Early Mechanical Environment Regulates the Success of Spontaneous Healing of the Completely Ruptured Anterior Cruciate Ligament

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Purpose of this study: The anterior cruciate ligament (ACL) has been believed to have a low spontaneous healing capacity; growing evidence has suggested that ACL could heal spontaneously. While the healed ACL has reduced mechanical properties and incomplete tissue maturation, the mechanisms underlying these problems remain unknown. We aimed to elucidate the effect of mechanical stresses during the early phase of spontaneous ACL healing.

Materials and Methods: Male and female C57BL/6 mice were subjected to ACL rupture and randomly classified into three groups: Tight-CATT; tightly controlled anterior tibial translation (ATT), Loose-CATT; loosely controlled ATT and mild increasing mechanical stress compared to Tight-CATT, and ACL-Rupture (ACL-R) group; not controlled ATT. Mice were sacrificed and analyzed immediately after injury and at 4 and 8 weeks. We evaluated the effect of controlling the braking force of the ATT of each knee, the success rate of the ACL healing, collagen maturation, COL1A1 expression in the healed ACL, and the mechanical properties of the healed ACL.

Results: 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 the mechanical properties of healed ACL did not differ between the Tight-CATT and Loose-CATT groups.

Conclusion: Our results suggested that loose ATT braking immediately after injury is a negative factor for the healing of the completely ruptured ACL, and that it may be necessary to apply higher mechanical stress in the later stages to achieve greater healing.

Keywords: Anterior cruciate ligament; Spontaneous healing; Braking; Mechanical stress; Mechanobiology

Introduction

The anterior cruciate ligament (ACL) is a critical connective tissue that provides static and dynamic stability of the knee joint[1–3], and ACL rupture is one of the most common knee injuries across all generations[4–6]. Due to the unique surrounding environment, within the joint capsule, an injured ACL is one of the most spontaneously non-healing connective tissues [7–9]. Therefore, surgical reconstruction is the current gold standard treatment for ACL-injured patients.

In contrast, over the past several decades, growing evidence has suggested that the ACL has the potential to heal spontaneously without surgical intervention[10–15]. Ihara et al. reported that ACLs healed spontaneously by minimizing abnormal sagittal deviation between the femur and tibia using a knee brace following ACL injury[10,11]. In addition, we previously showed that controlling anterior tibial translation following ACL injury enables spontaneous ACL healing in a rodent model[16]. We also found that ACLs could heal spontaneously regardless of the injury site[17]. However, spontaneously healed ACLs underwent anterior tibial displacement, formed scar tissue, and exhibited approximately 60% of the mechanical strength of normal tissue[16]. Therefore, the next step should identify the conditions that promote precision healing of the spontaneously healed ACL.

It is well unveilled that appropriate mechanical stress during ligament and tendon healing promotes tissue maturation and recovery of mechanical strength[18–20]. During the healing process of the medial collateral ligament (MCL), a ligament that constitutes the knee joint, mechanical stress improved the mechanical properties of healed tissue[21]. Furthermore, controlled and low-intensity mechanical stress would rescue ligament healing even during the acute phase after injury[22–24]. During the acute phase following ligament injury, granulation tissue infiltrates the injured site to form a provisional matrix, subsequently which collagen synthesis progresses[25]. The injured site also contains numerous cells, such as myofibroblasts and scleraxis (Scx) positive cells, which exhibit a mechanical stress response and contribute to collagen synthesis[9,26,27]. In fact, it was reported that low-intensity exercise after mouse Achilles tendon injury improved the mechanical properties of the healed tendon, even in the acute phase[28]. Thus, we hypothesized that during the early healing process, a mild increase in mechanical stress would promote collagen deposition and enhance mechanical strength in the healed ACLs. However, the influence of mechanical stress on the spontaneous healing process of the ACL remains undiscussed.

Therefore, the purpose of this study was to determine the acceptable mechanical stress condition during the acute phase of ACL injury. To achieve this goal, we

developed two types of controlled anterior tibial translation (CATT) mouse models using nylon sutures of two different diameters. Suture differences conferred varying degrees of anterior tibial translation (ATT) stress in murine knees. We assessed ACL healing success rates, mechanical strength, and collagen composition of the healed ACL under varying levels of mechanical stress.

