 months, n = 13; and EOM = 22 months, n = 10. All data are presented as the mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

3.2 Endurance Exercise Prevents Increased ECM Due to Frailty Syndrome

Experimental results show that the ECM area of the PL and QD muscles increases with age ($P < 0.05$) (Figure 5A-C), while the ECM of the tibialis anterior (TA) does not appear to be affected by age ($P > 0.05$) (Figure 5A and D). The impact of frailty syndrome on skeletal muscle ECM is significant. In the PL, TA, and QD muscles, the ECM in the FS group is significantly larger than in the OM group ($P < 0.05$) (Figure 5A-D). Additionally, after 16 weeks of endurance exercise, the ECM in the EOM group is significantly lower than in the OM and FS groups ($P < 0.05$) (Figure 5A-D). These findings indicate that aging is a key factor leading to abnormal ECM accumulation in skeletal muscles, and this negative effect is significantly exacerbated by frailty syndrome. Importantly, we found that 16 weeks of endurance exercise can prevent frailty syndrome by inhibiting abnormal ECM accumulation.



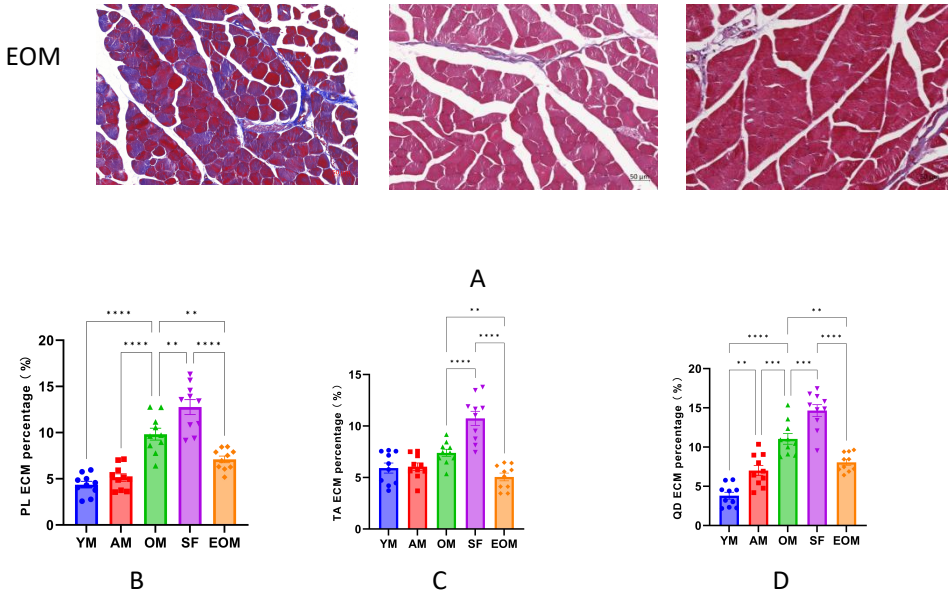
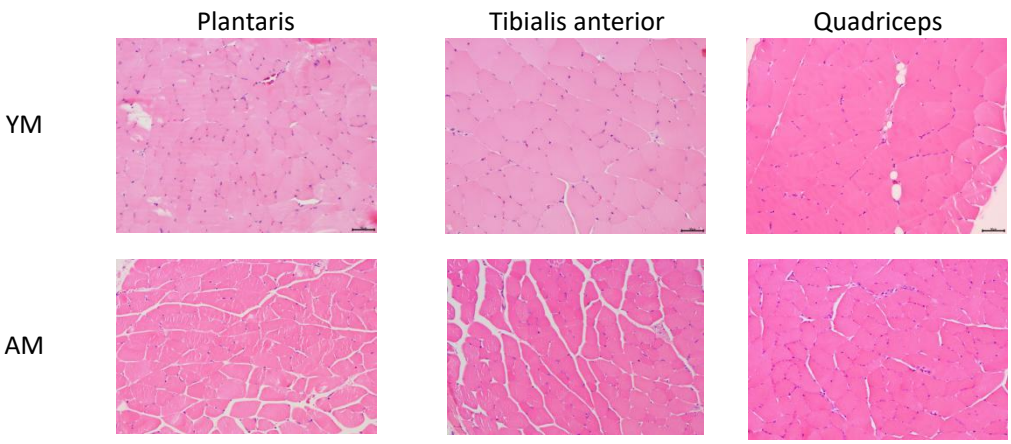


