Title

Substantial Braking Force of Anterior Tibial Translation Induces the Spontaneous Healing of Murine Anterior Cruciate Ligament

Author

Riku Saito[1], Kazuki Nakayama[1], Yuna Usami[1], Saaya Enomoto[2], Koyo Nogi[1], Takanori Kokubun[1, 3]

1. Graduate School of Health, Medicine, and Welfare, Saitama Prefectural University, Saitama, Japan
2. Saitama Rehabilitation Center
3. Department of Physical Therapy, School of Health and Social Services, Saitama Prefectural University, Saitama, Japan

Author Contributions

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.

Corresponding author

Takanori Kokubun, P.T., Ph.D.

Affiliation: Graduate School of Saitama Prefectural University

Postal address: 820 San-nomiya, Koshigaya-Shi, Saitama, 343-8540, Japan

Phone number: +81-48-973-4176

Fax number: +81-48-973-4176

Email address: kokubun-takanori@spu.ac.jp

Running Title

Braking force affects ACL healing.

Abstract

The anterior cruciate ligament (ACL) has been recognized to have no spontaneous healing capacity after complete rupture due to an intra-articular environment. Meanwhile, our group reported that a completely ruptured ACL could heal spontaneously by restabilizing the femur-tibia joint. Several clinical studies have recently reported that ruptured ACL could heal spontaneously by controlling abnormal joint movement. Appropriate mechanical stimulation on the ruptured ACL promotes spontaneous healing, whereas excessive mechanical stress may inhibit the healing response. Here, we investigated how much braking force to control anterior tibial translation (ATT) affects the healing response of the ACL using two different braking forces for controlled ATT (CATT) models. Male and female C57BL/6 mice were ruptured ACL and randomly classified into Tight-CATT, Loose-CATT, and ACL-Rupture (ACL-R) groups. The Tight-CATT group showed a higher success rate of ACL healing than the Loose-CATT group at 4 and 8 weeks. However, collagen maturation and mechanical properties on healed ACL did not differ between the Tight-CATT and Loose-CATT groups. Our results suggested that tight ATT braking immediately after injury is essential for successfully healing the completely ruptured ACL. On the other hand, tightly ATT in the early healing phase was insufficient to improve mechanical

strength and collagen maturation. Therefore, we should control mechanical stress tightly in the early phase of ACL spontaneous healing, and it would be necessary to apply higher mechanical stress in the later phases to achieve greater healing of the ACL.

Study Design: A controlled laboratory study.

Key terms:
Anterior cruciate ligament; spontaneous healing; conservative treatment; braking; mechanical stress

Introduction

The knee joint has unstable bone shape and structures; hence, the anterior cruciate ligament (ACL) provides joint stability when the knee joint moves. The ACL is the only tissue that controls the anterior translation of the tibia; therefore, once the ACL is injured, the knee joint loses the proper bone position and kinematics during joint motion in daily activities and sports. ACL injuries occur in a variety of sports, but ACL injury rates are reported to be 2-6 times higher in female athletes than in males due to sex differences in kinematics and hormonal factors.[1–4]

ACL is an intra-articular tissue; the injured ACL has low spontaneous healing capacity. The inside of the joint capsule is filled with synovial fluid and poor vascular supply,[5] leading to poor fibroblast activity in the healing site of the injured ACL.[6] Therefore, ACL reconstruction is recommended and the 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 Knee Injury and

Osteoarthritis Outcome Score (KOOS).[7] Thus, optimal treatment after ACL injury is debatable.

Several clinical studies have reported that ruptured ACL could heal spontaneously, as confirmed by MRI.[8–11] However, there is insufficient knowledge of appropriate post-injury management of knee joints to lead ACL to healing. Recently, 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.[12] They showed that 90% of patients had evidence of healing on MRI after a three-month post-injury. Our previous report using the rats model elucidated that the ACL could heal spontaneously due to only controlling abnormal anterior tibial translation after ACL injury.[13] Considering the role of mechanical stress, we should not immobilize the knee joint after ACL injury because appropriate mechanical loading promotes collagen remodeling during the ligament healing process.[14–16] On the other hand, excessive mechanical stress inhibits the healing of tendons and ligaments.[17] 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 ACL-injured patients.