Methods

Experimental Design

The University Animal Experiment Ethics Committee approved all experiments. We used male (n=66) and female (n=66) 8-9-week-old C57BL/6 mice (n=132). A total of 132 mice were ruptured the left ACL and then were randomly classified into three groups: Tight-CATT group (Figure 1B); controlled ATT with 3-0 nylon suture (n=57), Loose-CATT group (Figure 1C); controlled ATT with 4-0 nylon suture (n=55), and ACL-R (ACL-Rupture) group (Figure 1D); not controlled ATT (n=20). We used the right hindlimb 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. Three animals from both the Tight-CATT and Loose-CATT groups were excluded from the experiment due to tibial

fractures when the ACL ruptured, and the abnormal findings during housing. Thus, a total of 126 mice were included in this study and were sacrificed immediately after injury for anterior drawer testing and histology (n=20); at 4 (n=52) and 8 weeks (n=54), for anterior drawer testing, histology, immunofluorescence, and mechanical testing.

Nylon Suture Experiments

Before creating the CATT models, we measured the mechanical properties of each nylon suture using a mechanical testing machine (Univert. CellScale Biomaterial Testing, Canada). The purpose of this experiment was to investigate the differences 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 sutures at a preload of 0.1 N and a rate of 20 mm/min until they ruptured. During the test, load (N) and displacement (mm) were recorded, and we measured the load at failure (N) and the 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 seconds). We recorded the number of cycles at the point of rupture.

Procedure for 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[29]. We fixed the hindlimb with the knee joint at 90 degrees and ruptured the ACL by pushing the femur backward against the tibia. In non-invasive ACL ruptures, the ligament is generally ruptured in the mid-substance region (Figure 1E). Then, we created bone tunnels 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 tunnels to control the ATT. In the ACL-R group, we loosely looped a 3-0 nylon suture through the two tunnels to avoid controlling the ATT.

Anterior Drawer Testing

We performed anterior drawer testing to evaluate the braking force to control the ATT of each knee, carefully removing the muscle from the knee while retaining the suture. Then, we fixed the knee joint at 90 degrees on the self-made anterior drawer testing device. 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 X-ray images using ImageJ (National Institutes of Health, Bethesda, MD, USA) to evaluate the braking force exerted 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 potential characteristics of the knee stability with healed ACL. The knees in which the ACL was not healed in histology were excluded from the latter analysis.

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 12μm 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 stained sections with the BZ-X710 microscope (Keyence, Osaka, Japan). We determined whether the ACL was healed from HE images obtained at 4- or 8-weeks post-injury. We defined ACLs with observed continuity as "healed" (Figure 1F), and those with lost continuity as "non-healed" (Figure 1G). The success rate of ACL healing in each group was calculated from HE images. 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. A higher grayscale indicates higher ACL collagen maturation [27,28]. Using the BZ-X710 analysis software, the entire ACL areas in the PsR polarization images were defined as Regions of Interest (ROIs) and then converted to grayscale. We used the average grayscale value in the ROI as the representative value of the healed ACL.

Immunofluorescence

We performed immunofluorescence to evaluate the expression of type I collagen (COL1A1) 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., CA, USA). The anti-COL1A1 primary antibody was incubated with a Goat polyclonal antibody (1:200; AB758, Merck, Sigma-Aldrich, Taufkirchen, Germany) at 4°C overnight. The secondary antibody was incubated with anti-Goat Rabbit antibody Alexa Fluor 488 (1:500, ab150141, Abcam, Cambridge, UK) for 4 hours at room temperature. All sections were examined macroscopically using a BZ-X710 microscope under controlled imaging conditions.

Biomechanical Testing

The failure load testing was performed to evaluate the mechanical strength of the healed ACL. The harvested knee joints were dissected and frozen at -70°C. After thawing, all soft tissues were removed except the ACL, and the femur and tibia were fixed 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 force of the ACL.

Statistical Analysis

We performed statistical analyses of the results from nylon suture experiments, anterior drawer testing, grayscale analysis, 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. A two-way analysis of variance (ANOVA) was then performed to confirm significant differences among groups and to assess sex differences in the other results. Because all data were non-normally distributed, an Aligned Rank Transform (ART)[32,33] was performed before ANOVA. Based on the ANOVA results, the Steel-Dwass test was used for multiple comparisons among all groups when an 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 exhibited higher viscoelasticity and greater resistance to elongation. The 4-0 nylon sutures ruptured in 3 cycles at 90% of the failure load, 12 cycles at 80%, 107 cycles at 70%, and 791 cycles at 60%. The 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 group, and the ACL-R group showed the highest ATT. However, there were no significant differences among the groups or by sex (Figure 3C). In males 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. In contrast, in females, the ATT of the Tight-CATT showed a trend to be smaller than that of the Loose-CATT.

However, there were no significant differences among the groups or by sex (Figures 3D, E).

Secondly, for anterior tibial instability of the knee with a healed ACL, fewer knees were included in the Loose-CATT group because we excluded knees with non-healed ACLs from the CATT group in this analysis. 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. In females, the ATT of the Tight-CATT showed a trend to be smaller than that of the Loose-CATT. However, there were no significant differences among the groups or by sex (Figures 3F, G).

