ORIGINAL ARTICLE

Nr 2025;15 (2):211-221

Effects of Joint Immobilization and Treadmill Exercise on Muscle Atrophy after Anterior Cruciate Ligament Reconstruction in Rats

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DOI:
10.32098/mltj.02.2025.11

LEVEL OF EVIDENCE: N/A (Animal study)

SUMMARY

Objective. Muscle atrophy is frequently observed after anterior cruciate ligament (ACL) reconstruction. The effects of different rehabilitation protocols after ACL reconstruction on muscle atrophy are not well studied. We aimed to examine the effects of joint immobilization and treadmill exercise after ACL reconstruction on muscle atrophy.

Methods. After ACL reconstruction, rats were divided into no intervention, joint immobilization, or treadmill exercise (60 min/day, six days/week) groups. Untreated rats were used as controls. At two- or four-weeks post-surgery, muscle atrophy in the rectus femoris and semitendinosus as well as ankle plantar flexors was assessed.

Results. ACL reconstruction induced muscle atrophy at two weeks in the rectus femoris, semitendinosus, and plantar flexors. Muscle atrophy in the rectus femoris and plantar flexors spontaneously recovered to levels not significantly different from those in the control group at four weeks, while that in the semitendinosus persisted at four weeks. Four weeks of joint immobilization after ACL reconstruction did not affect the muscle atrophy progression until two weeks, but it hindered the recovery of muscle atrophy in the rectus femoris and plantar flexors during the remaining two weeks. Treadmill exercise did not affect muscle atrophy in all examined muscles.

Conclusions. Our results suggest that immobilization after ACL reconstruction should be limited to two weeks to avoid inhibition of recovery from muscle atrophy. Exercise may not be effective to alleviate muscle atrophy early after ACL reconstruction. However, since the exercise protocol differs from that used in humans, caution is needed when interpreting the results.

KEY WORDS
ACL reconstruction; joint immobilization; exercise; rehabilitation; muscle atrophy.

INTRODUCTION

Anterior cruciate ligament (ACL) injury is one of the most common sports-related knee injuries. Because ACL injury causes joint instability, re-stabilization by ligament reconstruction is the most common treatment. After ACL reconstruction, a decrease in muscle strength is frequently observed in knee extensors and flexors, as well as ankle plantar flexors (1). These decreases in muscle strength not only hinder return to sports (2) but also increase the risk of future osteoarthritis (3, 4). Muscle atrophy is a major cause of a decrease in muscle strength after ACL reconstruction, along with arthrogenic muscle inhibition (reflex inhibition of the muscle after joint injury and/or surgery) (4-6). Therefore, prevention and improvement of muscle atrophy are important issues during rehabilitation after ACL reconstruction.

Rehabilitation protocols after ACL reconstruction are roughly divided into traditional and accelerated rehabilitation (7). In the former, joint immobilization, as well as unweighting, is applied during the early post-surgery period to protect the reconstructed ligament, followed by gradual joint motion and weight bearing. When additional meniscus repair and medial collateral ligament therapy are

combined with ACL reconstruction, restriction of joint motion is unavoidable; time to full range of motion is approximately 5 weeks (8). In the latter, joint motion and weight bearing are started immediately after surgery to avoid the adverse effects of disuse. Regarding the effects of exercise on muscle atrophy, an animal study reported that treadmill exercise after ACL transection attenuated vastus medialis muscle fiber atrophy in rats (9). In humans, treadmill exercise with partial body-weight support after ACL reconstruction increased thigh circumference (an index of muscle mass in the thigh) compared with conventional physical therapy (10). Although these previous studies suggested that treadmill exercise after ACL injury and reconstruction improves muscle atrophy, it is known that muscle atrophy after ACL injury and/or reconstruction often continues even when actively engaging in muscle-strengthening exercises (11). The effect of exercise on muscle atrophy after ACL reconstruction is thus controversial.

It is believed that joint immobilization after ACL injury and reconstruction contributes to the development of muscle atrophy (6). In fact, previous studies showed that patients treated with ACL reconstruction followed by joint immobilization displayed muscle atrophy characterized by a decrease in thigh circumference and atrophy of muscle fibers in the vastus lateralis (12, 13). Unfortunately, these previous studies did not compare differences with and without immobilization; thus, it is unclear whether muscle atrophy was induced either by ACL reconstruction, joint immobilization, or a combination of these. Therefore, the effects of joint immobilization after ACL reconstruction on muscle atrophy are not well known.

In this study, we aimed to examine the effects of joint immobilization and treadmill exercise after ACL reconstruction on muscle atrophy using a rat model. We hypothesized that joint immobilization after ACL reconstruction facilitates muscle atrophy, while treadmill exercise reverses it.

MATERIALS AND METHODS

Experimental animals

Fifty male Wistar rats (eight-week-old, Japan SLC, Shizuoka, Japan) were randomly divided into the following four groups: control (n = 8), ACL reconstruction (ACLR; n = 14), ACL reconstruction plus immobilization (ACLR + IM; n = 15), and ACL reconstruction plus treadmill exercise (ACLR + EX; n = 13) (**figure 1**). These groups were further divided into two- and four-week groups (**figure 1**). In the control group, the right and left hindlimbs were considered as individual samples, and thus eight hindlimbs from four rats were used for analyses at each time point. Rats were housed in standard