Figure 5. Representative images of Masson's trichrome staining of the plantaris, tibialis anterior, and quadriceps extracellular matrixes of male C57BL/6N mice at different stages. (A) Representative images of Masson's trichrome staining of the plantaris, tibialis anterior. (B) Changes in the extracellular matrix of the plantaris in male C57BL/6N mice. (C) Changes in the extracellular matrix of the tibialis anterior in male C57BL/6N mice. (D) Changes in the extracellular matrix of the quadriceps in male C57BL/6N mice. YM = 2 months, n = 10; AM = 13 months, n = 10; OM= 22 months, n = 10, SF= 22 months, n = 10, and EOM = 22 months, n = 10. The extracellular matrix is indicated in blue and the myofiber in red. Scale bar=50 μm.

3.3 Endurance Exercise Prevents Decreased CSA Due to Frailty Syndrome

We found that the effects of aging on the CSA of different skeletal muscles vary, which may be determined by the functional roles of these muscles in daily activities. Importantly, we observed that in the TA and QD muscles, the CSA of the FS group was significantly lower than that of the OM group ($P < 0.05$) (Figure 6A, C-D). In contrast, in the PL muscle, the CSA of the FS group was significantly higher than that of the OM group ($P < 0.05$) (Figure 6A-B). Additionally, after 16 weeks of endurance exercise, the CSA of the EOM group was significantly higher than that of the OM and FS groups in all muscles except PL ($P < 0.05$) (Figure 6A-D). These data demonstrate that 16 weeks of endurance exercise can prevent frailty syndrome by increasing the CSA of skeletal muscles.



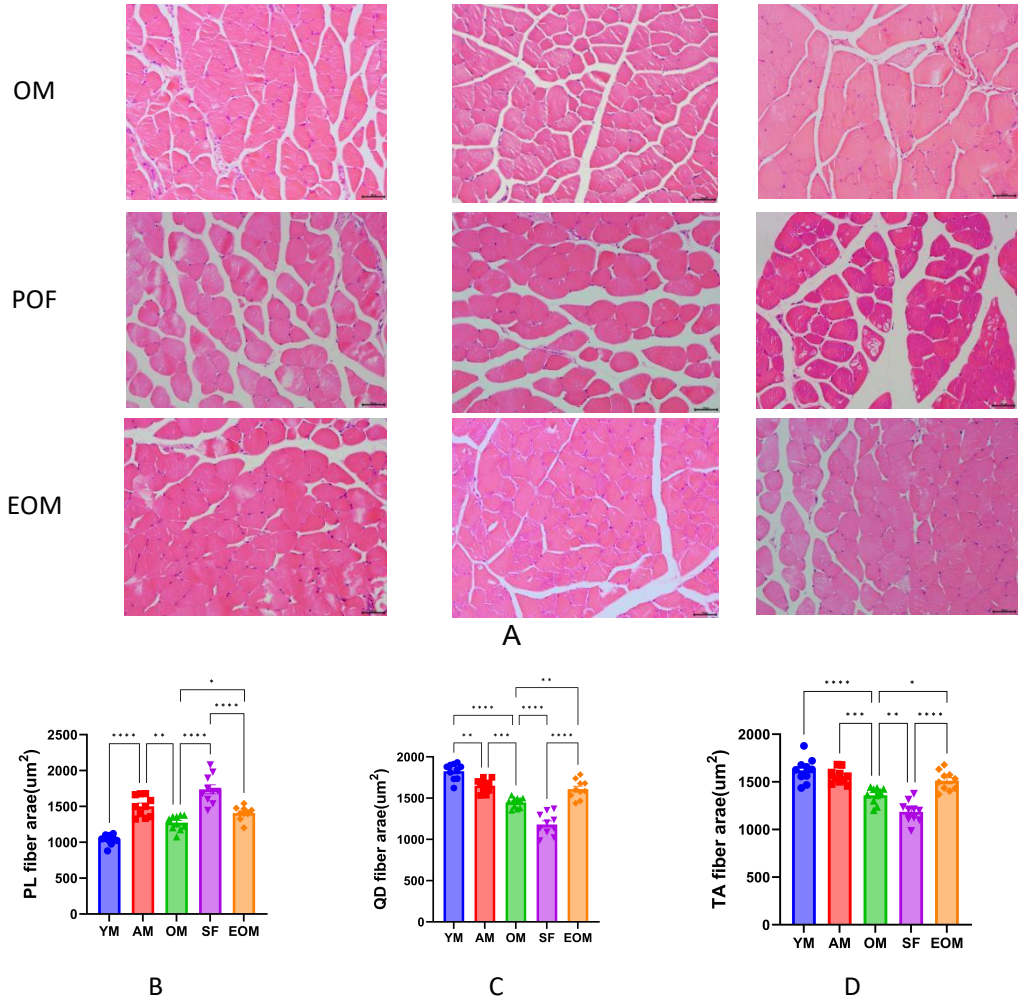
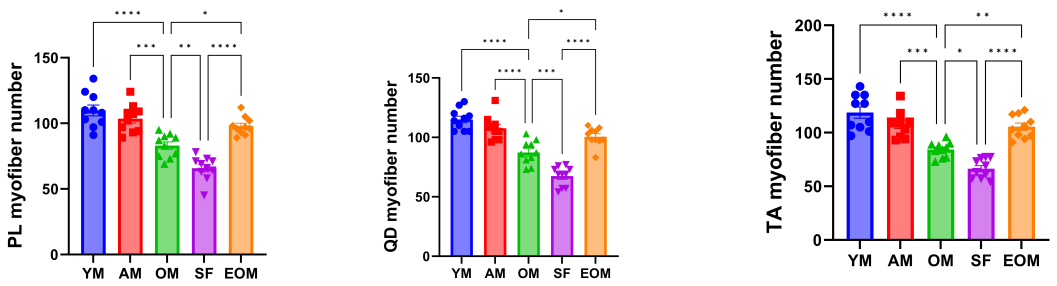