ACL Healing Success Rate and Collagen Maturation of the ACL

First, we reported the success rates of ACL healing in the Tight- and Loose-CATT groups at 4- and 8-week 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 a lower 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. 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 ACL remnant region. It was also observed at 4- and 8-week post-injury (Figure 4A-H).

In collagen maturation of the healed ACL, there are no interactions observed at both 4- and 8-week 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 5E, F).

Type I Collagen Content of Healing ACL

We performed immunofluorescence for COL1A1, a major ECM component, to assess ECM maturity in the healed ACL. The green area indicates COL1A1, and the blue area indicates the nucleus. In the intact ACL, we observed COL1A1 expression at the entire ACL region and nuclei aligned longitudinally of the ACL (Figure 6A, B). On the other hand, the healed ACLs of the Tight- and Loose-CATT groups showed limited expression outside the healed ACL and an irregular orientation of COL1A1 compared with the intact ACL. In addition, the Tight- and Loose-CATT groups showed higher and irregular nuclei aggregation throughout the ACL (Figure 6C-F). These collagen features

of the ACL were commonly observed at 4- and 8-weeks post-injury (Shown in Figure 6 is at 8 weeks post-injury).

Mechanical Properties of Healed ACL

We fixed the femur and tibia in 1.5-mm tubes (Figure 7A) and performed mechanical testing to evaluate the mechanical strength of the healed ACL. The number of ACLs for testing in the Loose-CATT group was fewer because only the knees with confirmed ACL re-connections were included when the knees were dissected. No interaction was observed at both 4- and 8-week 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 ($p < 0.05$) (Figure 7B), and at 8 weeks, intact had a significantly higher mechanical strength than the Tight-CATT group ($p < 0.01$) (Figure 7C). However, the healed ACLs in the Tight- and Loose-CATT groups exhibited similar mechanical strength and were approximately 50% of normal ACL strength.

Discussion

We investigated the effects of increased mild mechanical stress from the acute phase following ACL injury. The most important finding of this study was that loosening of the controlling ATT immediately after ACL injury is a negative factor for spontaneous healing of the ruptured ACL. The results of the Loose-CATT group showed that increased mechanical stress immediately after injury negatively affects ACL healing response. Conversely, counter to our hypothesis, the mechanical properties of healed ACLs in the Loose-CATT group were similar to those of the Tight-CATT group, and showed lower than those in the Intact group. There were no significant differences in mechanical properties and histological features between tight and loose conditions. Overall, our results suggest that appropriate force control may be necessary to bridge the gap of remnant ACLs and improve the success rate of healing of the injured ACL.

In this study, we developed CATT models with varying levels of ATT braking force, using two different types of nylon suture, immediately after ACL injury. Results of the nylon suture experiment showed that the 4-0 nylon suture used in the Loose-CATT group had higher suture elasticity compared to the 3-0 nylon suture used in the Tight-CATT group. Subsequently, we confirmed in the anterior drawer test that the

Loose-CATT group exhibited a lower braking force than the Tight-CATT group immediately after surgical intervention. Therefore, the healing tissue of the Loose-CATT group was defined as the group with early exposure to the higher mechanical stress in the early phase of ACL healing. This physiological situation in an injured ACL induces an inadequate environment for spontaneous healing of skeletal connective tissue in the Loose-CATT group in each phase. In the very early stages of ligament and tendon healing, the injured site is filled with granulation tissue and numerous cells, forming a mechanically fragile provisional matrix[25–27,34]. Increased ATT stress immediately after ACL injury may put a distance between the femur and tibia, inhibiting matrix formation or leading to the failure of the fragile matrix. In the next stage, loosening of the ATT would induce abnormal mechanotransduction during ACL healing. While proper mechanical stress after ligament and tendon injury promotes healing[18–21,35,36], excessive mechanical loading impairs maturation of tissue healing and biomechanical properties[37–39]. In our previous data, controlling the abnormal motion of the tibia after ACL injury can reduce MMPs that cause matrix degradation on the ACL[16,40,41]. Thus, tightly controlling the ATT would play an important role in reducing abnormal mechanotransduction during ACL healing. Future research focusing on changes during

the very acute phase of ACL healing may reveal critical mechanobiological mechanisms that inhibit ACL healing.

