Title

The braking force to Control the Abnormal Tibial Translation Affects the Spontaneous Healing Response for Complete Ruptured Anterior Cruciate Ligament in Mice Model

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All authors approved the final version to be published. Study design: RS and TK; data collection and statistical analysis: RS, KN, YU, SE, KN; manuscript composition RS, and TK.

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Running Title

Braking force affects ACL healing

Abstract

The anterior cruciate ligament (ACL) has been considered to not heal spontaneously after complete injury due to its unique intra-articular environment. However, several clinical and animal studies have reported that ruptured ACL could heal spontaneously by controlling abnormal joint movement. On the other hand, heled ACL has lower collagen maturity and mechanical properties than intact ACL. Appropriate mechanical stress on the ligament could promote spontaneous healing, whereas excessive mechanical stress probably inhibits the healing response. We investigated the effect of braking force to control anterior tibial translation (ATT) on the healing response of the ACL using two different controlled anterior tibial translation (CATT) models. Male and female C57BL/6 mice were ruptured ACL and randomly classified to CATT with 3-0 nylon suture (3-0 CATT) group, CATT with 4-0 nylon suture (4-0 CATT) group, ACL-rupture (ACL-R) group. We evaluated magnitude of ATT, success rate of the ACL healing, collagen maturity and mechanical property immediately after injury, at 4- and 8-weeks post-injury. 3-0 CATT group had better braking force of ATT than 4-0 CATT group immediately after injury and showed higher success rate than 4-0 CATT group females at 4 and 8 weeks. In contrast, collagen maturation and mechanical strength of the healed ACL showed no difference between 3-0 CATT and 4-0 CATT groups. Our

results suggested that firm ATT braking immediately after injury is necessary for successful healing of the injured ACL. On the other hand, increased ATT in the early phase was insufficient to improve mechanical strength and collagen maturation, and future studies is needed to elucidate the optimal timing of mechanical stress.

Study Design: A controlled laboratory study.

Key terms:

Anterior cruciate ligament; spontaneous healing; conservative treatment; braking; mechanical stress

Introduction

The anterior cruciate ligament (ACL) is essential to the knee joint stability and movement. The knee joint has unstable bone shape and structures, and the ACL is the only soft tissue that controls the anterior translation of the tibia.[1] Therefore, once ACL injured, the knee loses the proper bone position and kinematics during joint motion in daily activities and playing sports. ACL injuries used to be young and athlete injuries, but recently, the number of middle-aged or older ACL-injured patients has increased.[2] These patients need any type of treatment strategy, not only surgical reconstruction.

ACL in the intra-articular tissue, thus the injured ACL has low self-healing capacity. The environment of the joint capsule is filled with synovial fluid and poor vascular supply,[3] these leads poor fibroblast activity in the healing site of injured ACL.[4] Therefore, ACL reconstruction is recommended and most commonly selected treatment for ACL-injured patients. However, patients with a low activity level sometimes choose conservative treatment without ACL healing. Regarding these treatments, a 2-year follow-up comparing ACL reconstruction and conservative treatment reported no clear differences in knee joint function, covering pain, and difficulty in sports and recreational activities evaluated with the Kenn Injury and Osteoarthritis Outcome Score (KOOS).[5] Thus, optimal treatment after ACL injury is debatable.

Several clinical studies have reported that ruptured ACL could heal spontaneously, as confirmed by MRI.[6–9] However, there is insufficient knowledge of appropriate post-injury management of the ACL and knee joint to lead them to healing. Filbay et al. reported spontaneous ACL healing with the Cross Bracing Protocol (CBP), which constantly fixed the knee at 90 degrees in patients with ACL injuries.[10] They showed 90% of patients had evidence of healing on three-month MRI. In our previous report using the rats model, we elucidated that the ACL could heal spontaneously due to only controlling abnormal anterior tibial translation after ACL injury.[11] Regarding mechanical stress, we should not immobilize the knee joint after ACL injury because appropriate mechanical loading promotes collagen remodeling during the ligament healing process.[12–14] On the other hand, excessive mechanical stress inhibits the healing of tendons and ligaments.[15] Thus, understanding the acceptable range of mechanical stress, especially during the early phase of the ACL spontaneous healing response is important for establishing non-surgical treatment and appropriate management of the injured ACL.

In this study, we aimed to investigate the effect of braking force to control the anterior tibial translation (ATT) in mice on the healing response of the injured ACL. We created two controlled anterior tibial translation (CATT) models with altered anterior

tibial instability by controlling the anterior tibial translation using two different diameters of nylon sutures. Thus, we aimed to elucidate the acceptable mechanical stresses during early phase of the ACL spontaneous healing and to provide insight into the appropriate management after ACL injured patients.

Methods

Experimental Design

The University Animal Experiment Ethics Committee approved all experiments. We used male and female 8-9 weeks old C57BL/6 mice (n=125). A total of 125 mice were ruptured the left ACL and then randomly classified into three groups: 3-0 CATT group; controlled ATT with 3-0 nylon suture (n=53), 4-0 CATT group; controlled ATT with 4-0 nylon suture (n=52), and ACL-R (ACL-Rupture) group; not controlled ATT (n=20). We created the CATT and ACL-R model for the left hindlimb in all mice, with the right hindlimb used as the intact group. Mice were sacrificed immediately after injury (n=20) for anterior drawer testing and histology, at 4 (n=51) and 8 weeks (n=54) for anterior drawer testing, histology, immunofluorescence, and mechanical testing (Figure 1).

Nylon Suture Experiments

Prior to creating the CATT models, we measured the mechanical properties of nylon sutures using a mechanical testing machine, Univert (CellScale Biomaterial Testing, Canada). The purpose of mechanical testing was to understand the difference of mechanical properties between 3-0 and 4-0 nylon sutures (Akiyama Medical MFG, Co., LTD., Tokyo, Japan). Each nylon suture was measured in a double layer, since a nylon suture was looped outside the joint to control ATT in the CATT model. In the failure testing, we stretched the nylon suture at preload of 0.1 N and a rate of 20 mm/min until the nylon sutures ruptured. During the test, the load (N) and displacement (mm) were recorded, and we measured the load to failure (N) and stiffness (N/mm). Then we performed the cyclic test to evaluate viscoelasticity. Nylon sutures were repeated 5 seconds of stretching up to 90%, 80%, 70%, and 60% of the failure load of each nylon sutures, followed by 5 seconds of relaxation (1 cycle: 10 sec). We recorded the number of cycles at the point of rupture.