Figure 6. Representative images showing H&E staining of plantaris, tibialis anterior, and quadriceps cross-sectional areas in male C57BL/6N mice at various stages. (A) Changes in the cross-sectional area of the plantaris in male C57BL/6N mice. (B) Changes in the cross-sectional area of the tibialis anterior in male C57BL/6N mice. (C) Changes in the cross-sectional area of the quadriceps in male C57BL/6N mice. YM = 2 months, n = 10; AM = 13 months, n = 10; OM= 22 months, n = 10, SF= 22 months, n = 10, and EOM = 22 months, n = 10. Scale bar=50 μm.

3.4 Endurance Exercise Prevents Decreased Fiber Number Due to Frailty Syndrome

Our data show that aging leads to a significant reduction in the number of skeletal muscle fibers ($P < 0.05$) (Figure 7A-C). Compared to OM, FS exhibited an even lower number of skeletal muscle fibers ($P < 0.05$) (Figure 7A-C). Additionally, 16 weeks of endurance exercise EOM increased the number of skeletal muscle fibers, significantly surpassing both the FS and OM group ($P < 0.05$) (Figure 7A-C). These experimental results reveal that 16 weeks of endurance exercise can mitigate the negative effects of frailty syndrome on skeletal muscles by increasing the number of muscle fibers.