Interestingly, we also found sex differences in ACL healing success rates. It is well 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[1,42,43]. On the other hand, it is still controversial whether there is a sex dimorphism in the ligaments and tendons healing mechanisms[44–46]. Surprisingly, our findings showed that ACL healing success rates were higher in females than in males. While the similar ATT braking force in the male and female Tight-CATT group was observed immediately after surgical intervention, the ATT braking of the male Tight-CATT group decreased after 4 weeks post-injury, compared with the female. These results may be attributable to differences in cage activity between male and female mice. Previous studies have reported that the amount of spontaneous exercise in wheel-running is higher in C57BL/6 female mice[47]. Additionally, a study examining home-cage activity levels found that females exhibited significantly higher activity in the dark time[48]. In contrast, males exhibit more aggressive behaviours, such as fighting and mounting, when kept with two or more animals[49]. In this study, we housed 2-4 mice in a cage for both sexes. Based on these facts, male mice might exhibit more intense and irregular knee joint motion in a cage

than females. Additionally, standing on two limbs in a fighting or defensive posture is more unstable than walking on four limbs[50,51], which would increase stress on the knee joints, particularly the healing ACL. Thus, the aggressive behavioural characteristics of male mice may have contributed to the reduced ACL healing rate. A clinical study found a lower success rate of spontaneous healing in younger ACL-injured patients because they have higher activity levels compared with middle-aged and older patients[11]. Thus, this finding underscores the importance of balancing the quality and quantity of exercise during ACL healing. Further study is needed to clarify the relationship between behavioural characteristics of the mice and ACL healing.

We hypothesized that the increased mechanical stress on the healing ACL immediately after injury would promote collagen maturation and mechanical strength, even if it led to a low success rate in ACL healing. However, against our expectations, there was no difference in the quality of the healed ACL between the Tight- and Loose-CATT groups. The healed ACLs in Tight- and Loose-CATT groups showed similar collagen maturation and mechanical strength, and inferior properties compared to the intact ACL. Accordingly, tight ATT braking is important for achieving a higher ACL-healing success rate in the early phase. On the other hand, it is suggested that an increase in mechanical stress at the level of loosening does not promote ACL healing.

Previous studies have shown that low-intensity exercise during the healing process of the supraspinatus tendon is beneficial for maintaining structure and promoting repair in the early stages of healing, but is insufficient to promote long-term healing[20]. In ACL-reconstructed mice, proper timing of mechanical load was more effective at promoting healing than immediate postoperative or delayed mechanical stress[35]. This study revealed that the mild increase in mechanical force induced by loosening is insufficient to improve ECM synthesis or the mechanical strength of the healed ACL. Although our results suggest the importance of tight control of abnormal tibial translation in the early phase after ACL injury, the optimal mechanical force for promoting ACL healing remains unclear. Future study will focus on this point.

This study has several limitations. First, mice are a commonly used model for research on ligament and tendon healing; however, the structure and alignment of the mouse knee, which is always flexed, differ from those of the human knee, which is typically extended. Therefore, the mice are not a model that fully reproduces human conditions of ACL healing. Second, the present data were evaluated immediately after injury and at 4- and 8-weeks after injury. It was impossible to evaluate how differences in the ATT stress affect 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 during which orthotic management is necessary following ACL injury. Finally, the current study examined, first, how increased mechanical stress affects ACL healing and subsequent properties. Hence, it was unable to elucidate in detail the mechanisms by which increased mechanical stress impaired ACL healing in the early stages of healing. In the future, we will analyze biochemical data and intracellular changes up to 4 weeks after ACL injury to elucidate the mechanism of mechanical transduction in ACL healing.

In this study, we investigated the effects of controlling abnormal tibial translation braking forces on the spontaneous-healing success rate of the ACL and the quality of the healed ACL in mice using CATT models with different sutures. The results showed that loose braking of the ATT immediately after injury is a negative factor for the healing of the completely injured ACL. Conversely, the increasing mechanical force induced by loosening is insufficient to improve the ECM synthesis or mechanical properties of the healed ACL. Still, the mechanism underlying spontaneous ACL healing remains unclear; controlling the mechanical force on the ACL should play a key role in ACL healing and maturation. We provided essential insights into the state of ACL healing, which are critical for elucidating healing mechanisms and informing conservative rehabilitation in skeletal connective tissue beyond the ACL healing.

Funding

This work was supported by JSPS KAKENHI [21H03306, 19KK0411] (to TK), the grant from Tokyo Physical Therapist Association [05094] (to RS) and the attack grant from Saitama Prefectural University (to RS).

Declaration of Interests

No potential conflict of interest is reported by the authors.

Acknowledgments

We would like to thank the members of Kokubun Laboratory at the Saitama Prefectural University for their cooperation.

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Table Legend

Table 1. We defined ACL spontaneous healing from HE-stained images. The Tight-CATT group showed a higher healing success rate than the Loose-CATT group at 4 and 8 weeks after injury.