In this study, we created two controlled anterior tibial translation (CATT) models with altered anterior tibial instability using two different diameters of nylon sutures. We investigated the effect of braking force to control the anterior tibial translation (ATT) in mice on the healing response of the injured ACL. Our goal in the current study is to elucidate the acceptable mechanical stresses during the early phase of spontaneous healing because excessive mechanical stress during the ACL healing process may inhibit healing, while stress restriction may make the healing ligaments fragile.

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: Tight-CATT group, controlled ATT with 3-0 nylon suture (n=53), Loose-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. All mice were housed in

plastic cages under a 12-h light/ dark cycle. The mice were allowed unrestricted movement within the cage and had free access to food and water. 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 1A).

Nylon Suture Experiments

Before 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 this experiment was to understand the difference in 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 a 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 with 5 seconds of stretching up to 90%, 80%, 70%, and 60% of the failure load of each nylon

suture, 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 according to a previous study.[18] We fixed the hindlimb with the knee joint at 90 degrees and ruptured the ACL by pushing the femur backward against the tibia (Figure 1B-C). Then, we created bone holes at the femoral condyle and proximal tibia using a 26G needle (Terumo Co., Ltd., Tokyo, Japan). In the Tight- and Loose-CATT groups, we tightly looped a single 3-0 or 4-0 nylon suture through the two holes to control the ATT (Figure 1F). 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 the 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 to

control ATT. Then, we fixed the knee joint at 90 degrees on the self-made anterior drawer testing device (Figure 3A). 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 (Figure 3B-E). 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. 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 the 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 0 on the grayscale (Figure 5A-C). A 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 (Figure 5D). 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 (Agilent Technologies, Inc., USA). The primary antibody was incubated with Goat polyclonal antibody (AB758, Sigma-Aldrich) at 4°C overnight. The 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 (Figure 7A). 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, grayscale, 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 significant differences between the group and sex differences in the other results. Because all data were non-normally

distributed, an Aligned Rank Transform (ART)[19,20] 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 ($p < 0.05$) (Figure 2A, 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 2C).

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 timepoints ($p < 0.01$). Immediately after injury, the ATT was smallest in the intact and Tight-CATT groups. The Loose-CATT group showed a higher ATT than the Tight-CATT groups, and the ACL-R group showed the highest ATT. However, there were no significant differences among all groups and sex (Figure 3F). At 4 and 8 weeks after injury, the ATT was smallest in the intact group, followed by the Tight-CATT and Loose-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 3G, H).

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 Tight-CATT and Loose-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 3I, J).

Histology

First, we showed the success rates of ACL healing with both Tight- and Loose-CATT groups at 4- and 8-weeks post-injury (Table 1). The Tight-CATT group showed

a higher healing rate for both males and females (42.9~100%), while the Loose-CATT group showed less healing rate (17~57.1%) and no healed ACL in the ACL-R group (0%). Next, we showed HE-stained images of the healed ACL (Figure 4). Compared to the intact ACL with parallel and regular nuclear arrangement, the healed ACL showed heterogeneous nuclear aggregation not only in the healed region but also in the remnant ACL region. It was also observed at 4- and 8-weeks post-injury.

In collagen maturation of the healed ACL, there are no interactions observed at both 4- and 8-weeks post-injury. On the other hand, a significant main effect was observed in the group: the Tight-CATT group had a significantly lower grayscale than intact ($p < 0.001$) at 4 weeks, and the Tight- and Loose-CATT groups showed a significantly lower grayscale than intact ($p < 0.01$) at 8 weeks (Figure 5D, 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 6). In the intact ACL, we observed type I collagen orientation and nuclear alignment along the ACL. On the other hand, the healed ACL of Tight- and Loose-CATT groups showed an irregular orientation of type I

collagen compared to intact. In addition, the Tight- and Loose-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 Tight-CATT ($p < 0.01$) and Loose-CATT group ($p < 0.05$), and at 8 weeks, intact had a significantly higher mechanical strength than the Tight-CATT group ($p < 0.01$). However, the healed ACLs in the Tight- and Loose-CATT groups showed similar mechanical strength and were approximately 50% of the normal ACL (Figure 7).