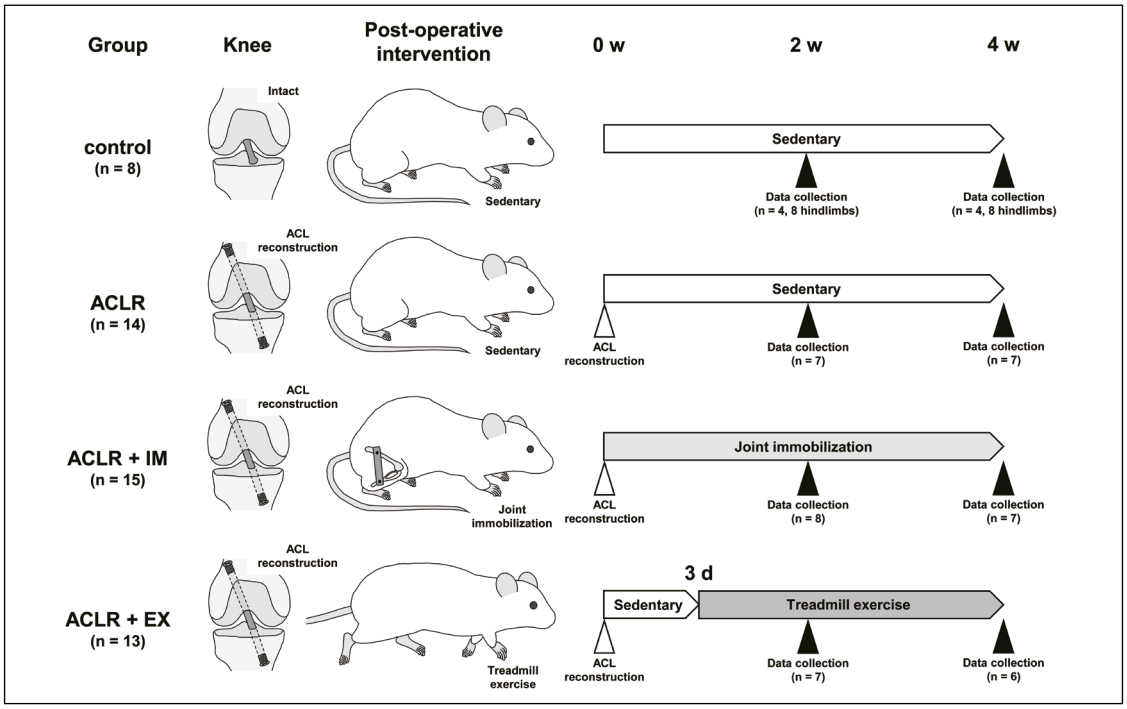


Figure 1. Experimental protocol.

cages in a temperature-controlled room (20-25 °C) with a 12 h light/dark cycle. Standard rodent food and water were provided *ad libitum*. This experimental design was approved by the animal experimentation committee of Hiroshima International University (AE20-022, date of approval: March 19, 2020).

ACL reconstruction surgery

ACL reconstruction surgery was performed on the right knees of rats, except those in the control group, using methods described previously (14, 15). Rats were anesthetized by an intraperitoneal injection of ketamine and xylazine (80 and 10 mg/kg, respectively), and the tail tendons were collected to prepare a quadruple-bundle autograft. The knee was opened by the medial parapatellar approach, and the ACL was transected. Bone tunnels from the antero-medial side of the proximal tibia to the lateral side of the distal femur were drilled using a 0.8 mm diameter Kirschner wire, and the autograft was passed to the bone tunnels. After the proximal end of the autograft was fixed to the femur using stainless steel interference screw (0.8 mm diameter and 2.0 mm length, TE-00001; Matsumoto, Chiba, Japan), the autograft was tensioned, and the distal end was fixed to the tibia using stainless steel interference screw. Finally, the knee was closed. Previous studies found that this ACL reconstruction method could partially reduce joint instability (14, 15).

Joint immobilization

Joint immobilization was performed for rats in the ACLR + IM group using methods described previously (16, 17). Immediately after ACL reconstruction surgery, 0.8 mm diameter Kirschner wires were screwed into the proximal femur and distal tibia, and were fixed with wire and resin (PROVINICE FAST; Shofu, Kyoto, Japan) at a knee flexion angle of 140°. During joint immobilization, rats were able to put weight on the immobilized hindlimb and could walk freely using all four limbs.

Treadmill exercise

Rats in the ACLR + EX group performed treadmill exercise using methods described previously (18-21). Rats walked on a rodent treadmill machine (Rat runner; Agawa, Shimane, Japan) at 12 m/min. The intervention consisted of six sessions of 10-min walks separated by 1 min intervals, so rats walked a total of 60 min/day. Exercise was started three days post-surgery and was performed six days a week.

Assessment of muscle atrophy

At the end of the experimental period, the body weight of the anesthetized rats was measured, and the rats were sacrificed by exsanguination. The ankle plantar flexors (*i.e.*, gastrocnemius, soleus, and plantaris) were collected, and the wet weights of these muscles were measured using an electronic balance (GX-200; A&D, Tokyo, Japan). Relative muscle wet weight (*i.e.*, muscle wet weight/body weight) was calculated to be correct for the differences in body size. The rectus femoris and semitendinosus were sampled and were rapidly frozen in isopentane cooled by liquid nitrogen. Using a cryostat, 10-μm-thick transverse sections were cut from the mid-belly portion. Sections were stained with hematoxylin and eosin, and the central region of each muscle was photographed at 10× magnification. The cross-sectional area (CSA) of randomly selected 100 muscle fibers were manually measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) in a blinded fashion.