	4 weeks				**8 weeks**			
	Tight-CATT		**Loose-CATT**		**Tight-CATT**		**Loose-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

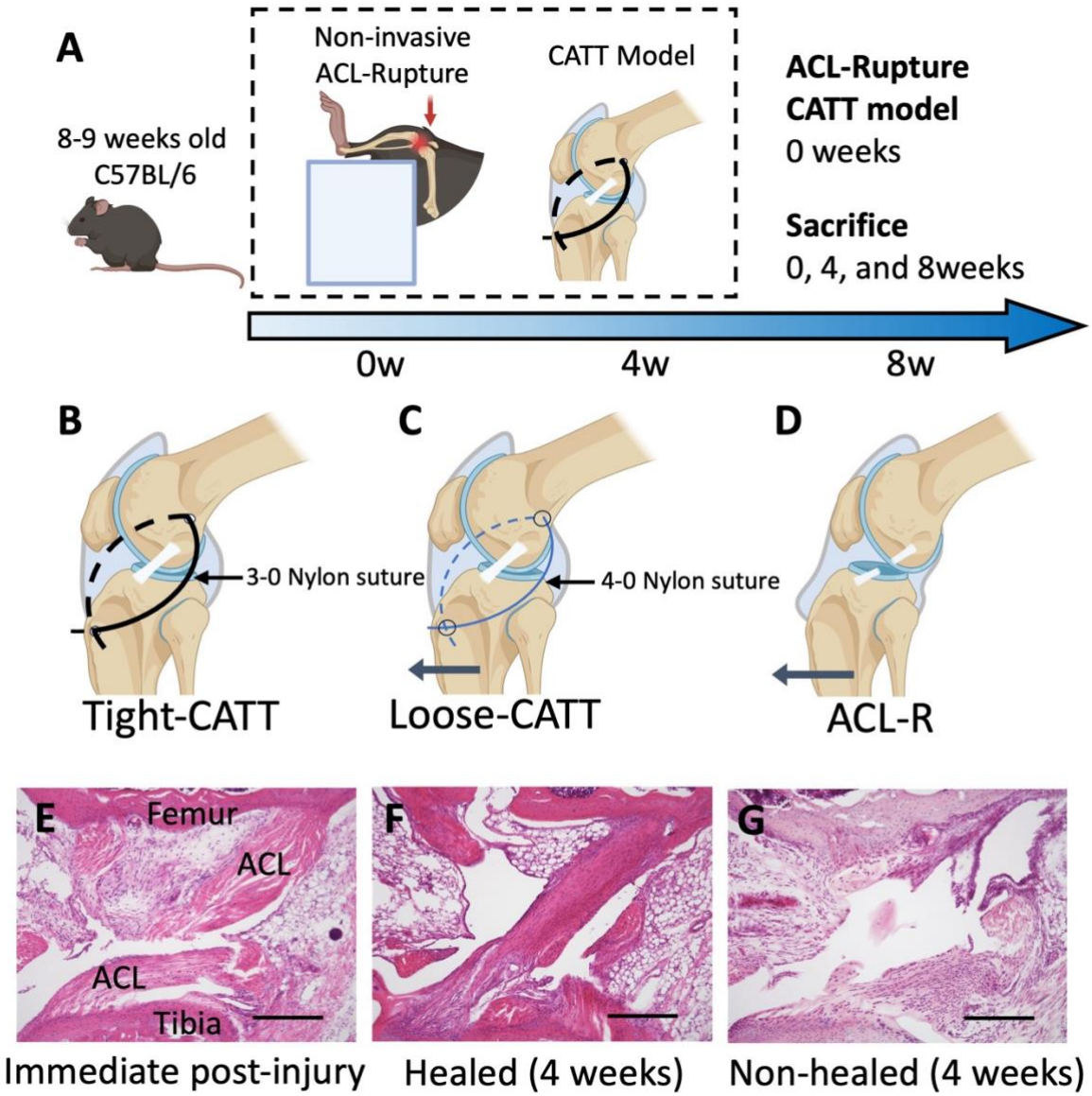

Figure 1. Study design: Mice were ruptured ACL non-invasively and created the CATT model immediately post-injury and sacrificed post-injury immediately, at 4- and 8-weeks post-injury (A). Mice were randomly classified into the Tight-CATT (B), Loose-CATT (C), and ACL-R (D) groups. The images of the ACL immediately after injury (E), the healed ACL (F), and the non-healed ACL (G) (Scale bar = 300 μm). This schematic was made using https://www.biorender.com/