Procedure of Creating the Model

All mice were anesthetized with a combination of anesthetics (medetomidine, 0.375 mg/kg; midazolam, 2.0 mg/kg; and butorphanol, 2.5 mg/kg) for sedation and pain

control, and then we ruptured the ACL non-invasively (Figure 2A-B) according to a previous study.[16] We fixed the hindlimb with the knee joint at 90 degrees and ruptured the ACL by pushing the femur backward against the tibia (Figure 2A-B). Then, we created bone holes at the femoral condyle and proximal tibia using a 26G needle (Terumo Co., Ltd., Tokyo, Japan). In the 3-0 and 4-0 CATT groups, we tightly looped a single 3-0 or 4-0 nylon suture through the two holes to control the ATT (Figure 2E). In the ACL-R group, we loosely looped a nylon suture through the two holes not to control the ATT.

Anterior Drawer Testing

We performed anterior drawer testing to evaluate the braking force to control ATT of the knee. We harvested knee joint samples immediately after the mice were sacrificed. We removed the muscle from the knee while retaining the suture used for controlling ATT. Then, we fixed the knee joint in 90-degree on the self-made anterior drawer testing device (Figure 4A). The tibia was pulled forward with a 0.05 kgf constant force spring (Sanko Spring Co., Ltd., Fukuoka, Japan), and X-ray images of the sagittal plane of the knee joint were taken. We measured ATT from the X-ray images using ImageJ (ImageJ; National Institutes of Health, Bethesda, MD, USA) to

evaluate the braking force by nylon sutures. The ATT was defined as the perpendicular line from the most prominent point of the femoral condyle to the long axis of the tibia (Figure 4B). We then removed the nylon suture and again measured the ATT to evaluate the anterior tibial instability of the ACL healed knees.

Histology

After anterior drawer testing, knee samples were immediately fixed (24-48h) in 4% paraformaldehyde and decalcified with 10% ethylenediaminetetraacetic acid (Sigma-Aldrich Japan, Tokyo, Japan) for 2 weeks. Finally, they were immersed in 5% and 30% sucrose for 4 and 12 hours, respectively, and then freeze-embedded using OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Each sample was sectioned at 12 μm using a Cryostar NX70 (Thermo Fisher Scientific, Kanagawa, Japan). We performed Hematoxylin & Eosin (HE) staining to observe ACL continuity and morphological features and Picrosirius Red (PsR) staining to evaluate collagen maturity of the healed ACL. We observed the ACL with a BZ-X710 microscope (Keyence, Osaka, Japan). We defined the healed ACL if the ACL reconnection was confirmed in the HE image. PsR images converted to grayscale images (256 degrees) showed collagenous tissue as 1-255 on the grayscale and non-collagenous tissue as on the

grayscale. Higher gray scale indicates higher ACL collagen maturation. Using the BZ-X710 analysis software, the entire ACL areas of the PsR polarization images were specified as Region of Interest (ROI) and then converted to grayscale images. We used the average of the grayscale in the ROI as the representative value of the healed ACL.

Immunofluorescence

We performed immunofluorescence to evaluate the expression of type 1 collagen in the healed ACL. ACL cryosections were blocked at room temperature for 1 hour using DAKO Antibody Diluent with Background Reducing Components (Aglient Technologies, Inc., USA). The primary antibody was incubated with Goat polyclonal antibody (AB758, Sigma-Aldrich) at 4°C overnight. Secondary antibody was incubated with anti-Goat Rabbit antibody Alexa Flour 488 (ab150141, abcam) for 4 hours at room temperature. All the stained sections were observed macroscopically with a microscope BZ-X 710 under controlled imaging conditions.

Biomechanical Testing

We performed failure testing to evaluate the mechanical strength of the healed ACL. The harvested knee joints were removed from the muscle and frozen at -70°C.

After thawing, we removed all soft tissues except the ACL and fixed the femur and tibia in 1.5-mm tubes. The tubes were then fixed to the mechanical testing machine Univert in the extended position. The ACL was stretched at a preload of 0.1 N and a rate of 10 mm/min until it was ruptured. We recorded the load to failure of the ACL.

Statistical Analysis

We performed statistical analysis on the results of nylon suture experiments, anterior drawer testing, gray scale, and mechanical testing using R. The Shapiro-Wilk test was used to verify the normality of all the analyzed data. Mann-Whitney U-tests were performed for the data from the nylon suture experiments. The two-way analysis of variance (ANOVA) was then performed to confirm for significant differences between the group and sex difference the other results. Because all data were non-normally distributed, an Aligned Rank Transform (ART)[17,18] was performed before ANOVA. Based on the results of ANOVA, the Steel-Dwass test was performed as a multiple comparison between all groups when the interaction was found. When no interaction was found, multiple comparisons were performed within factors for which a main effect was found. Statistical significance was set at $p < 0.05$.

Results

Mechanical Properties of Two Nylon Sutures

The results showed that the 3-0 nylon sutures had better mechanical properties. The load to failure and stiffness were significantly higher for 3-0 nylon sutures ($p < 0.05$) (Figure3A, B). In cyclic testing, the 3-0 nylon sutures showed higher viscoelasticity and resistance to elongation. The 4-0 nylon sutures ruptured in 3 cycles at 90% of the maximum load, 12 cycles at 80%, 107 cycles at 70%, and 791 cycles at 60%. The double 3-0 nylon sutures ruptured in 6 cycles at 90% and in 197 cycles at 80%. 3-0 nylon sutures did not rupture even after 2,000 cycles at loads lower than 70% (Figure 3C).