Discussion

The first objective of this study was to elucidate whether the difference in braking force to control the ATT affects the healing response of the completely injured ACL. The results of the current study showed that the Tight-CATT group had a higher success rate of ACL healing. The Tight-CATT group showed a similar braking force of the ATT as the intact group, which had a trend higher braking force than the Loose-CATT group immediately after injury. However, after 4 weeks post-injury, the Tight-CATT group showed decreased braking force, which was not significantly different from the Loose-CATT group. These results indicate that controlling the ATT immediately after ACL injury is one of the most critical key factors for spontaneous healing of the ruptured ACL. A clinical study of ACL non-surgical treatment reported that 90% of ACL-injured patients had healed ACL with knee joints immobilized at 90 degrees by orthotics.[12] 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-aged and older patients.[21] In addition, it was reported that early exercise intervention during the supraspinatus tendon healing process induced tendon degeneration.[22] Accordingly, the lower ACL healing success rate in the Loose-CATT group is probably caused by the mechanical stress associated with increased ATT. 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.[23] 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 the tight braking force of ATT is important in the early healing process after ACL injury.

In this study, we found sex differences in the success rate of ACL healing. It is known that there are sex differences in ACL injury mechanisms, and the ACL injury rate in women is 2~6 times higher than that in men.[1] Women are more likely to have knee ligament laxity, which can lead to decreased knee joint stability during exercise.[24] However, the ACL healing success rate of the Tight-CATT group was higher in females than in males. The ATT braking by the nylon suture may have loosened more easily in males, which would have affected these results. The similar ATT braking force in male and female Tight-CATT group showed immediately after injury. However, the ATT braking of the male Tight-CATT group decreased after 4 weeks post-injury, which was lower than that of female. 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.[25] 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.[26] In contrast, males exhibit more aggressive behaviors, such as fighting and mounting, when kept with 2 or more animals.[27] In this study, we housed 2-4 mice in 1 cage for both sexes. Based on this knowledge, male mice may show more intense activity than females in a cage, which may induce the CATT to loosen and high mechanical stress on the ACL in male mice.

The second objective of this study was to elucidate whether the difference in mechanical stress in the early phase affects collagen maturation and mechanical strength of the healed ACL in mice. As mentioned above, we suggested that tight 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 the healing process is negative. However, we observed ACL healing in several knees of the Loose-CATT group, although the success rate was low. In general, mechanical stress on the ligaments promotes fibroblast activation and ECM synthesis, such as collagen.[15] It is known that the promotion of ECM synthesis improves the mechanical strength of the ligaments. Based on this knowledge, the healed ACL in the Loose-CATT group might have a greater quality of the healed ACL, such as ECM maturation and mechanical strength, although it had a lower healing success rate. Once the injured ACL

in the Loose-CATT group was fortunately healed, it was exposed to higher mechanical stress during the early ACL healing process. Therefore, we performed ACL mechanical testing, picrosirius red staining, and immunofluorescence at 4 and 8 weeks to understand the quality of the healed ACL in each group. As the results, the healed ACLs in Tight- and Loose-CATT groups showed similar collagen maturation and mechanical strength, and which were lower quality of the ACL compared to the intact ACL. In addition, the expression of type I collagen, a protein that contributing to the mechanical properties in the ligaments and tendons showed that it was localized along the ACL in the intact ACL, while irregular and poor collagen localization was observed in both Tight- and Loose-CATT groups. To summarize these results, against our expectations, there was no difference in the quality of the healed ACL between the Tight- and Loose-CATT groups. The reason for these results is probably that the mechanical stress on the healed ACL was similar in the Tight- and Loose-CATT groups after 4 weeks post-injury. Anterior drawer testing showed ATT was increased in both the Tight- and Loose-CATT groups after 4 weeks post-injury compared with immediately after injury. Increased ATT after 4weeks post-injury should led to mechanical stress on the healed ACL, but it was insufficient 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.[28] 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 proper timing of mechanical stress was more effective in promoting healing than immediate postoperative and delayed mechanical stress.[25] Thus, there is an optimal amount and timing of mechanical stress loading during tendon and ligament healing processes. In future studies, 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 at 4 and 8 weeks after injury. It was impossible 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 would 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, applying a higher load of mechanical stress using exercise would be necessary 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 models with different braking sutures. The results showed that tight ATT braking immediately after injury is necessary for the 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. This study suggested that tight 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. We provide insight into the appropriate management after ACL-injured patients in this study.