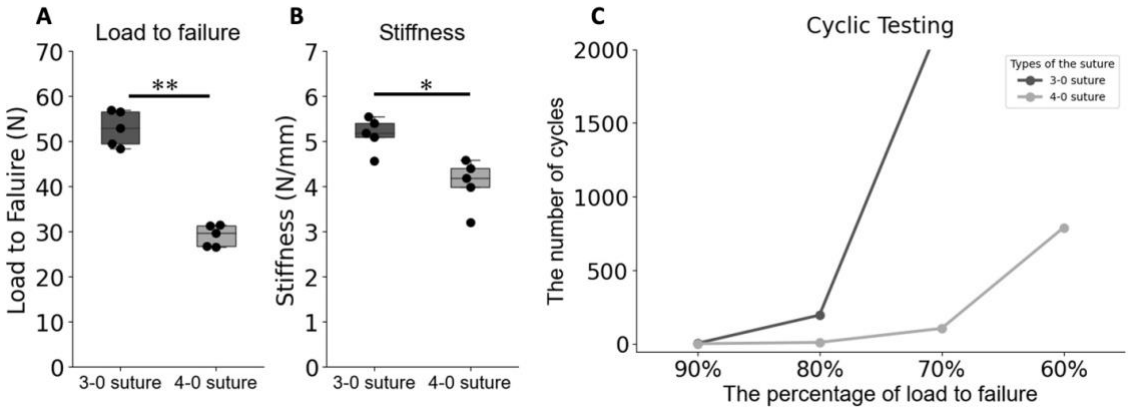

Figure 2. The load to failure (A) and stiffness (B) of the nylon suture in failure testing, and the number of cycles at the point of suture rupture in cyclic testing (C) were recorded (*$p < 0.05$, **$p < 0.01$).

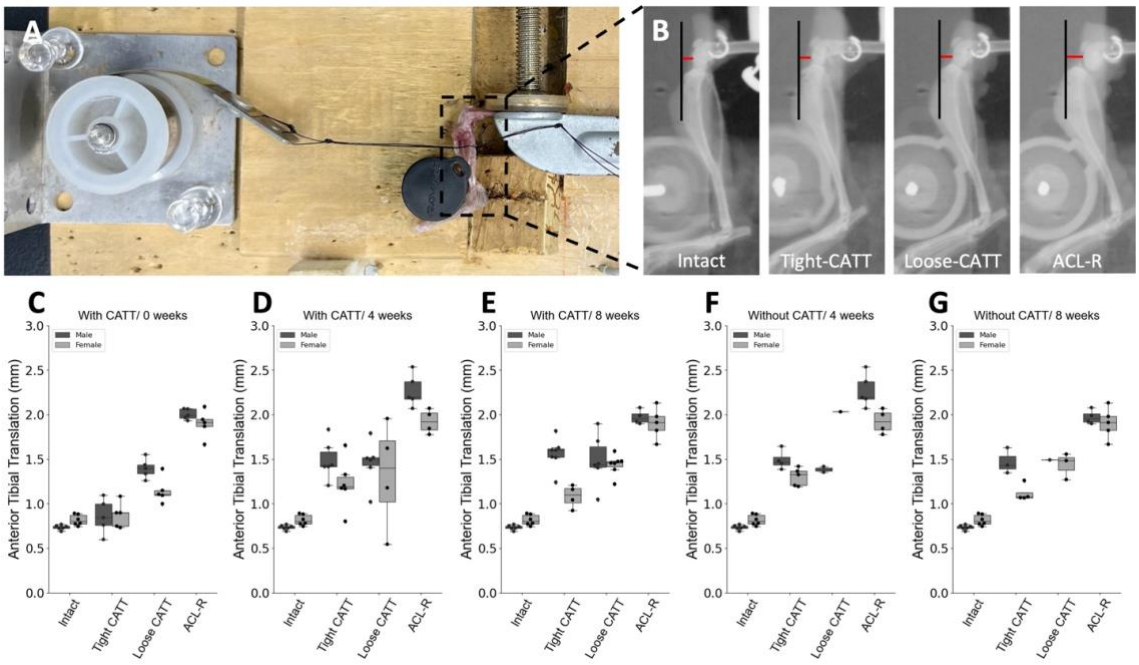

Figure 3. Anterior drawer testing was performed with the knee joint fixed at 90° via the self-made anterior drawer device (A). Representative X-ray images of each group (B). We evaluated the braking force of the ATT for each group immediately after injury (C), at 4 (D) and 8 weeks (E), and anterior tibial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