Magnitude of Anterior Translation of Tibia

First, in the knee joints with nylon sutures for controlling ATT, interactions between groups and sex were observed at all timepoint ($p < 0.01$). Immediately after injury, the ATT was smallest in the intact and 3-0 CATT groups. The 4-0 CATT group showed the higher ATT compared with intact and 3-0 CATT group, and the ACL-R group showed the highest ATT. However, there were no significant differences among all groups and sex (Figure 4D). At 4 and 8 weeks after injury, the ATT was smallest in

the intact group, followed by the 3-0 CATT and 4-0 CATT groups, which showed similar ATT. The ACL-R group had the highest ATT. However, there were no significant differences among all groups and sex (Figure 4E, F).

Secondly, for anterior tibial instability of the ACL healed knees, interactions between groups and sex were observed at 4 weeks and 8 weeks ($p < 0.001$). In males at 4 and 8 weeks, intact had the smallest ATT, and the 3-0 CATT and 4-0 CATT groups had similar ATT but higher ATT than intact. ACL-R showed the highest ATT. However, there were no significant differences among all groups and sex (Figure 4G, H).

Histology

First, we showed the success rates of the ACL healing with both 3-0 and 4-0 CATT groups at 4- and 8-weeks post-injury (Table 1). The 3-0 CATT group showed a higher healing rate for both males and females (42.9~100%), while 4-0 CATT group showed less heling rate (17~57.1%), and no healed ACL in ACL-R group (0%). Next, we showed HE-stained images of the healed ACL (Figure 5). We observed ACL spontaneously healing at 4 weeks after injury in both males and females. Compared to the intact ACL with parallel and regular nuclear arrangement, the healed ACL showed

heterogeneous nuclear aggregation not only at the healed region but also the remnant ACL region. It was also observed at 4- and 8-weeks post-injury.

In collagen maturation of the healed, there are no interaction was observed at both 4- and 8-weeks post-injury. On the other hand, a significant main effect was observed in the group: at 4 weeks, the 3-0 CATT group had a significantly lower gray scale than intact ($p < 0.001$), and at 8 weeks, still the 3-0 and 4-0 CATT groups showed a significantly lower gray scale than intact ($p < 0.01$) (Figure 6D, E).

Type I Collagen content of Healing ACL

We performed immunofluorescence of type I collagen, the main component of the ECM, to evaluate the ECM of the healed ACL. The green area indicates type I collagen and the blue area indicates the nucleus (Figure 7). In the intact ACL, we observed type I collagen orientation and nuclear alignment along the ACL. On the other hand, the healed ACL of 3-0 CATT and 4-0 CATT groups showed an irregular orientation of type I collagen compared to intact. In addition, the 3-0 and 4-0 CATT groups showed higher and irregular nuclei aggregation throughout the ACL.

Mechanical Properties of Healed ACL

We performed mechanical testing to evaluate the mechanical strength of the healed ACL. No interaction was observed at both 4- and 8-weeks post-injury. On the other hand, a significant main effect was observed in the group: at 4 weeks, intact had a significantly higher mechanical strength than the 3-0 CATT ($p < 0.01$) and 4-0 CATT group ($p < 0.05$), and at 8 weeks, intact had a significantly higher mechanical strength than the 3-0 CATT group ($p < 0.01$). However, the healed ACLs in the 3-0 CATT and 4-0 CATT groups showed similar mechanical strength and were approximately 50% of the normal ACL (Figure 8).

Discussion

The objectives of this study were to elucidate whether the difference of braking force to control the ATT affect the healing response of the completely injured ACL. And also, whether the difference of mechanical stress in the early phase affects to collagen maturation and mechanical strength of the healed ACL in mice. The results of the current study showed that the 3-0 CATT group with firmly braked ATT had a higher success rate of ACL healing. This means that the control of the ATT after ACL injured knee is one of the most critical key factors for the spontaneous healing of the ruptured ACL. On the other hand, the 4-0 CATT group with increased mechanical stress in the early phase did not show improvement in the quality of the healed ACL such as collagen maturation and mechanical strength.

In this study, we created the CATT model using 3-0 and 4-0 nylon sutures. We then confirmed the braking force with these sutures of the ATT ex vivo using anterior drawer testing. As a result, the 3-0 CATT group showed the similar braking force of the ATT as the intact group, which had a trend higher braking force than the 4-0 CATT group immediately after injury. However, at 4- and 8-weeks post-injury, the 3-0 CATT group showed a trend toward decreased braking force, which was no difference to the 4-0 CATT group. Prior to mice experiments, we evaluated the mechanical properties of

each nylon suture. The 3-0 nylon sutures with larger diameters showed higher mechanical strength in failure testing and better viscoelastic properties in cyclic testing. These results indicate that the difference in nylon suture elasticity is probably one of the factors that caused the difference in braking force between the 3-0 and 4-0 CATT groups. In addition, differences in the diameter of the nylon suture relative to the bone hole probably also affect the difference in braking force. We used the same 26G needle to create the bone holes in the femur and tibia in the 3-0 CATT and 4-0 CATT models. The 3-0 CATT group with a larger diameter suture had a smaller gap after passing through the bone holes, whereas the 4-0 CATT group had a larger gap. This may have been another reason for the difference in braking force between the 3-0 and 4-0 CATT. Since the result of HE-staining showed that the 3-0 CATT group had a higher success rate of ACL healing than 4-0 CATT group at 4 weeks and 8 weeks, we found that firm ATT braking at early phase post-injury is one of the most important factors for spontaneous healing of the ACL. Even mild anterior tibial instability, such as in the 4-0 CATT group, inhibited spontaneous healing of the ACL. In a clinical study of ACL conservative treatment, it was reported that 90% of ACL-injured patients had healed ACL with knee joints immobilized at 90 degrees by orthotics.[10] On the other hand, another study showed a lower rate of successful healing in younger ACL-injured

patients because they have higher activity levels compared with middle-age and older patients.[19] In addition, it was reported that early exercise intervention during the supraspinatus tendon healing process induced degeneration of the tendon.[20] Therefore, the mechanical stress on the ACL probably needs to be controlled during the early phase of the ACL spontaneous healing. During the healing process of the MCL, vascular-rich granulation tissue invades the rupture area of the ligament to close and then gradually replace with fibrous tissue.[21] Thus, the increase in ATT probably led to the failure of healing by pulling apart the ACL remnant and inhibiting the invasion of granulation tissue, or by re-rupturing the very fragile granulation tissue. Therefore, we showed that firm braking of the ATT is important in the early healing process after ACL injury.