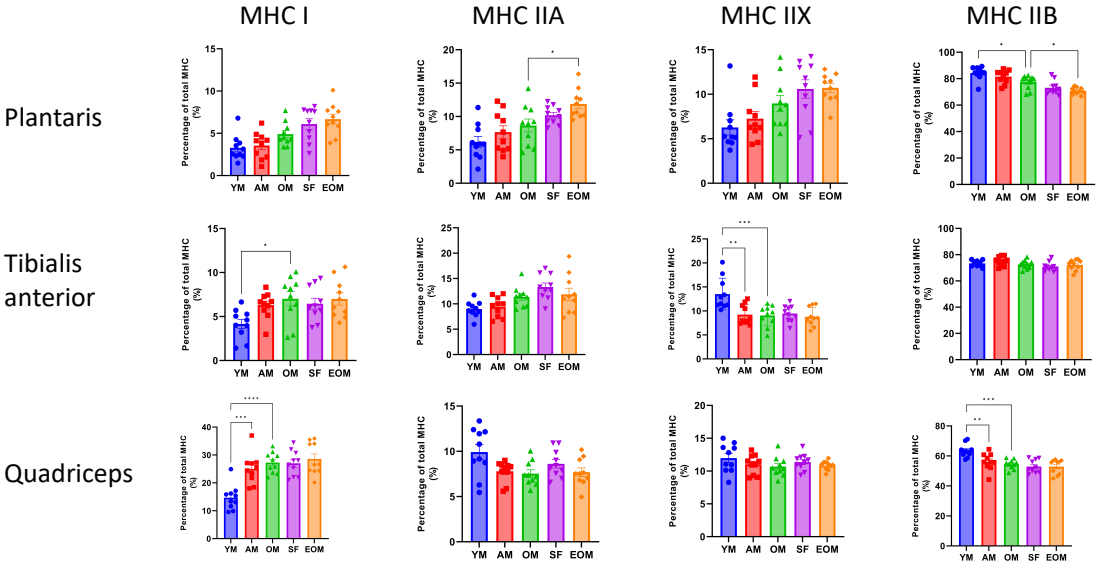


A B C

*Figure 7. Changes in the myofiber numbers of the plantaris, tibialis anterior, and quadriceps muscles in male C57BL/6N mice at different stages. (A) Changes in the myofiber number of the plantaris in male C57BL/6N mice. (B) Changes in the myofiber number of the tibialis anterior in male C57BL/6N mice. (C) Changes in the myofiber number of the quadriceps in male C57BL/6N mice. YM = 2 months, n = 10; AM = 13 months, n = 10; OM= 22 months, n = 10, SF= 22 months, n = 10, and EOM = 22 months, n = 10. All data are presented as the mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).*

3.5 Frailty Syndrome does not Affect MHC Isoforms.

We used an anti-MHC (Myosin 4 Monoclonal Antibody, Thermo, #14-6503-82) to examine the band positions in the gel, confirming that the bands observed in silver staining correspond to MHC (S1). Aging leads to an increase in the proportion of MHC I and a decrease in the proportion of MHC IIX in TA ($P < 0.05$) (Figure 8). Similarly, in QD, aging significantly increases the proportion of MHC I ($P < 0.05$) (Figure 8). We also found that frailty syndrome does not significantly affect the proportion of MHC in skeletal muscles ($P > 0.05$) (Figure 8). 16 weeks of endurance exercise resulted in a significant decrease in the proportion of MHC IIB only in TA ($P < 0.05$) (Figure 8). These data indicate that frailty syndrome does not affect the proportion of MHC in skeletal muscles.



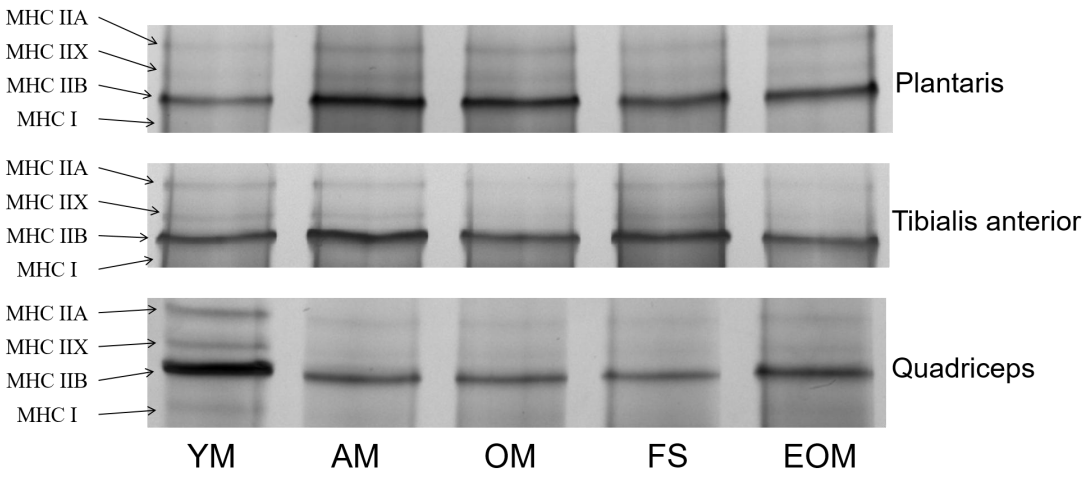


*Figure 8. Changes in the myosin-heavy-chain isoform compositions of the plantaris, tibialis anterior, and quadriceps muscles in male C57BL/6N mice at different stages. YM = 2 months, n = 10; AM = 13 months, n = 10; OM= 22 months, n = 10, SF= 22 months, n = 10, and EOM = 22 months, n = 10. All data are presented as the mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

4. Discussion

The increase in the aging population is a global trend. Frailty syndrome, caused by aging, has become a significant challenge to "healthy aging" and imposes a substantial burden on society. Therefore, research on frailty syndrome and preventive measures is urgently needed. We found that frailty syndrome leads to skeletal muscle dysfunction by increasing body weight and ECM accumulation, while decreasing CSA and muscle fiber quantity. However, endurance exercise can prevent those negative effects of frailty syndrome on skeletal muscle. These findings provide scientific data for the prevention, diagnosis, treatment, and post-recovery evaluation of frailty syndrome in the future.