Gene expression analysis

In this study, the degree of muscle atrophy in the rectus femoris was affected by post-operative intervention (please see the Results section below), and therefore, gene expression analysis was performed using the rectus femoris. Part of the rectus femoris was immersed in RNAlater reagent (Thermo Fisher Scientific, Waltham, MA, USA) and was stored at -80 °C until RNA extraction. Using TRIzol reagent (Thermo Fisher Scientific) and the RNeasy mini kit (Qiagen, Hilden, Germany), total RNA was extracted. Then, cDNA was synthesized using the total RNA and the SuperScript III First-strand synthesis system (Thermo Fisher Scientific). Quantification of gene expression levels of *insulin-like growth factor-1* (*IGF-1*), *muscle RING finger-1* (*MuRF-1*), and *atrogin-1* was performed by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). TaqMan primer and probe sets for *IGF-1* (Rn00710306_m1), *MuRF-1* (Rn00590197_m1), *atrogin-1* (Rn00591730_m1), and *S18* (Rn01428913_gH) were obtained from Applied Biosystems. *S18* rRNA was used as an internal control. Gene expression levels were quantified using the comparative cycle threshold method.

Statistical analysis

Statistical analyses were performed using Dr. SPSS II for Windows (SPSS Japan, Tokyo, Japan). Two-way analyses of variance (ANOVAs) were used to detect significant main effects and interactions. If significant main effects, but not interactions, were detected, Bonferroni tests were performed to compare among levels. If significant interactions were detected, Bonferroni tests were performed to detect simple main effects. A P-value of less than 0.05 was considered statistically significant.

RESULTS

One rat in the ACLR + IM group that was to be sacrificed at two weeks post-surgery died due to anesthesia during surgery, and was therefore excluded from the analyses.

Body weight

A significant interaction between time and intervention ($p < 0.001$) and main effects of both time ($p < 0.001$) and intervention ($p < 0.001$) were detected. At two weeks post-surgery, body weight was significantly lower in all ACL reconstructed groups than in the control group ($p \leq 0.003$, **figure 2**). Among ACL reconstructed groups, there were no significant differences in body weight at two weeks post-surgery ($p \geq 0.448$). At four weeks post-surgery, body weight in all groups significantly increased compared with two weeks post-surgery ($p \leq 0.002$). Body weight in the ACLR and ACLR + EX groups was comparable to that in the control group ($p = 1.000$), while that in ACLR + IM group was still significantly lower than that of the control group ($p < 0.001$). Among ACL reconstructed groups, body weight in the ACLR + IM group was significantly lower than those in the ACLR and ACLR + EX groups ($p < 0.001$).

Muscle wet weight

For the wet weight of the gastrocnemius muscle, a significant interaction between time and intervention ($p < 0.001$) and main effects of both time ($p < 0.001$) and intervention ($p < 0.001$) were detected. At two weeks post-surgery, gastrocnemius muscle wet weight was significantly lower in all ACL reconstructed groups compared to the control group ($p < 0.001$, **figure 3A**). Among ACL reconstructed groups, there were no significant differences in gastrocnemius muscle wet weight at two weeks post-surgery ($p \geq 0.169$). At four weeks post-surgery, gastrocnemius muscle wet weight significantly increased in the control, ACLR, and ACLR + EX groups ($p \leq 0.016$), but not in the ACLR + IM group ($p = 0.427$), compared to two weeks post-surgery. Compared with the control group, gastrocnemius muscle wet weight in the ACLR + EX group was not significantly different ($p = 0.560$), while those in the ACLR and ACLR + IM groups were still significantly lower ($p \leq 0.043$). Among ACL reconstructed groups, gastrocnemius muscle wet weight in the ACLR + IM group was significantly lower than those in the ACLR and ACLR + EX groups. Similar results were obtained when muscle wet weight was corrected by body weight (*i.e.*, relative wet weight) (**figure 3B**). However, the significant difference between the control and ACLR groups at four weeks post-surgery was not observed for relative wet weight ($p = 0.080$). Relative wet weight in the gastrocnemius muscle in the ACLR + IM group was significantly lower than that of all other groups at four weeks post-surgery ($p < 0.001$).

For soleus muscle wet weight, a significant interaction between time and intervention ($p < 0.001$) and main effects of both time ($p < 0.001$) and intervention ($p < 0.001$) were detected. At two weeks post-surgery, soleus muscle wet weight in all ACL reconstructed groups was significantly lower than that in the control group ($p < 0.001$, **figure 3C**). Among ACL reconstructed groups, there were no significant differences in soleus muscle wet weight at two weeks post-surgery ($p = 1.000$). At four weeks post-surgery, soleus muscle wet weight significantly increased in the ACLR and ACLR + EX groups ($p \leq 0.001$), but not in the control and ACLR + IM groups ($p \geq 0.531$), compared to two weeks post-surgery. Soleus muscle wet weight in the ACLR and ACLR + EX groups recovered to levels comparable to that in the control group ($p \geq 0.811$), but was still significantly lower in the ACLR + IM group compared to the control group at four weeks post-surgery ($p < 0.001$). Among ACL reconstructed groups, soleus muscle wet weight was significantly lower in the ACLR + IM group than in the ACLR and ACLR + EX groups ($p \leq 0.001$). Similar results were obtained for relative wet weight (**figure 3D**). However, the significant difference between the ACLR and ACLR + IM groups at four weeks post-surgery was not observed for relative wet weight ($p = 0.125$).

For plantaris muscle wet weight, a significant interaction between time and intervention ($p < 0.001$) and main effects

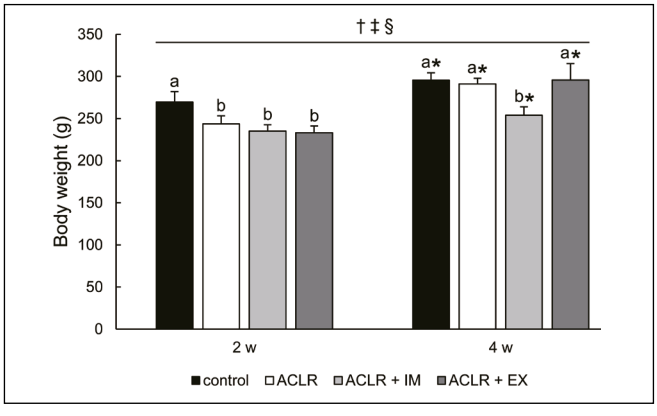


Figure 2. Body weight.