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Table Legend

Table 1. We defined ACL spontaneous healing from HE stained images. Tight-CATT group showed higher healing success rate than Loose-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

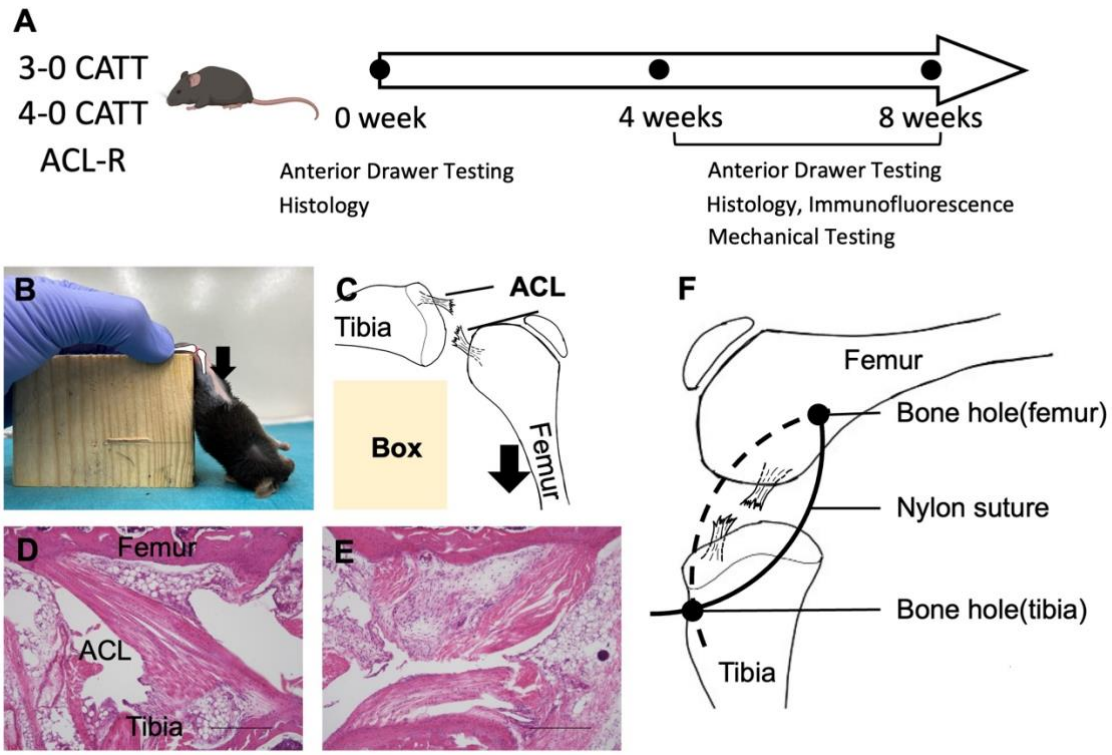

Figure 1. C57BL/6 mice were ruptured the ACL and classified to Tight-CATT, Loose-CATT and ACL-R group. Mice were analyzed via anterior drawer testing and histology immediately after injury, and anterior drawer testing, histology, and mechanical testing at 4- and 8-weeks post-injury (A). Non-invasive 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. (B, C) HE images of intact ACL (D) and immediately after ACL rupture (E) (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 (F).