4.1 Appropriateness of an Frailty Syndrome Mouse Model

Frailty syndrome is defined as a clinically recognizable state of increased vulnerability resulting from aging-associated declines across multiple physiological systems, which reduces an individual's ability to cope with everyday or acute stressors (Xue, 2011). Fried and colleagues proposed a clinical phenotype of frailty syndrome, characterized by weakened grip strength, low walking speed, unintentional weight loss, reduced physical activity, and fatigue (Fried et al., 2001). Alternatively, frailty has been operationalized as a risk index by calculating the cumulative number of deficits over time, known as the "Frailty Index (FI)." This index includes disabilities, diseases, physical and cognitive impairments, psychosocial risk factors, and geriatric syndromes such as falls, delirium, and incontinence (Mitnitski et al., 2001). Compared to Fried phenotype of frailty syndrome, the FI is a more sensitive predictor of adverse health outcomes because it provides a more nuanced grading of risk. Additionally, the FI is robust in clinical inference concerning the number and actual composition of items included (Rockwood et al., 2007).

However, the Fried frailty syndrome phenotype aligns better with our experimental design. Firstly, the Fried frailty syndrome phenotype is based on five specific and easily measurable indicators: weight loss, endurance, grip strength, walking speed, and physical activity level. These indicators are typically easy to measure in animal experiments using standard methods such as weight scales,

grip strength meters, and voluntary running wheel devices. Secondly, the indicators of the Fried frailty syndrome phenotype directly reflect the physiological and behavioral state of the animals. These indicators are analogous to those used in human studies, making the results more comparable and translatable to human research. Finally, the Fried frailty syndrome phenotype is highly adaptable and can be adjusted to fit the characteristics of different animal species. For instance, grip strength tests can be replaced with other muscle strength tests, and walking speed tests can be modified to suit the specific behaviors of the animals being studied.

We want to emphasize that the onset of frailty syndrome is a continuous process (Ahmed et al., 2007), currently broadly categorized into three stages: pre-frailty, frailty, and frailty complication (Ahmed et al., 2007; Lang et al., 2009). Research has shown that patients in the pre-frailty stage are more likely to progress to the frailty and frailty complication stages compared to non-pre-frailty patients (Gill et al., 2006). Additionally, there is potential for reversal during the pre-frailty stage (Gill et al., 2006). The final stage of frailty syndrome involves complications that ultimately lead to patient mortality (Robertson & Montagnini, 2004). To our knowledge, this is the first study investigating the impact of frailty syndrome on skeletal muscle histology. This research will provide scientific data to support future exploration of the different stages of frailty syndrome.

In our frailty syndrome mouse model, we followed the selection method described by Thompson et al. (Baumann et al., 2018, 2019; Kwak et al., 2020). Briefly, in cohort of old mice (22-months-old), those ranking in the bottom 20% for walking speed, grip strength, endurance, or physical activity were considered frailty-positive. However, differing from the Fried frailty syndrome phenotype, instead of unintentional weight loss, we determined that old mice with a high body weight, ranking in the top 20%, were positive for frailty syndrome.

Thompson et al. had already established high body weight as a criterion for predicting frailty syndrome in mice (Baumann et al., 2018, 2019; Kwak et al., 2020). Briefly, high body weight has a significant negative impact on the lifespan of mammals (Economos, 1980). A typical example is the Ames dwarf, which has a longer lifespan (Bartke et al., 2003; Brown-Borg et al., 1996), and calorie restriction, which leads to lower body weight and increases both average and maximum lifespan (Masoro, 1993; Weindruch, 1989). Additionally, an increase in body weight is positively correlated with mortality in mice (Miller et al., 2000; Miller et al., 2002). For more detailed information, please refer to the excellent papers by Thompson et al. (Baumann et al., 2018, 2019; Kwak et al., 2020).