Values are means ± standard deviations; †significant interactions between time and intervention; ‡significant main effect of time; §significant main effect of intervention; *significant difference compared to two weeks post-surgery. Different letters indicate statistically significant differences between groups at the same timepoints, such that groups not sharing the same letter are significantly different from one another.

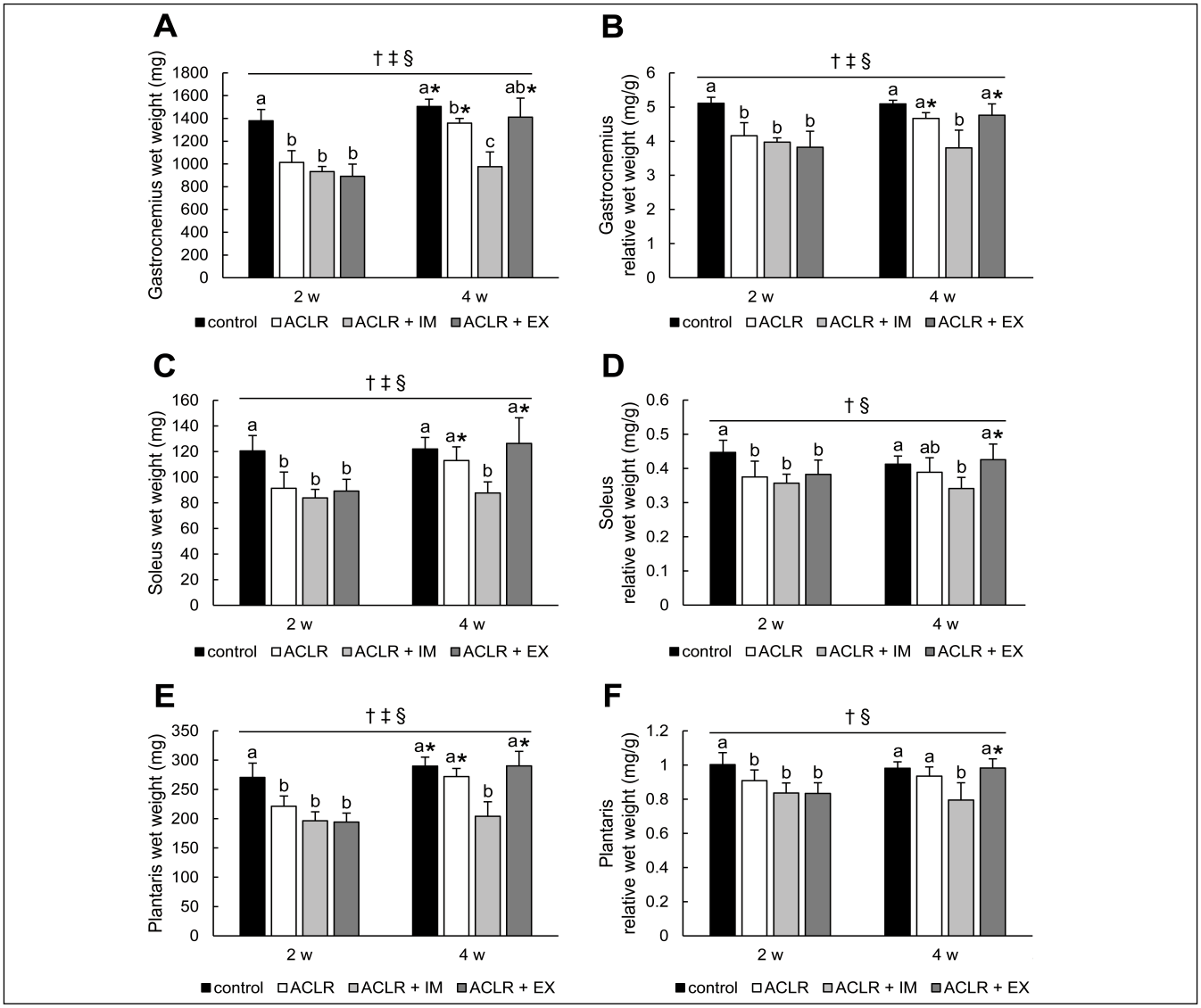


Figure 3. Muscle wet weight of the plantar flexors.

(**A**) Gastrocnemius wet weight, (**B**) gastrocnemius relative wet weight, (**C**) soleus wet weight, (**D**) soleus relative wet weight, **(E)** plantaris wet weight, and (**F**) plantaris relative wet weight. Values are means ± standard deviations; †significant interactions between time and intervention; ‡significant main effect of time; §significant main effect of intervention; *significant difference compared to two weeks post-surgery. Different letters indicate statistically significant differences between groups at the same timepoints, such that groups not sharing the same letter are significantly different from one another.

of both time ($p < 0.001$) and intervention ($p < 0.001$) were detected. At two weeks post-surgery, plantaris muscle wet weight was significantly lower in all ACL reconstructed groups than in the control group ($p < 0.001$, **figure 3E**). Among ACL reconstructed groups, there were no significant differences in plantaris muscle wet weight at two weeks post-surgery ($p \geq 0.070$). At four weeks post-surgery, plantaris muscle wet weight in the control, ACLR, and ACLR + EX groups ($p \leq 0.049$), but not in the ACLR + IM group ($p = 0.466$), significantly increased compared to two weeks post-surgery. Compared with the control group, plantaris muscle wet weight in the ACLR and ACLR + EX groups were not significantly different ($p \geq 0.466$), but was significantly lower in the ACLR + IM group ($p < 0.001$). Among ACL reconstructed groups, plantaris muscle wet weight in the ACLR + IM group was significantly lower than that of the ACLR and ACLR + EX groups ($p < 0.001$). Similar results were also obtained for relative wet weight (**figure 3F**).