It is known that there are sex differences in ACL injury mechanisms, and the ACL injury rate in women is 2~8 times higher than that in men.[22] Women are more likely to have knee ligament laxity, which can lead to decreased knee joint stability during exercise.[23] These sex differences in ACL injury mechanisms[24–26] may also affect the ACL healing process, so we also investigated sex differences in the ACL healing. The ACL healing success rate of the 3-0 CATT group was higher in females than in males. The ATT braking by the nylon suture may have loosened more easily in male,

which would have affected these results. Anterior drawer testing with nylon suture showed that the ATT braking force in the 3-0 CATT group both males and females was similar to that of intact immediately after injury. On the other hand, the ATT of male 3-0 CATT group was higher than that of female at 4 weeks post-injury, although there was no significant differences. These results suggested that the nylon sutures of the male CATT group were more likely to loosen, and it had a negative effect on the ACL healing. It may be caused by the difference in cage activity between male and female mice. Previous studies have reported that the amount of spontaneous exercise in wheel running in C57BL/6 mice is higher in females.[27] In addition, a study investigating the amount of home-cage activity showed that the amount of activity in the dark zone was significantly higher in females.[28] In contrast, male is known to exhibit more aggressive behaviors such as fighting and mounting when kept with 2 or more animals.[29] In this study, we housed both each sex mice with 2-4 mice. Based on these knowledges, male mice may have been higher intense activity than female in cage, which may have caused the CATT to loosen and higher mechanical stress on the ACL in male.

As mentioned above, we suggested that firm ATT braking is necessary to achieve a higher spontaneous healing success rate after ACL injury. Thus, in terms of healing success rate, increased mechanical stress in the early phase of healing process had a

negative effect. On the other hand, we observed healing of the ACL even in the 4-0 CATT group with higher ATT. In general, mechanical stress on the ligaments promotes fibroblast activation and ECM synthesis such as collagen.[13] It is known that the promotion of ECM synthesis improves the mechanical strength of the ligaments. Based on these knowledges, once the injured ACL in 4-0 CATT group was healed, it exposed higher mechanical stress during the early ACL healing process. Healed ACL in 4-0 CATT group might have grater quality of the healed ACL such as ECM maturation and mechanical strength, although had a lower healing success rate. Therefore, we performed ACL mechanical testing, picrosirius red staining, and immunofluorescence at 4 and 8 weeks to understand the mechanical strength and collagen maturation of the ACL in each group. We evaluated the maturity of collagen, the main component of the ECM, from the grayscale of the picrosirius red staining. In addition, we evaluated expression of type I collagen, the most abundant ECM in intact ligaments, with immunofluorescence. The intact ACL showed that type I collagen was localized along the ACL, while irregular and poor collagen localization was observed in both 3-0 and 4-0 CATT groups. In addition, the mechanical testing results showed that the 3-0 and 4-0 CATT groups had similar mechanical strength of the healed ACL and only 50% strength compared with intact ACL at both 4 and 8 weeks after injury. To summarize

these results, against our expectations, there was no difference in collagen maturation and mechanical strength between the 3-0 and 4-0 CATT groups. The reason for these results is probably that the mechanical stress on the healed ACL was similar in the 3-0 and 4-0 CATT groups after 4 weeks post-injury. Anterior drawer testing showed that ATT was lower in the 3-0 CATT group than in the 4-0 CATT group immediately after injury, while ATT increased in both the 3-0 and 4-0 CATT groups after 4 weeks post-injury, which indicated no difference in mechanical stress in each group. Moreover, the ACL mechanical strength of both groups was lower than intact. Increased ATT after 4weeks post-injury lead mechanical stress on the healed ACL, but it was not sufficient to promote healing of the ACL. Low-intensity exercise during the healing process of the supraspinatus tendon is reported to be beneficial in maintaining structure and promoting repair in the early stages of healing but is insufficient to promote long-term healing.[30] In addition, there is an optimal timing for mechanical stress loading to promote tendon and ligament healing. In ACL-reconstructed mice, it was reported that moderate timing of loading was more effective in promoting healing than immediate postoperative and delayed mechanical stress.[27] Thus, there is an optimal amount and timing of mechanical stress loading during tendon and ligament healing processes. In this study, it was found that mechanical stress by increasing ATT in the early phase was not sufficient to

promote healing. Therefore, in the future study, we need to investigate the influence of exercise intervention, i.e., rehabilitation, on promoting healing after spontaneous ACL healing.

In this study, we have several limitations. First, the present data were evaluated immediately after injury, and early phase of ACL healing at 4 weeks, and at 8 weeks. It is not possible to evaluate differences in ATT and the healing process from 0 to 4 weeks. Therefore, we will analyze at finer time points in the future to identify when CATT is no longer necessary. We will then be able to determine the period when orthotic management is necessary after ACL injury. Second, differences in the braking force of the ATT in anterior drawer testing are ex vivo data and do not completely show the differences in stress on the ACL in vivo. Although we evaluated the mechanical stress load by the difference of ATT, we will evaluate the knee joint motion during walking and the amount of activity in the cage as a future study. Then, we can analyze in detail the mechanical stress loaded on the ACL by the difference in braking force. Third, in this study, mechanical stress on the healed ACL did not improve mechanical strength sufficiently. Therefore, it would be necessary to apply a higher load of mechanical stress using exercise in future studies.

In this study, we investigated the effects of different ATT braking forces on the ACL spontaneous healing success rate and mechanical strength of the healed ACL in mice CATT model with different braking sutures. The results showed that firm ATT braking immediately after injury is necessary for successful healing of the injured ACL. On the other hand, increased ATT in the early phase was insufficient to improve mechanical strength and collagen maturation. These results suggested that firm ATT braking is required on ACL healing at the early phase of post-injury, and then higher mechanical loading may be necessary to achieve higher ACL properties at the remodeling phase when the ACL has healed.

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Table Legend

Table 1. We defined ACL spontaneous healing from HE stained images. 3-0 CATT group showed higher healing success rate than 4-0 CATT group at 4 and 8 weeks after injury.