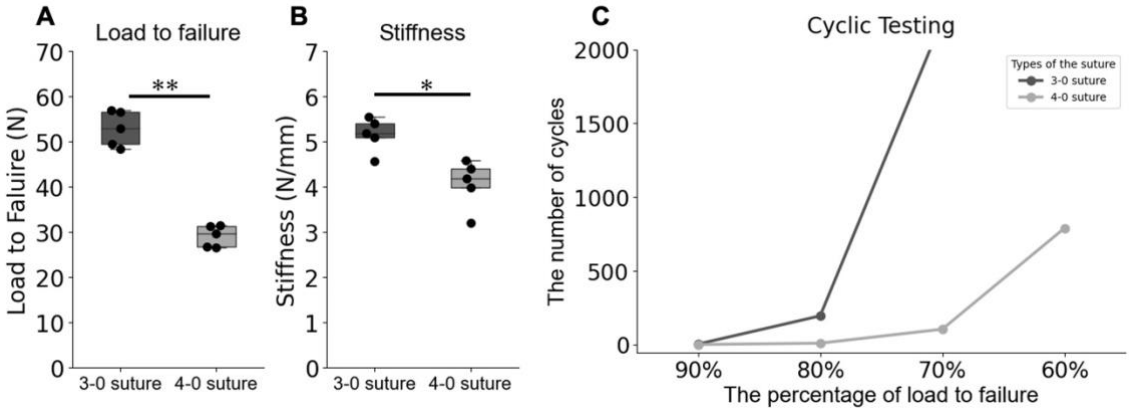

Figure 2. The load to failure (A) and stiffness (B) of the nylon suture in failure testing, and the number of cycles at the point of suture rupture in cyclic testing (C) were recorded. (*$p < 0.05$, **$p < 0.01$)

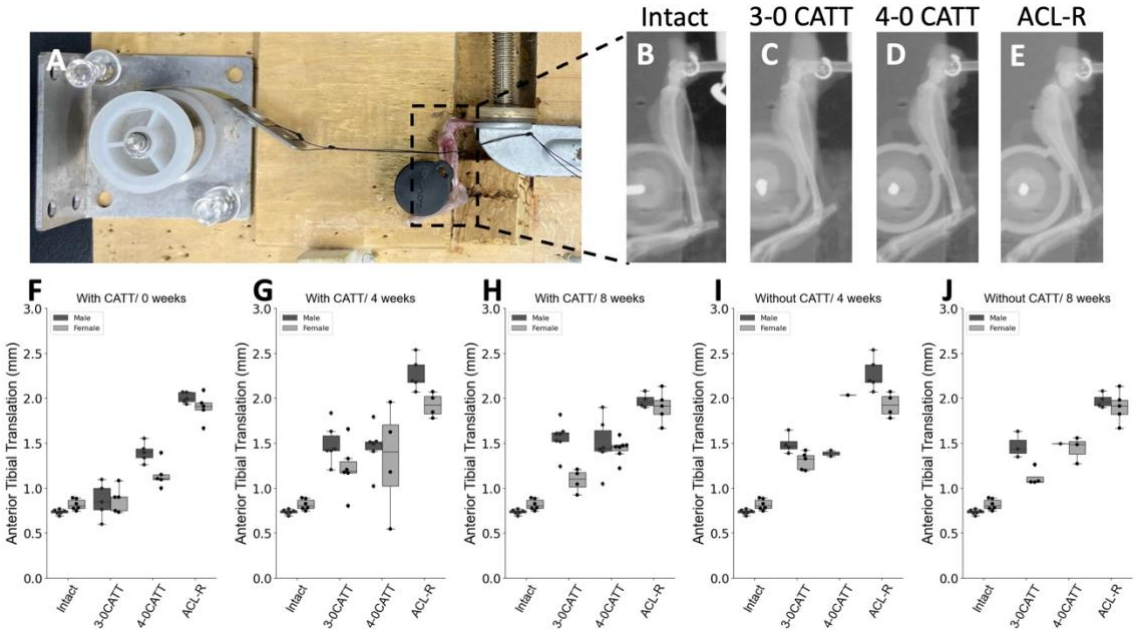

Figure 3. Anterior drawer testing was performed with the knee joint fixed at 90° via the self-made anterior drawer device (A). Representative X-ray images of each group (B-E). We evaluated the braking force of the ATT for each group immediately after injury (F) and at 4 (G) and 8 weeks (H), and anterior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