Muscle fiber CSA

Small muscle fibers (~2,000 mm²) increased, while large muscle fibers (~5,000 mm²) decreased, in the rectus femoris of all ACL reconstructed groups compared with the control group at two weeks post-surgery (**figure 4A-D**, **I**). At four weeks post-surgery, the frequency distribution of muscle fiber CSA in the ACLR and ACLR + EX groups were similar to that in the control group, while in the ACLR + IM group, the CSA of large muscle fibers decreased (**figure 4E-H**, **J**). Figure 4K shows the average muscle fiber CSA in the rectus femoris. A significant interaction between time and intervention ($p < 0.001$) and main effects of both time ($p < 0.001$) and intervention ($p < 0.001$) were detected. At two weeks post-surgery, muscle fiber CSA in the rectus femoris was significantly lower in all ACL reconstructed groups than in the control group ($p \leq 0.001$). Among ACL reconstructed groups, there were no significant differences in muscle fiber CSA in the rectus femoris ($p \geq 0.165$). At four weeks post-surgery, rectus femoris muscle fiber CSA significantly increased in the ACLR and ACLR + EX groups ($p < 0.001$), but not in the control and ACLR + IM group ($p \geq 0.289$), compared to two weeks post-surgery. Compared with the control group, rectus femoris muscle fiber CSA in the ACLR and ACLR + EX groups were not significantly different ($p \geq 0.601$), but was significantly lower in the ACLR + IM group ($p < 0.001$). Among the ACL reconstructed groups, rectus femoris muscle fiber CSA in the ACLR + IM group was significantly lower than that of the ACLR group ($p = 0.049$).

In the semitendinosus, the frequency distribution of muscle fiber CSA in all ACLR reconstructed groups showed increased small muscle fibers (~2,000 mm²) and decreased large muscle fibers (~3,000 mm²) at both time points (**figure 5A-J**). **Figure 5K** shows the average muscle fiber CSA in the semitendinosus. A significant main effect of intervention ($p < 0.001$) was detected. Compared with the control group, semitendinosus muscle fiber CSA in all ACLR reconstructed groups was significantly lower ($p < 0.001$). Semitendinosus muscle fiber CSA was similar among ACL reconstructed groups ($p = 1.000$). The interaction between time and intervention ($p = 0.825$) and the main effect of time ($p = 0.099$) were not significant.

Gene expression levels in the rectus femoris

For the gene expression of *IGF-1*, a significant interaction

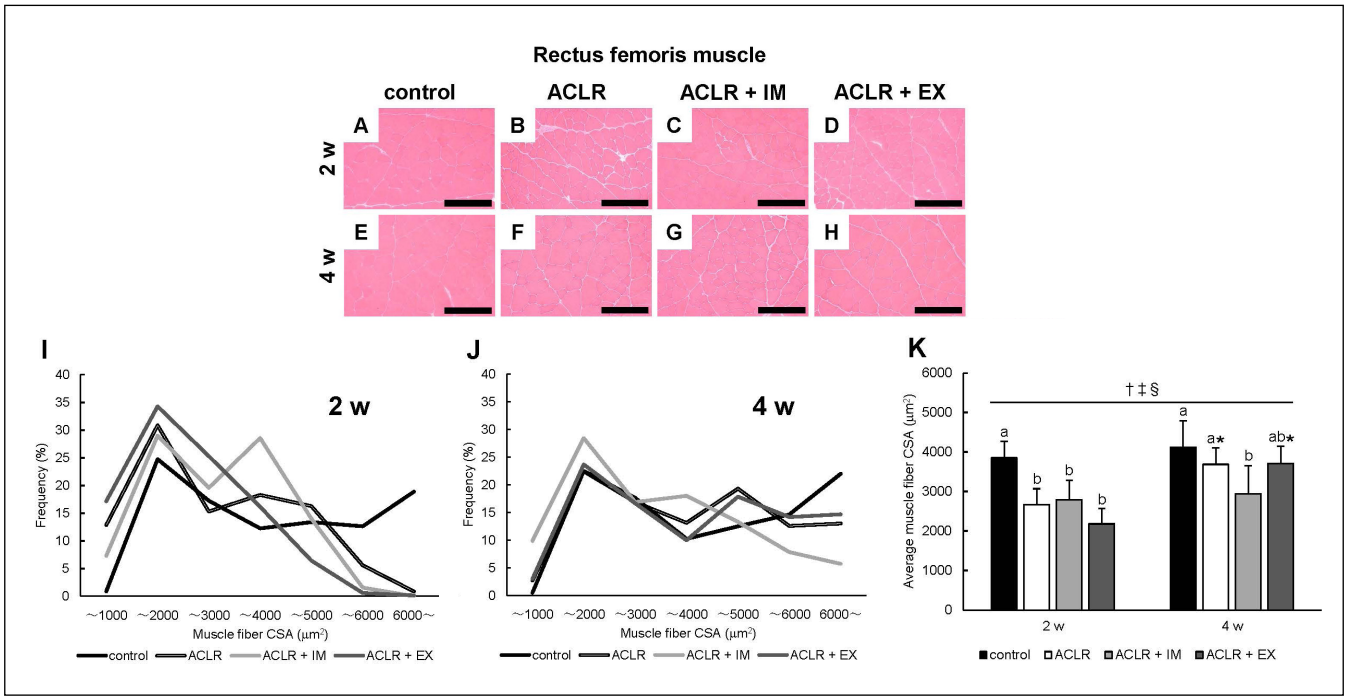


Figure 4. Rectus femoris muscle fiber cross-sectional area.