	4 weeks				8 weeks			
	3-0 CATT		4-0 CATT		3-0 CATT		4-0 CATT	
	Male	Female	Male	Female	Male	Female	Male	Female
Healed	5	4	2	1	3	4	1	4
Non-healed	2	1	3	3	4	0	5	3
Success rates of ACL healing (%)	71.4	80.0	40.0	25.0	42.9	100.0	16.7	57.1

Figure Legend

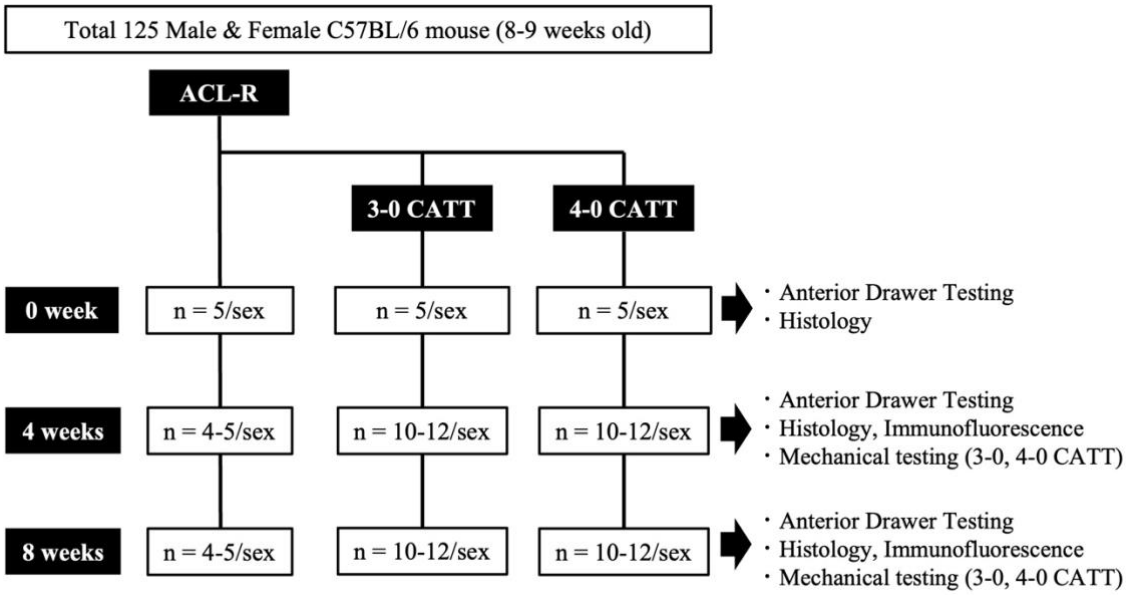

Figure 1. Experimental Design. Male and female 8-9 weeks old C57BL/6 mice were ruptured the ACL and classified to 3-0 CATT, 4-0 CATT and ACL-R group. Mice were

analyzed via anterior drawer testing and histology immediately after injury, and anterior drawer testing, histology, and mechanical testing at 4- and 8-weeks post-injury.

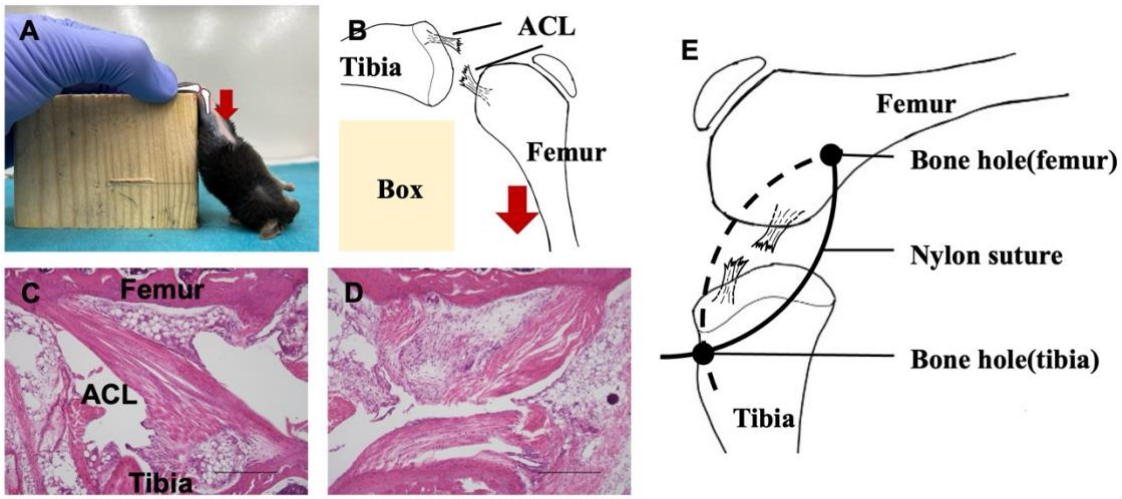

Figure 2. Non-invasive ACL-rupture. We fixed the hindlimb with the knee joint at 90 degrees and ruptured the ACL by pushing the femur backward against the tibia. (A, B) HE images of intact ACL (C) and immediately after ACL rupture (D) (Scale bar = 300 μm). Controlled Anterior Tibial Translation (CATT) model. Tightly looped by single 3-0 or 4-0 nylon suture through the femoral and tibial bone holes to control the ATT.