The 20% cut-off point is widely recognized in research for diagnosing frailty syndrome in both humans and mice (Baumann et al., 2018, 2019, 2020; Fried et al., 2001; Kwak et al., 2020). For a detailed explanation of the use of the 20% cut-off point, refer to the excellent review by Thompson et al. (Baumann et al., 2020). The specific selection process for the frailty syndrome mice is detailed in the supplementary materials (S2).

Selecting 22-month-old mice as experimental subjects is intended to better simulate the characteristics of human frailty syndrome. A 22-month-old mouse roughly corresponds to a human aged 60-65 years (Dutta & Sengupta, 2016; Justice et al., 2016), which is the period when frailty syndrome typically emerges during normal aging (Calado et al., 2016; Fried et al., 2001). Moreover, because frailty syndrome is thought to be reversible, this age provides adequate time to implement possible life changing interventions.

4.2 Lean Mass Ratio in Frailty Syndrome

Although the lean mass ratio decreased with age, no significant difference was noted between OM and FS group. This may be related to the high body weight observed in the frailty syndrome mouse model. High body weight potentially causes ectopic fat deposition in muscle tissue, known as myosteatosis (fat infiltration of skeletal muscle), and increased ECM (see below) in skeletal muscle potentially exacerbates the risk of myosteatosis. Thus, we speculate that frailty syndrome may lead to a rebound in lean mass ratio by inducing myosteatosis.

Moreover, the skeletal muscle of mice with frailty syndrome possibly also undergo prolonged heavy weight-bearing to maintain basic vital activities (e.g., postural maintenance). The compensatory effects of weight gain-induced increases in skeletal muscle mass and strength have also been identified in previous studies (Abdelmoula et al., 2012; Duché et al., 2002). Notably, endurance exercise has been shown to exert a beneficial effect in preventing frailty syndrome by significantly reducing body weight and increasing the lean mass ratio.

4.3 ECM in Frailty Syndrome

The ECM of FS group was significantly larger than that of OM, which to our knowledge is the first time it has been reported. While it is not clear how frailty syndrome affects ECM, some studies have found that both aging (Clemens et al., 2021; Liou et al., 2013) and high body weight (Pincu et al., 2015) lead to increased ECM. The abnormal increase in the ECM in frailty syndrome might have resulted from a combination of aging and high body weight. Specifically, aging and obesity lead to the excessive accumulation of ECM components such as collagen and fibrinogen through oxidative stress and inflammatory responses (Ruiz-Ojeda et al., 2019). These changes are mediated by alterations in the activity of matrix metalloproteinases (MMPs) and the activation of hypoxia-inducible factor (HIF-1α) (Brown et al., 2016). Therefore, the abnormal increase in the ECM may be one of the histological characteristics of frailty syndrome. Excessive ECM accumulation accelerates fat infiltration into skeletal muscle, which may be responsible for the abnormal lean mass ratio in frailty syndrome.

Endurance exercise can prevent frailty syndrome by improving the skeletal muscle ECM condition in aging individuals. There are numerous reports stating that endurance exercise can promote ECM remodeling and improve its health status (Csapo et al., 2020; Song et al., 2006). A healthy ECM not only efficiently transmits muscle strength (Csapo et al., 2020) and provides mechanical support to nerves and blood vessels (Sheard & Anderson, 2012) but also promotes the proliferation and differentiation of satellite cells (Csapo et al., 2020) and counteracts age-related muscle stiffness (Csapo et al., 2020). An unhealthy ECM state is one of the negative factors contributing to or causing frailty syndrome. Therefore, based on these data, endurance exercise may prevent frailty syndrome by remodeling and improving the ECM.

4.4 CSA in Frailty Syndrome

The change in the CSA of the PL muscle was unexpected. The fluctuating trend in the CSA of the PL during aging was consistent with that in previous studies (Lee et al., 2019; Mobley et al., 2017). The growth and development peak of the PL occurs at approximately 12 months of age (Mobley et al., 2017); therefore, the increase in CSA occurs at the onset of early aging. According to a previous study, the CSA of the PL decreases with increasing age in the second half of aging (Lee et al., 2019). Unexpectedly, frailty syndrome mice exhibited an abnormal increase in the PL CSA. This phenomenon has also been observed in other skeletal muscles (Sayed et al., 2016; Sheard &

Anderson, 2012); nonetheless, to the best of our knowledge, this is the first time it has been found in the PL in frailty syndrome.