Representative images of the hematoxylin and eosin-stained rectus femoris in the control (**A, E**), ACLR (**B, F**), ACLR + IM (**C, G**), and ACLR + EX (**D, H**) groups. (**A-D, E-H**) Sections at two- and four-weeks post-surgery, respectively. Scale bars = 200 μm. (**I, J**) Distributions of muscle fibers according to the cross-sectional area at two- and four-weeks post-surgery, respectively. (**K**) Average muscle fiber cross-sectional area. Values are means ± standard deviations; †significant interactions between time and intervention; ‡significant main effect of time; §significant main effect of intervention; *significant difference compared to two weeks post-surgery. Different letters indicate statistically significant differences between groups at the same timepoints, such that groups not sharing the same letter are significantly different from one another.

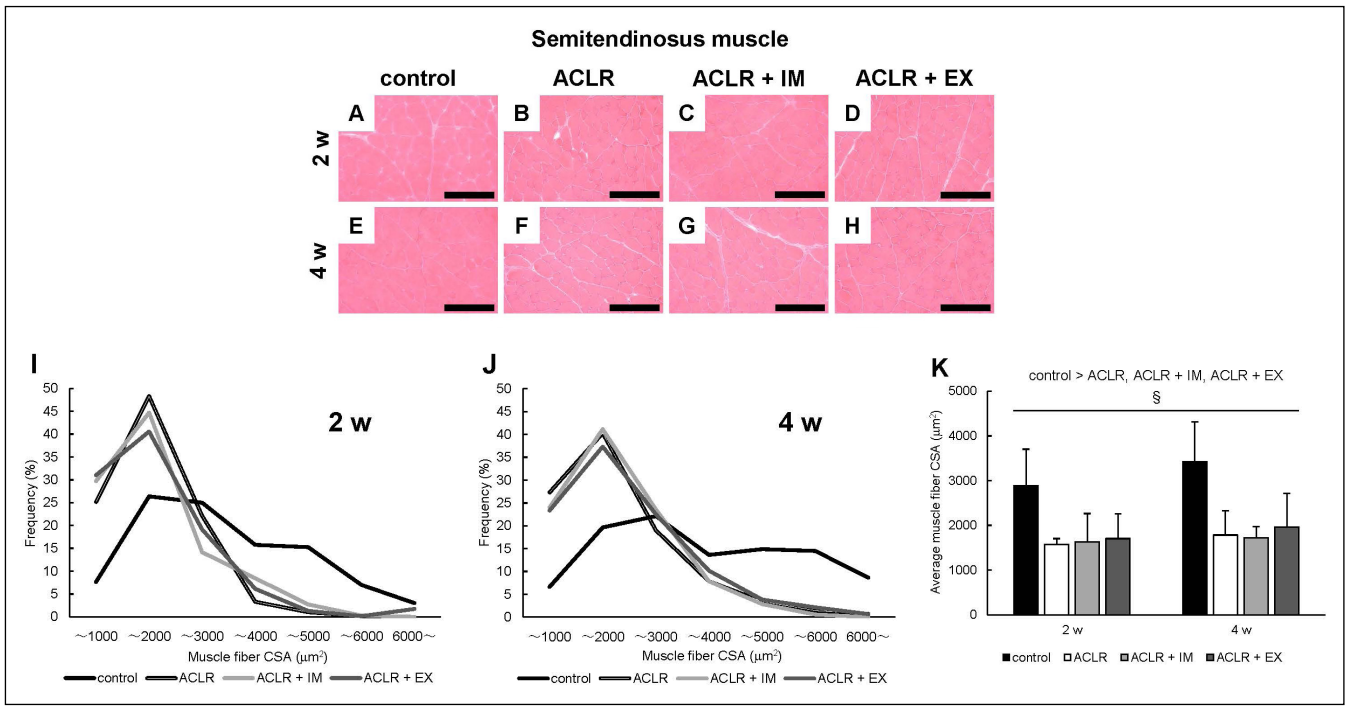


Figure 5. Semitendinosus muscle fiber cross-sectional area.
Representative images of the hematoxylin and eosin-stained semitendinosus rectus femoris in the control (**A, E**), ACLR (**B, F**), ACLR + IM (**C, G**), and ACLR + EX (**D, H**) groups. (**A-D, E-H**) Sections at two- and four-weeks post-surgery, respectively. Scale bars = 200 μm. (**I, J**) Distributions of muscle fibers according to the cross-sectional area at two- and four-weeks post-surgery, respectively. (**K**) Average muscle fiber cross-sectional area. Values are means ± standard deviations; §significant main effect of intervention. groups not sharing the same letter are significantly different from one another.

between time and intervention ($p < 0.001$) and a main effect of intervention ($p < 0.001$) were detected. At two weeks post-surgery, the gene expression of *IGF-1* was significantly lower in the ACLR + IM groups than in the control group ($p = 0.004$, **figure 6A**). Among ACL reconstructed groups, there were no significant differences in the gene expression of *IGF-1* ($p \geq 0.423$). At four weeks post-surgery, the gene expression of *IGF-1* significantly increased in the ACLR group ($p < 0.001$) and significantly decreased in the control and ACLR + IM groups ($p \leq 0.045$) compared to two weeks post-surgery. Compared with the control group, the gene expression of *IGF-1* was significantly higher in the ACLR group ($p < 0.001$) and was significantly lower in the ACLR + IM group ($p = 0.011$). Among ACL reconstructed groups, the gene expression of *IGF-1* was significantly lower in the ACLR + IM and ACLR + EX groups than in the ACLR group ($p < 0.001$). The main effect of time was not significant ($p = 0.746$).