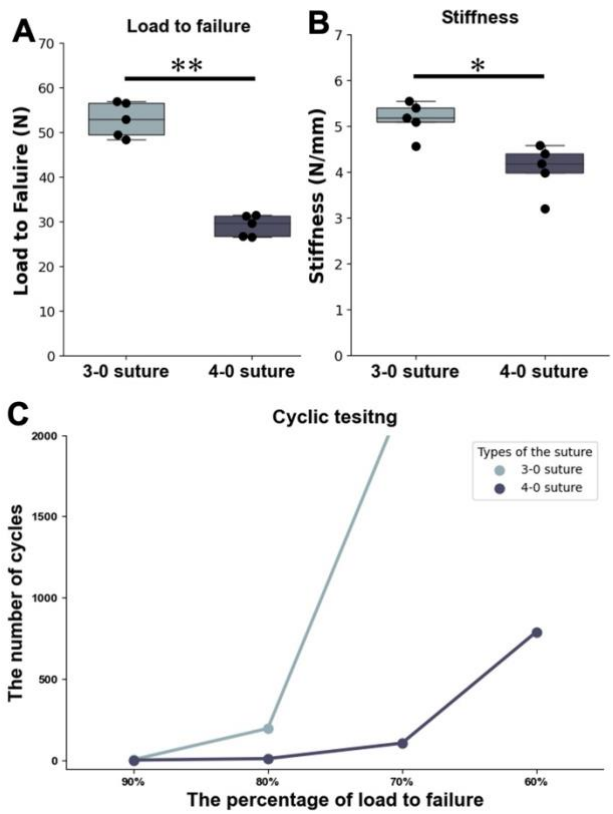

Figure 3. Nylon suture experiments. The load to failure (A) and stiffness (B) of the nylon suture in failure testing, and the number of cycles at the point of suture rupture in cyclic testing (C) were recorded. (*$p < 0.05$, **$p < 0.01$)

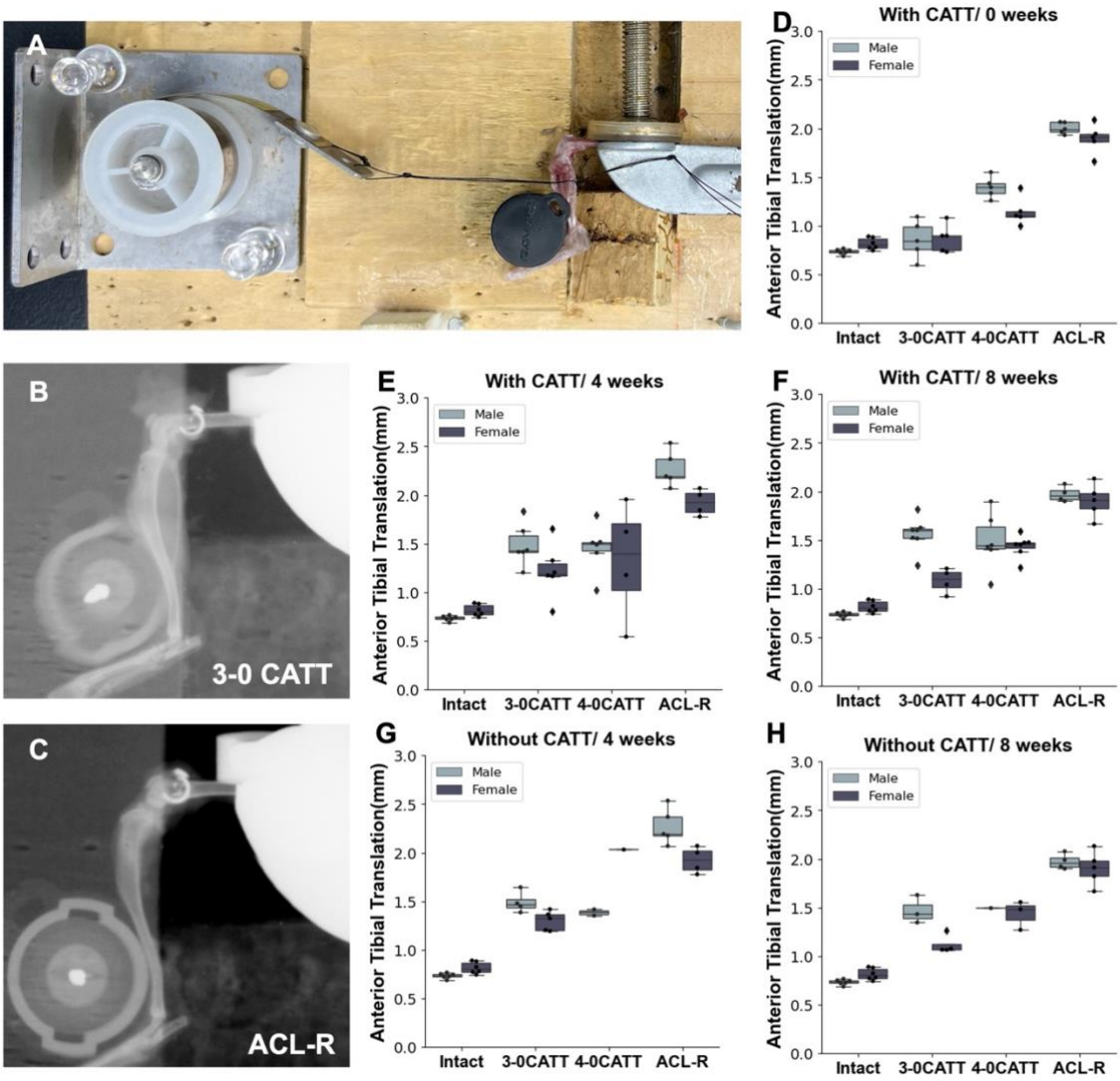

Figure 4. Anterior drawer testing was performed with the knee joint fixed at 90° via the self-made anterior drawer device (A). Representative X-ray images of 3-0 CATT group (B) and ACL-R group (C). We evaluated the braking force of the ATT for each groups immediately after injury (D) and at 4 (E) and 8 weeks (F), and anterior tibial instability of ACL healed knee at 4 (G) and 8 weeks (H).