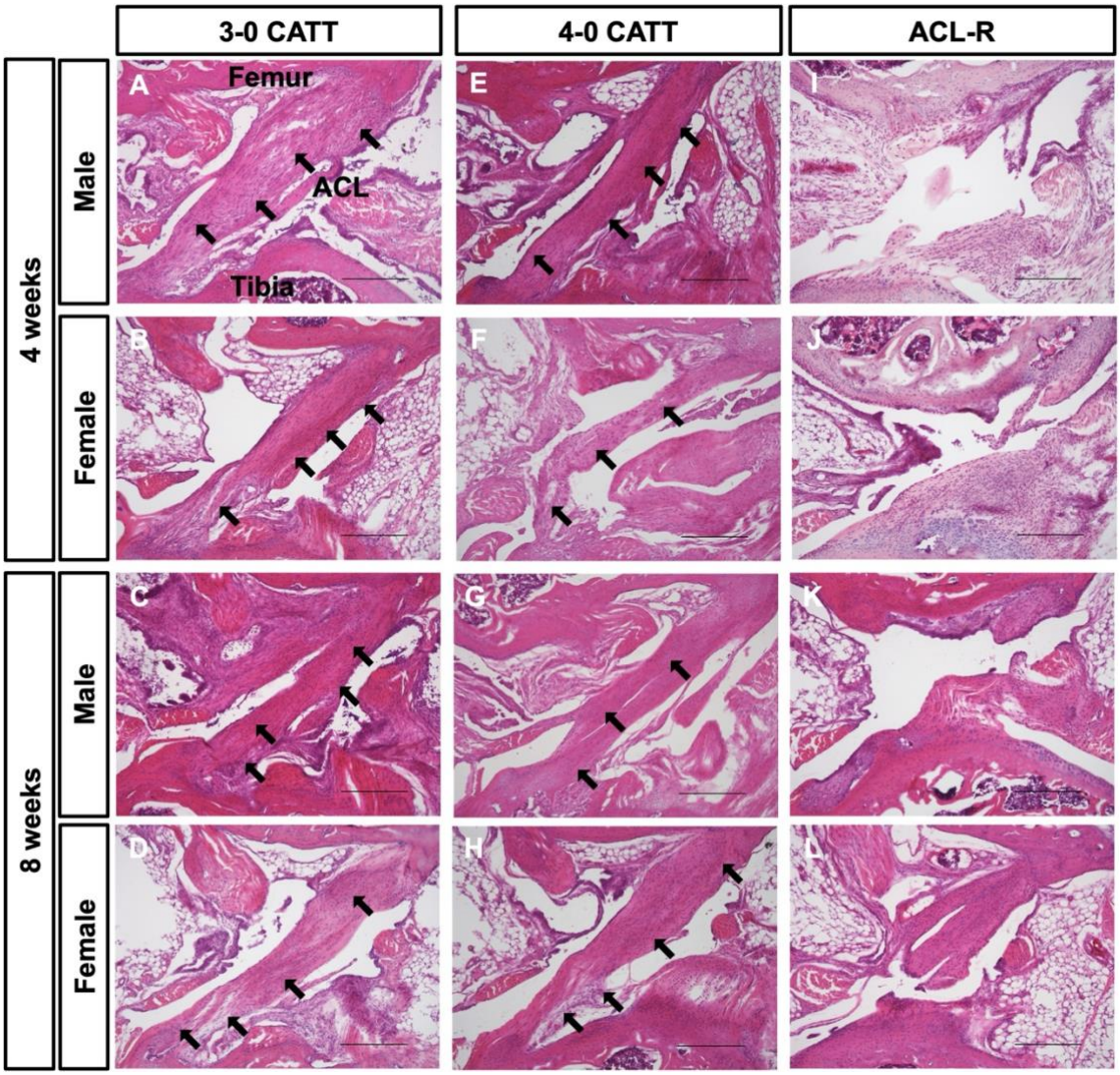

Figure 4. HE stained images of the Tight-CATT (A, B, C, and D), Loose-CATT (E, F, G, and H), ACL-R groups (I, J, K, and L) at 4 and 8 weeks (Scale bar = 300 μm). The arrows point to the healing region of the healed ACL.