For the gene expression of *MuRF-1*, a main effect of intervention was detected ($p < 0.001$). Compared with the control group, the gene expression of *MuRF-1* was significantly higher in the ACLR and ACLR + IM groups ($p \leq 0.009$, **figure 6B**). Among ACL reconstructed groups, the gene expression of *MuRF-1* was significantly higher in the ACLR + IM group than in the ACLR and ACLR + EX groups ($p \leq 0.026$). The interaction between time and intervention ($p = 0.062$) and the main effect of time ($p = 0.940$) were not significant.

For the gene expression of *atrogin-1*, a significant interaction between time and intervention ($p < 0.001$) and main effects of both time ($p = 0.004$) and intervention ($p = 0.003$) were detected. At two weeks post-surgery, the gene expression of *atrogin-1* was significantly higher in the ACLR + IM groups than in the ACLR group ($p = 0.017$, **figure 6C**). At four weeks post-surgery, the gene expression of *atrogin-1* significantly increased in the control and ACLR groups ($p \leq 0.020$) and significantly decreased in the ACLR + EX group ($p = 0.023$) compared to two weeks post-surgery. Compared with the control group, the gene expression of a*trogin-1* was significantly higher in the ACLR group ($p < 0.001$) and was significantly lower in the ACLR + EX group ($p = 0.030$). Among ACL reconstructed groups, the gene expression of *atrogin-1* was significantly lower in the ACLR + IM and ACLR + EX groups than in the ACLR group ($p < 0.001$).

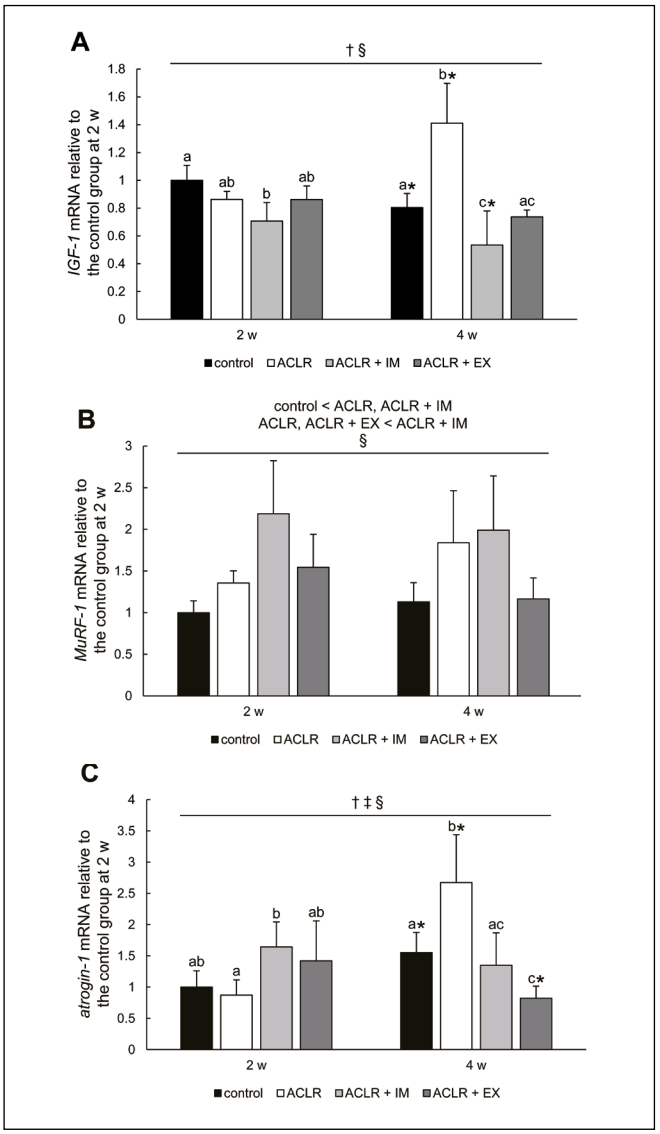


Figure 6. Gene expression in the rectus femoris: (**A**) *IGF-1*, (**B**) *MuRF-1*, and (**C**) *Atrogin-1*.
Values are means ± standard deviations; †significant interactions between time and intervention; ‡significant main effect of time; §significant main effect of intervention; *significant difference compared to two weeks post-surgery. Different letters indicate statistically significant differences between groups at the same timepoints, such that groups not sharing the same letter are significantly different from one another.

DISCUSSION

In this study, we aimed to examine the effects of joint immobilization and treadmill exercise after ACL reconstruction on muscle atrophy. While two weeks of joint immobilization after surgery did not affect muscle atrophy, an additional two weeks of immobilization hindered the recovery of muscle atrophy in the rectus femoris and plantar flexors. Additional treadmill exercise did not improve muscle atrophy after ACL reconstruction.

In the ACLR group, muscle atrophy was detected in the rectus femoris, semitendinosus, and plantar flexors at two weeks post-surgery. Atrophy in the rectus femoris and plantar flexors spontaneously recovered to levels that were not significantly different from that in the control group by four weeks, while atrophy in the semitendinosus did not recover by four weeks. Muscle mass is regulated by the balance between protein synthesis and breakdown. In the rectus femoris, a significant main effect of intervention was detected in the gene expression of *MuRF-1*, which is involved in protein breakdown, and expression level was significantly upregulated in the ACLR group compared to the control group. In contrast, gene expression of *IGF-1*, which is involved in protein synthesis, showed no significant difference between the control and ACLR groups at two weeks post-surgery. These results suggest that atrophy in the rectus femoris observed in the ACLR group at two weeks post-surgery was mainly due to increased protein breakdown. At four weeks post-surgery, despite atrophy-related genes (*i.e.*, *MuRF-1* and *atrogin-1*) were upregulated, gene expression of *IGF-1* was also upregulated in the ACLR group compared to the control group. The resulting increase in protein synthesis might contribute to the recovery from muscle atrophy.