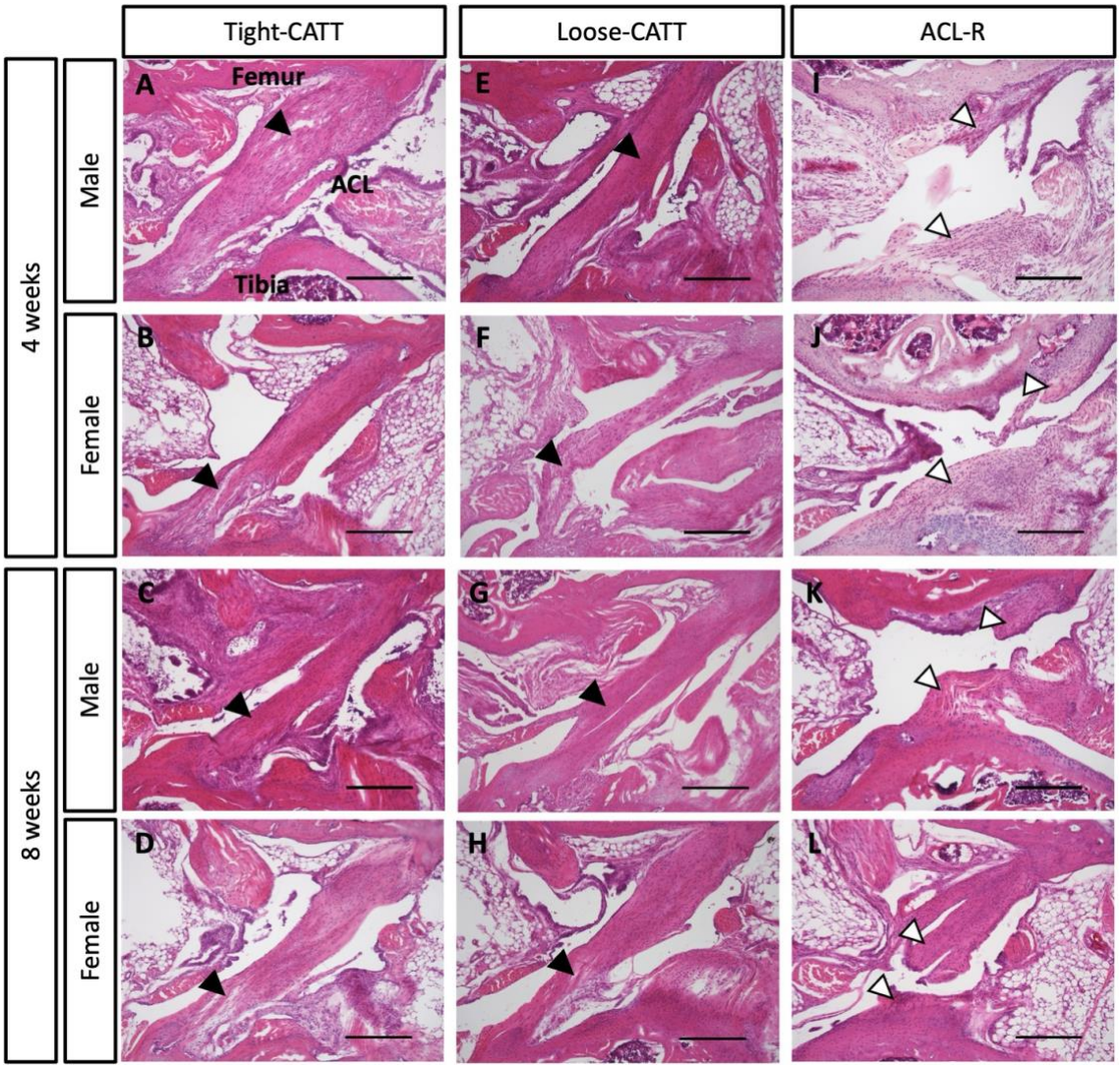

Figure 4. HE stained images of the Tight-CATT (A, B, C, and D), Loose-CATT (E, F, G, and H), ACL-R groups (I, J, K, and L) at 4 and 8 weeks (Scale bar = 300 μm). The black arrows point to the healing region of the healed ACL and the white arrows point to the non-healed ACL.