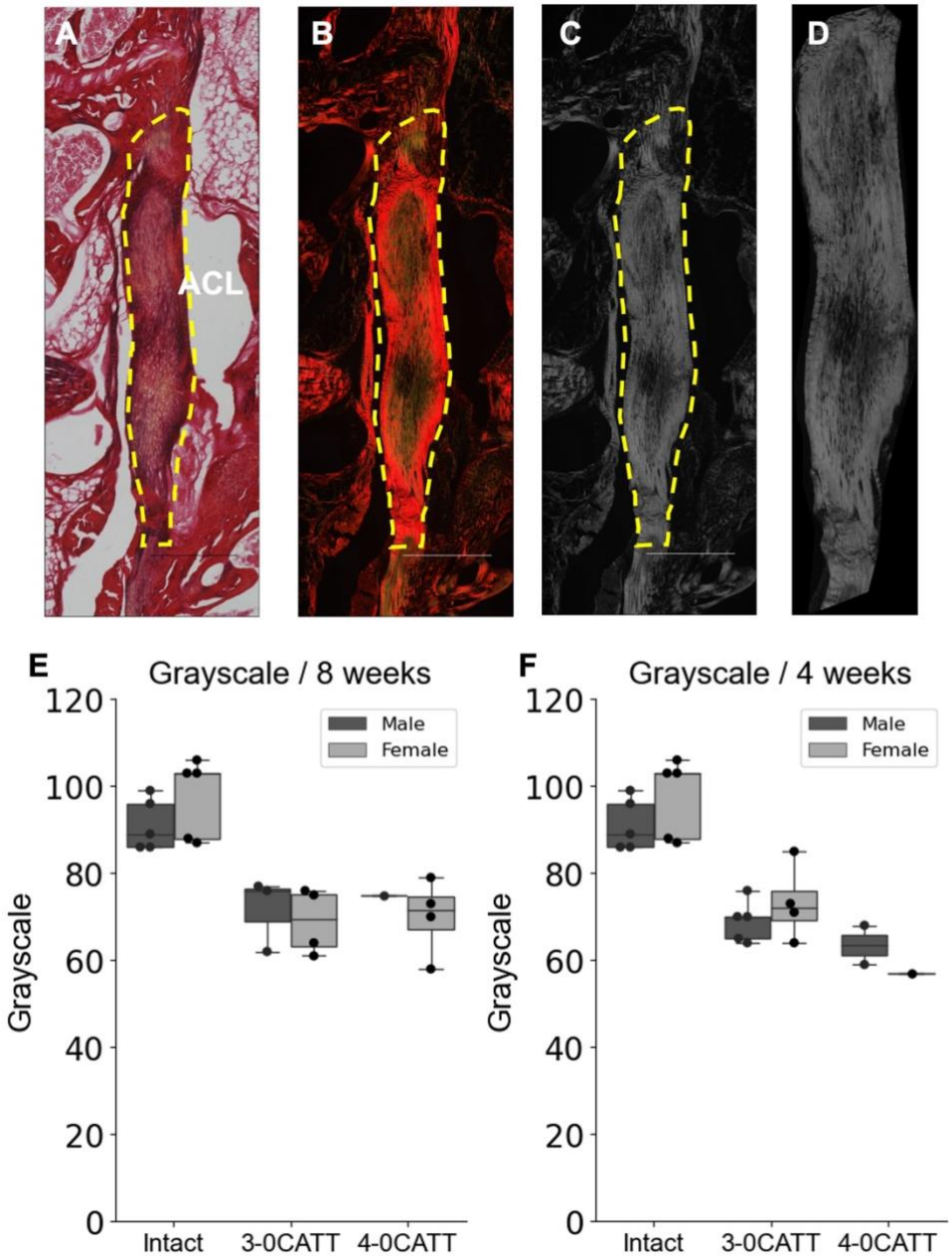

Figure 5. Picrosirius red stained (A) and polarized image (B) of the ACL. Picrosirius red images were converted to grayscale images (C) and specified ACL area as ROI (D). Collagen maturation of the ACL was evaluated at 4 (E) and 8 weeks (F) after injury (Scale bar = 300 μm).