Joint immobilization after ACL reconstruction did not affect muscle atrophy until two weeks, as muscle fiber CSAs in the rectus femoris and semitendinosus and the wet weights of the plantar flexors were not different between the ACLR and ACLR + IM groups at two weeks post-surgery. At four weeks post-surgery, however, muscle fiber CSA in the rectus femoris and the wet weights of the plantar flexors in the ACLR + IM group were significantly lower than that in the ACLR group. These results suggest that immobilization hinders the recovery of muscle atrophy in the rectus femoris and planter flexors between two and four weeks, and immobilization after ACL reconstruction should be limited to two weeks to avoid inhibition of recovery from muscle atrophy.

In the ACLR + IM group, gene expression of *MuRF-1* in the rectus femoris was significantly upregulated compared to the ACLR group, although gene expression of *atrogin-1* was significantly lower than that in the ACLR group at four weeks post-surgery. In addition, gene expression of *IGF-1* was significantly lower in the ACLR + IM group than in the ACLR group at four weeks post-surgery. As a result of these changes, the net balance between protein synthesis and breakdown in the ACLR + IM group might be tilted

towards breakdown compared to the ACLR group, impeding recovery from muscle atrophy. In contrast to the rectus femoris and plantar flexors, atrophy of the semitendinosus was not affected by immobilization. Our results suggest that the muscle mass of the hamstrings is regulated by different mechanisms than the quadriceps and plantar flexors, and different treatment strategies may be needed depending on the targeted muscle.

Treadmill exercise did not affect the muscle atrophy, as all parameters of muscle atrophy were not different between ACLR and ACLR + EX groups at both two and four weeks post-surgery. In support of our results, a recent review by Lepley *et al*. stated that extensive strengthening exercises are not effective for muscle atrophy after ACL injury and reconstruction (11). Because the degrees of muscle atrophy in all examined muscles at two weeks post-surgery were not different among the ACLR, ACLR + IM, and ACLR + EX groups, factors other than activity level will determine the degree of muscle atrophy up to two weeks post-surgery. A previous study reported that the administration of the non-steroidal anti-inflammatory drug celecoxib partially prevented atrophy of the rectus femoris at one week after ACL reconstruction in rats (22). Thus, to reduce muscle atrophy early after ACL reconstruction, emphasis may be placed on other treatments, such as anti-inflammatory treatments, rather than aggressive exercise.

This study has some limitations. First, most ACL reconstruction surgeries are performed arthroscopically in human patients (23), but we selected open surgery due to technical reasons. Previous studies reported that arthrotomy (opening the joint) can induce muscle atrophy in the rectus femoris and semitendinosus (22, 24). Thus, muscle atrophy detected in this study might be overestimated. Second, the immobilization position was different from that used in the clinical setting. Rat knees were immobilized at a flexion angle of 140°, while human knees are immobilized at a flexion angle of 0 to 60° if immobilization is applied after ACL reconstruction (7). This difference in immobilization position might affect muscle atrophy, because it is generally considered that muscle atrophy is more prominent when the muscle is immobilized at a shortened position than at a stretched position (25, 26). However, a previous study indicated that muscle mass in the thigh and knee extensor and flexor strength after ACL reconstruction were not different between the patients immobilized in the knee flexion position *vs* extension position (27). Third, we did not examine the effects of remobilization on muscle atrophy. Whether the effects of immobilization on muscle atrophy persist after remobilization is an open question. Fourth, the effects of graft harvesting from the patella or hamstring tendons could not be considered, because we used tail tendon autografts. Graft harvesting from the patellar and hamstring tendons is known to adversely affect muscle mass and strength in the quadriceps and hamstrings, respectively (28-30). Fifth, muscle wet weight was used as an index for muscle atrophy in the plantar flexors. Muscle wet weight may be affected not only by muscle fiber size, but also by other factors such as the number of muscle fiber and water and connective tissue content. However, a previous study reported a strong positive correlation between muscle wet weight and muscle fiber CSA in rat skeletal muscle (24). Thus, we consider that muscle wet weight was an appropriate index for muscle atrophy. Sixth, we used a small animal model, and there may be some differences from human patients. It is known that recovery of muscle strength after ACL reconstruction is faster in the hamstrings than in the quadriceps (31). In our study, however, atrophy in the rectus femoris spontaneously recovered by four weeks, while atrophy in the semitendinosus did not recover by four weeks. Additionally, in human patients, rehabilitation typically begins with isometric strengthening exercises to improve muscle strength (32-34), whereas we employed treadmill exercise in this study. This difference in exercise protocols may have influenced the results.

CONCLUSIONS

Four weeks of joint immobilization after ACL reconstruction did not affect the progression of muscle atrophy until two weeks, but it hindered the recovery of muscle atrophy in the rectus femoris and plantar flexors during the remaining two weeks. Thus, immobilization after ACL reconstruction should be limited to two weeks for the recovery of muscle atrophy. Additional treadmill exercise after ACL reconstruction did not improve muscle atrophy. To reduce muscle atrophy early after ACL reconstruction, emphasis may be placed on treatments other than aggressive exercise.

FUNDINGS

This study was supported by JSPS KAKENHI (Grant Number 20K19400) and the Nakatomi Foundation.

DATA AVAILABILITY

Data are available under reasonable request to the corresponding author.

CONTRIBUTIONS

AK: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, writing - original draft, writing - review & editing. KY: writing - original draft, writing - review & editing. JO: conceptualization, investigation, methodology, project administration, writing - original draft, writing - review & editing.

ACKNOWLEDGEMENTS

We acknowledge technical assistance from Jukiya Iwazawa, Ikuma Ueura, Shun Ezumi, Takumi Funakoshi, Takuma Maeno, and Yu Muraoka.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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