We speculate that the loss and reduced proportion of smaller fibers in the PL lead to an increase in the mean area of muscle fibers or a compensatory mechanism that attempts to compensate for the loss of muscle strength to maintain the basal vital activity of the organism (Sayed et al., 2016; Sheard & Anderson, 2012). This increase in the CSA of the PL in old exercise group has similarly been reported in other studies (Lee et al., 2019; Masschelein et al., 2020). Moreover, during the experiment, we observed that mice with frailty syndrome had increased resting time and decreased physical activity, exacerbating their sedentary status. Furthermore, the PL is more involved in postural maintenance than the QD and TA (Akay et al., 2014; Charles et al., 2018), possibly explaining the abnormal increase in the CSA of the PL in frailty syndrome because of compensatory effects, a phenomenon not apparent in the QD and TA.

The significant reduction in the CSA of QD and TA may result from multiple negative factors induced by frailty syndrome. Frailty syndrome leads to a decrease in skeletal muscle CSA through various molecular biological mechanisms. Firstly, autophagy dysregulation associated with frailty and aging affects the clearance of muscle proteins and organelles, leading to a decline in muscle mass and function. Frail elderly individuals exhibit elevated levels of the autophagy protein LC3-I, which may indicate weakened autophagosome formation (Aas et al., 2019). Additionally, frailty syndrome is associated with an imbalance between muscle protein synthesis and degradation. Dysregulation of skeletal muscle protein metabolism in the elderly leads to a significant decline in muscle mass (Grimby, 1995). Finally, increased levels of oxidative stress and chronic inflammation in frail elderly individuals damage muscle cells and tissues, further contributing to the decline in muscle mass and function (Lang et al., 2010). Therefore, we believe that the significantly reduced CSA is one of the histological characteristics of frailty syndrome.

Our experimental results indicate that endurance exercise significantly increases skeletal muscle CSA. This finding aligns with previous studies (Clark et al., 1989; Coggan et al., 1992; Lewis et al., 2015; Wang et al., 2023). Endurance exercise promotes the increase in skeletal muscle fiber CSA through various molecular biological mechanisms. Endurance exercise activates the AMPK signaling pathway, enhancing mitochondrial biogenesis and oxidative metabolic capacity. This process involves AMPK-mediated upregulation of PGC-1α, a key regulator of mitochondrial biogenesis (Hoppeler et al., 2011). PGC-1α facilitates the adaptive changes in muscle fibers by regulating the expression of genes associated with mitochondrial biogenesis and muscle fiber type conversion, maintaining the CSA and function of muscle fibers (Ruas et al., 2012).

Additionally, the mTOR signaling pathway supports muscle fiber growth and maintenance by promoting protein synthesis, particularly during prolonged and high-intensity endurance training (Hoppeler et al., 2011). Autophagy mechanisms help maintain cellular homeostasis and function by removing damaged organelles and proteins, thus supporting muscle health and functional recovery during endurance exercise (Kwon et al., 2018)Finally, endurance exercise increases the expression of vascular endothelial growth factor (VEGF), promoting capillary formation in muscles, enhancing oxygenation, and supporting the growth and metabolic needs of muscle fibers (Kon et al., 2014). These mechanisms collectively contribute to the significant increase in skeletal muscle fiber CSA, improving oxidative metabolic capacity and overall muscle function through endurance exercise. Therefore, we believe that the CSA reduction caused by frailty syndrome can be prevented through endurance exercise.

4.5 Myofiber Number in Frailty Syndrome

Our results indicated that myofiber number to be significantly reduced in FS compared with that in OM group, possibly because of the simultaneous activation of apoptotic pathways by obesity (Heo et al., 2018) and aging (Ziaaldini et al., 2015). Specifically, aging and obesity lead to an increase in pro-apoptotic factors (e.g., P53 and Bax) and a decrease in anti-apoptotic factors (e.g., Bcl-2) in skeletal muscle, potentially inducing a decrease in myofiber number with aging and obesity (Agrawal et al., 2024; Lin et al., 2017; Yahagi et al., 2003; Ziaaldini et al., 2015). Therefore, we consider the reduction in skeletal muscle fiber quantity to be one of the histological characteristics of frailty syndrome.