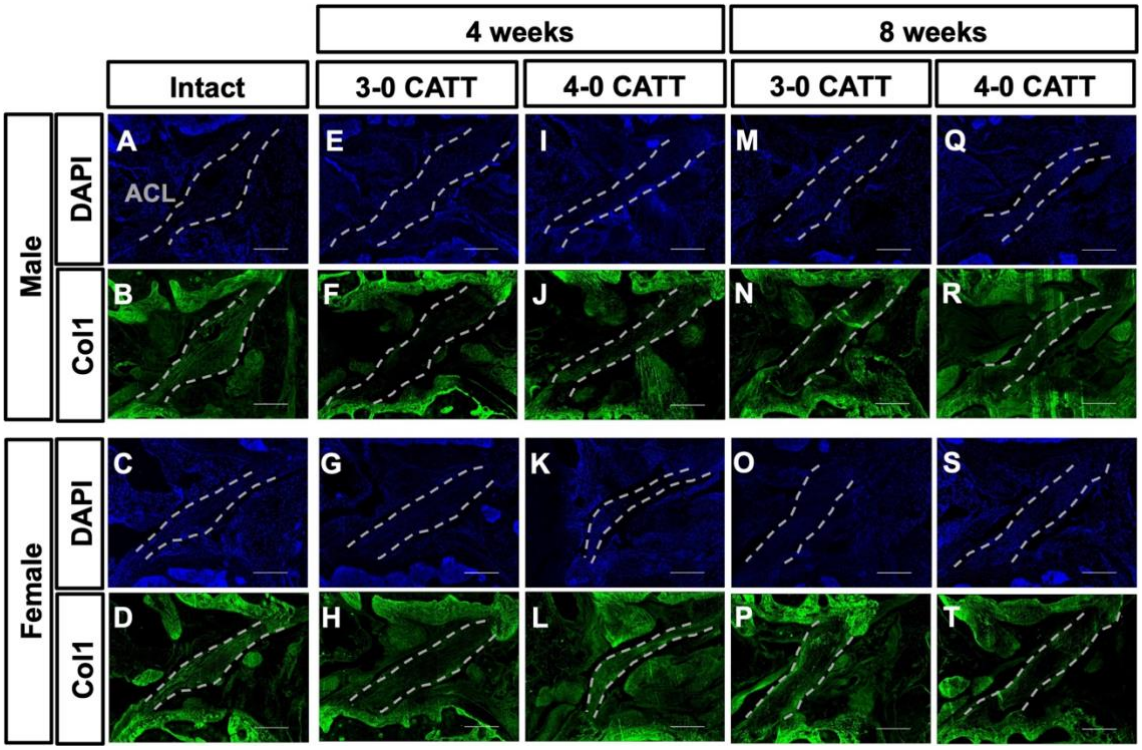

Figure 6. Immunofluorescence images of the intact ACL, Tight-CATT, Loose-CATT groups at 4 and 8 weeks: DAPI (blue) and Collagen type I (green). The images were taken with a 20× magnification lens (Scale bar = 300 μm).

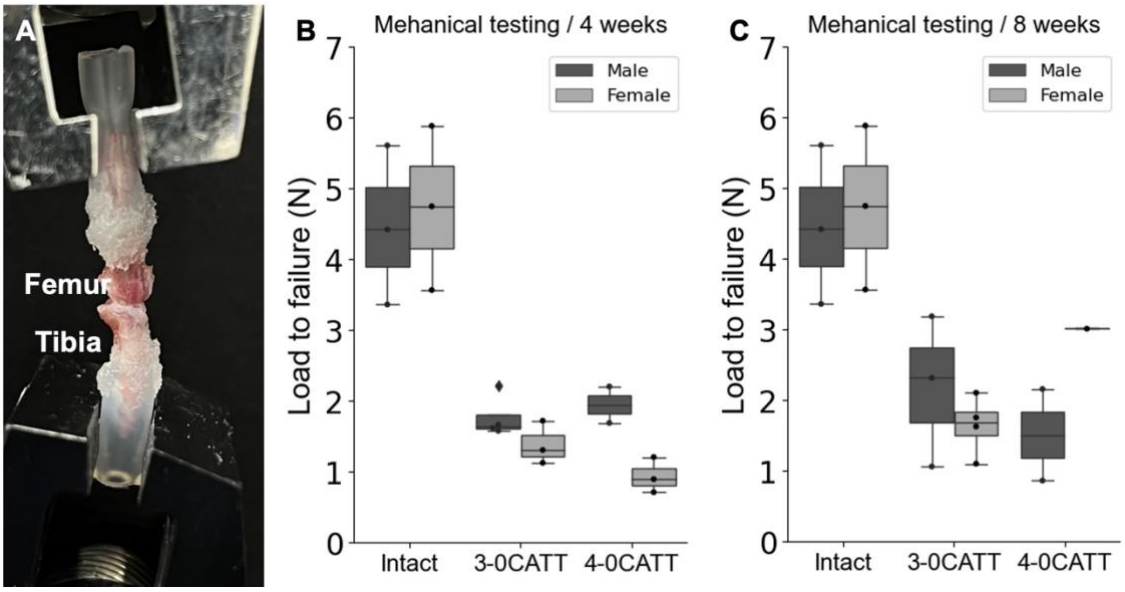

Figure 7. To perform biomechanical testing, the soft tissues of the knee except the ACL were dissected and the femur and tibia were fixed in tubes (A). The load to failure (A) of the healed ACL at 4 (B) and 8 (C) weeks after injury were recorded.