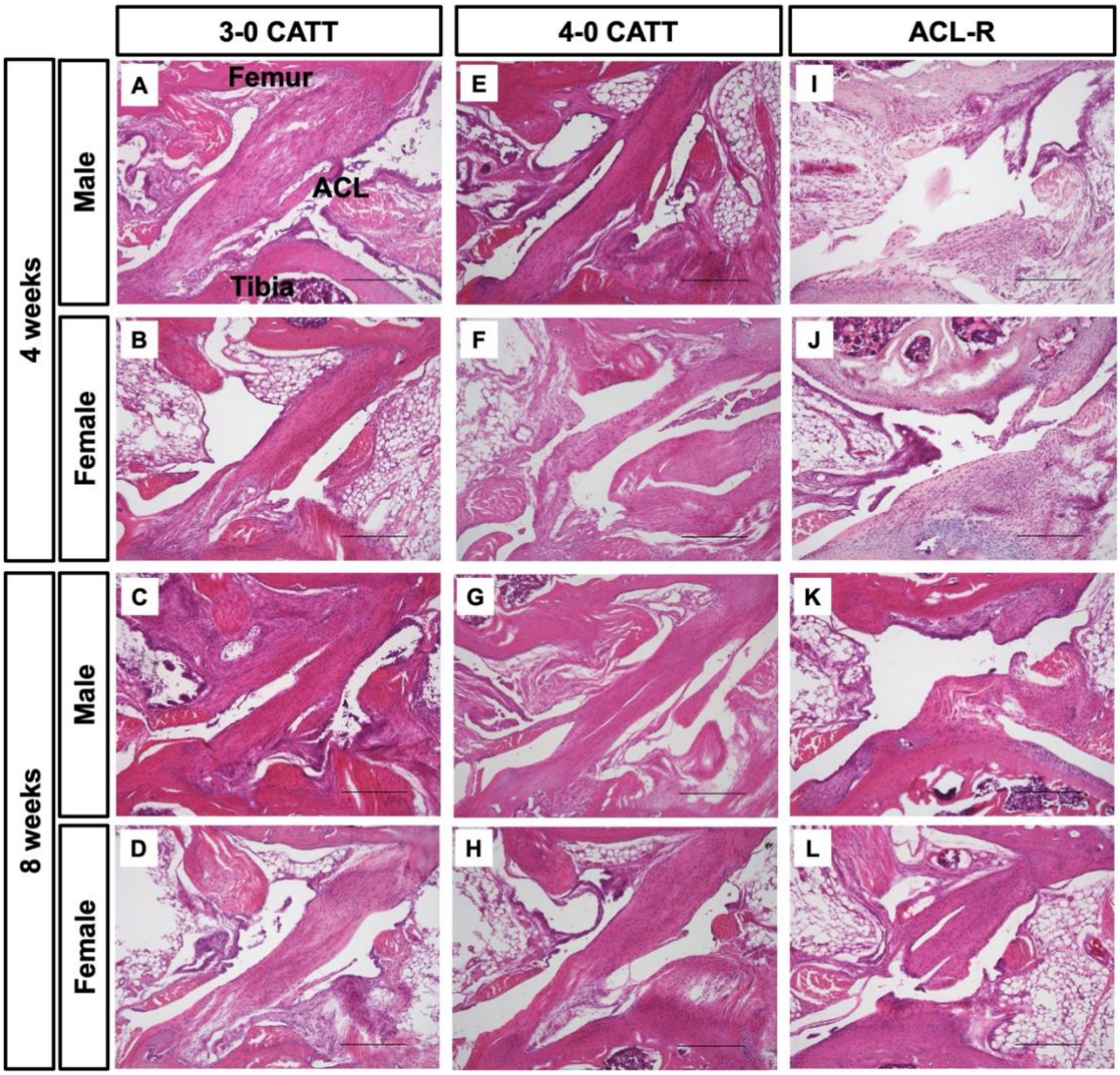

Figure 5. HE stained images of the 3-0 CATT (A, B, C, and D), 4-0 CATT (E, F, G, and H), ACL-R groups (I, J, K, and L) at 4 and 8 weeks (Scale bar = 300 μm).