After 16 weeks of endurance exercise, the number of skeletal muscle fibers significantly increased, consistent with the findings of previous studies (Gonyea et al., 1986; Hoffman et al., 2013; Luo et al., 2023; Waters et al., 2004). Endurance exercise may promote the increase in skeletal muscle fiber number through various molecular biological mechanisms. Firstly, endurance exercise activates the AMPK signaling pathway, leading to the upregulation of PGC-1 α , which plays a crucial role in mitochondrial biogenesis and muscle fiber type conversion (Hoppeler et al., 2011). Secondly, calmodulin-dependent kinases (CaMKs) such as CaMKII are important in regulating oxidative enzyme expression, mitochondrial biogenesis, and muscle fiber-specific protein expression. They can decode frequency-dependent information to activate adaptive responses, thus promoting the increase in muscle fiber number and function (Chin, 2005). Finally, autophagy is critical in supporting muscle adaptive changes. Endurance exercise increases autophagic flux, helping to clear damaged organelles and proteins, maintaining cellular homeostasis and function (Sanchez et al., 2014), thereby promoting an increase in muscle fiber number. These mechanisms collectively contribute to a significant increase in the number of skeletal muscle fibers, enhancing overall muscle endurance and health. Therefore, the reduction in skeletal muscle fiber number caused by frailty syndrome can be prevented through endurance exercise.

4.6 Myosin Heavy Chains in Frailty Syndrome

Skeletal muscle MHC content potentially reflects its physiological characteristics. We found aging to reduce the expression of fast MHC isoforms (e.g., IIb and IIx), exhibiting consistency with mainstream findings (Balagopal et al., 2001; Lopez et al., 2000; Uchitomi et al., 2019). Recent studies on skeletal muscle MHC isoforms in mice with frailty syndrome are inadequate, and our data did not reveal significant differences in MHC content between old control and pfrailty syndrome. Aging lead to decreased fast MHC isoforms and gene expression (Balagopal et al., 2001; Ciciliot et al., 2013; Uchitomi et al., 2019). Despite some controversy, studies have found that obesity increases fast MHC isoforms and gene expression in humans and rodents (Almond & Enser, 1984; Houmard et al., 2002; Lillioja et al., 1987). We hypothesize that the unchanged skeletal muscle MHC content in mice with frailty syndrome emanates from the combined effects of aging and high body weight. Furthermore, changes in MHC content that are not detected in extremely old and obese mice also exist (Ballak et al., 2014; Turpin et al., 2009). No definite conclusions have been drawn yet, and a future in-depth study on this topic is worthwhile.

Only the slow MHC isoforms of the PL increased significantly after endurance exercise. Fiber diameter and area were negatively correlated with oxidative capacity, with small muscle fibers possessing higher oxidase activity and mitochondrial density than large muscle fibers, regardless

of fiber type (Umek et al., 2019; van Wessel et al., 2010). Based on our data, the mean CSA of the PL was smaller than that of the QD and TA, potentially explaining the changes in the MHC content of the PL after endurance exercise. This is because more oxidase activity is activated in the PL after endurance exercise than in the QD and TA, thus increasing oxidative fibers and slow MHC isoforms.

5. Limitations

In the histological experiments, skeletal muscle was not examined at high resolution using transmission electron microscopy (TEM). TEM allows for the examination of ultrasmall changes in skeletal muscle fibers and organelles in frailty syndrome mice. The present study investigated the histological characteristics of frailty syndrome mice. In the future, the specificity of protein and gene expression in mice with frailty syndrome should be further investigated.

6. Ethical approval and consent to participate

All the procedures followed in this experiment were approved by the Institutional Animal Care and Use Committee (IACUC) of Hanyang University (HYU 2017-0265A; 2019-0017A).

7. Consent for publication

Not applicable.

8. Availability of data and materials

The data used to support the findings of this study are presented here. Any further data requirements are available from the corresponding author upon request.

9. Competing interests

No conflicts of interest, financial or otherwise, have been declared by the author(s).

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11. Author's contributions

F.J.J. and J.H.K. conceived and designed the study; F.J.J., H.S.L., and H.s.L. performed the experiments; F.J.J. and H.s.L. analyzed the data; F.J.J. and J.H.K. interpreted the experimental results; F.J.J. prepared the figures; F.J.J., J.H.K. drafted the manuscript; F.J.J., H.S.L., and J.H.K. edited and revised the manuscript; J.H.K. mobilized funds; F.J.J., H.S.L., H.s.L., and J.H.K. approved the final version of the manuscript.

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