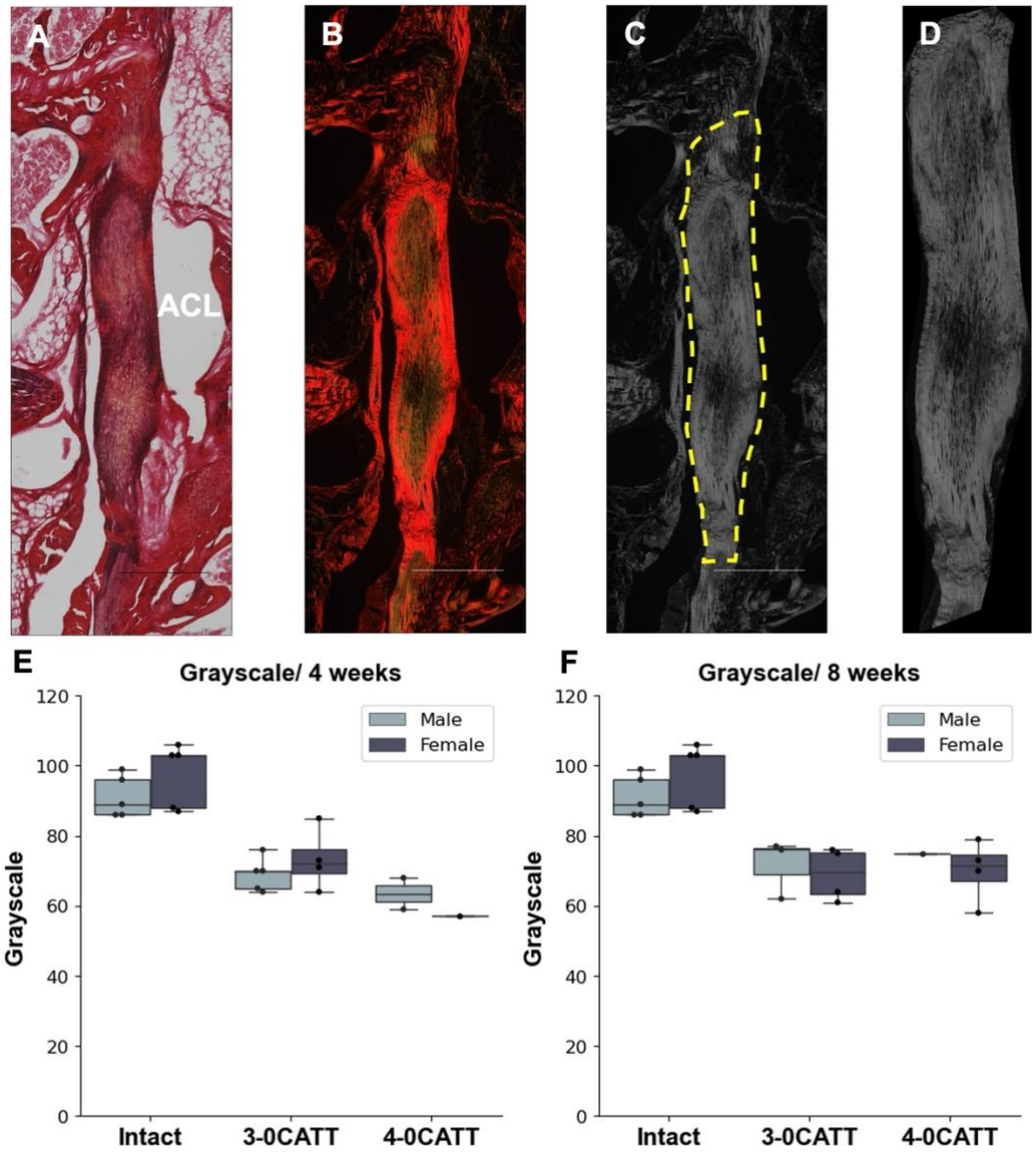

Figure 6. Picrosirius red stained (A) and polarized image (B) of the ACL. Picrosirius red images were converted to grayscale images (C) and specified ACL area as ROI (D). Collagen maturation of the ACL was evaluated at 4 (E) and 8 weeks (F) after injury (Scale bar = 300 μm).

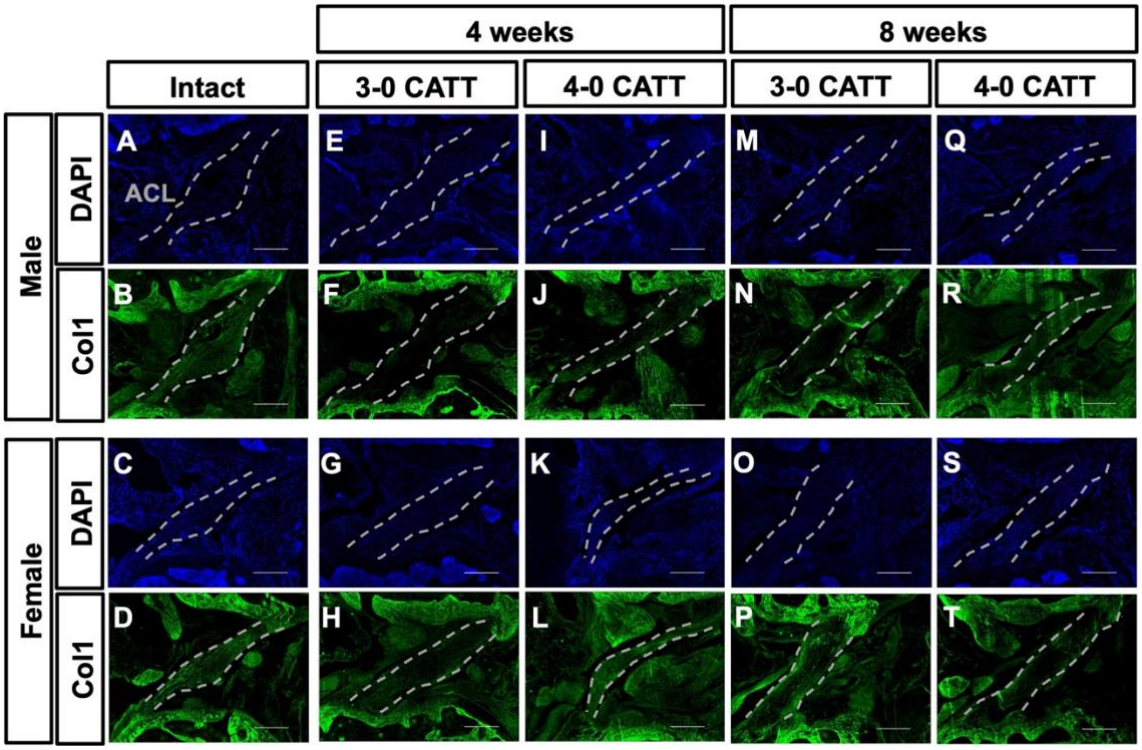

Figure 7. Immunofluorescence images of the intact ACL, 3-0 CATT, 4-0 CATT groups at 4 and 8 weeks: DAPI (blue) and Collagen type I (green). The images were taken with a 20× magnification lens (Scale bar = 300 μm).

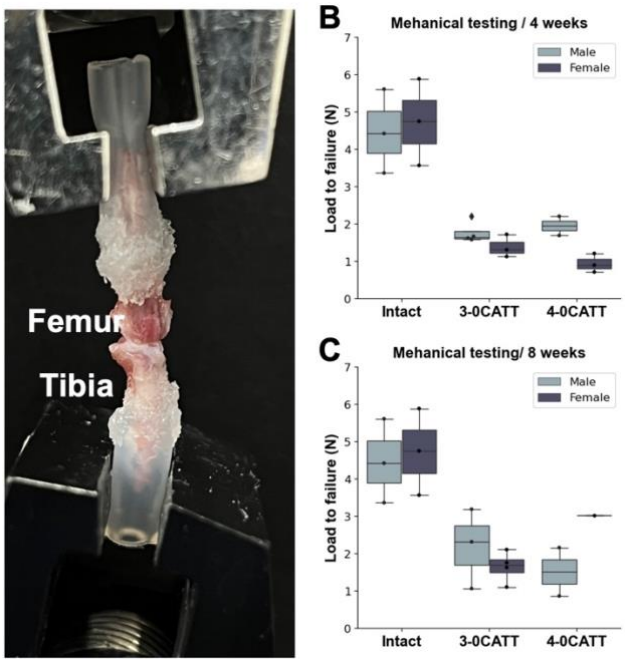

Figure 8. To perform biomechanical testing, the soft tissues of the knee except the ACL were dissected and the femur and tibia were fixed in tubes (A). The load to failure (A) of the healed ACL at 4 (B) and 8 (C) weeks after injury were recorded.