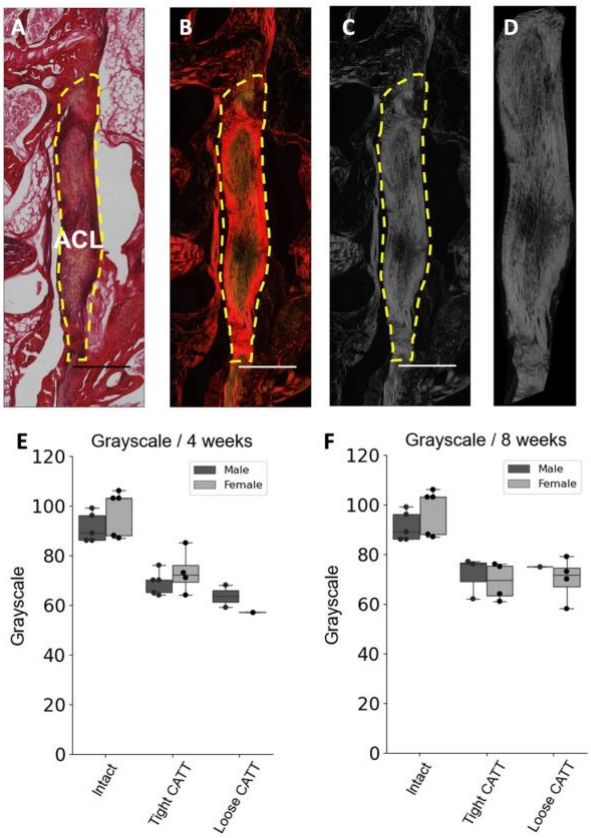

Figure 5. Picrosirius red stained (A) and polarized image (B) of the ACL. Picrosirius red images were converted to grayscale images (C) and the specified ACL area was specified as ROI (D). Collagen maturation of the ACL was evaluated at 4 (E) and 8 weeks (F) post-injury (Scale bar = 300 µm).

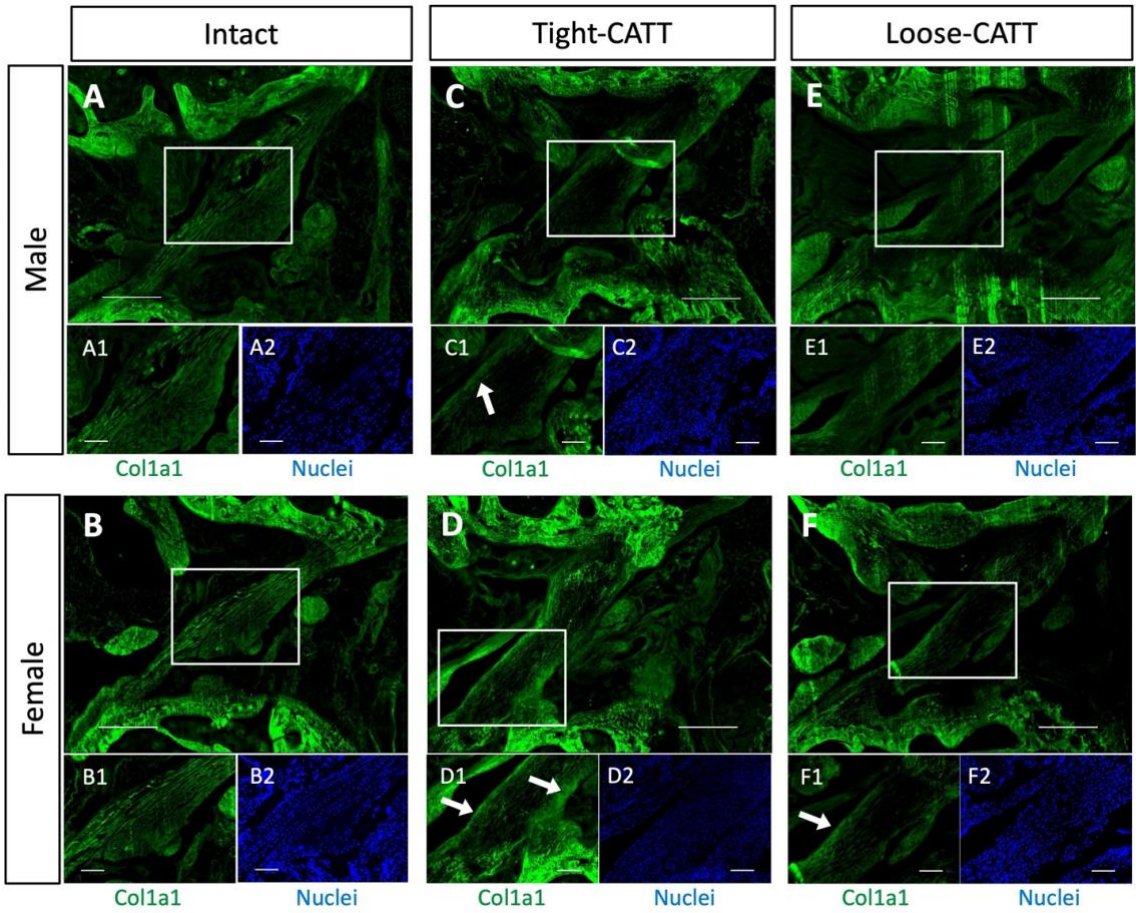

Figure 6. Immunofluorescence images targeted COL1A1 of the intact ACL (A, B), healed ACL of the Tight-CATT (C, D), and Loose-CATT (E, F) groups at 8 weeks (Scale bar = 300μm) and showing mid-substance of the ACL: COL1A1 (green) and DAPI (blue) (Scale bar = 100μm). Arrows pointed to areas of high COL1A1 expression.

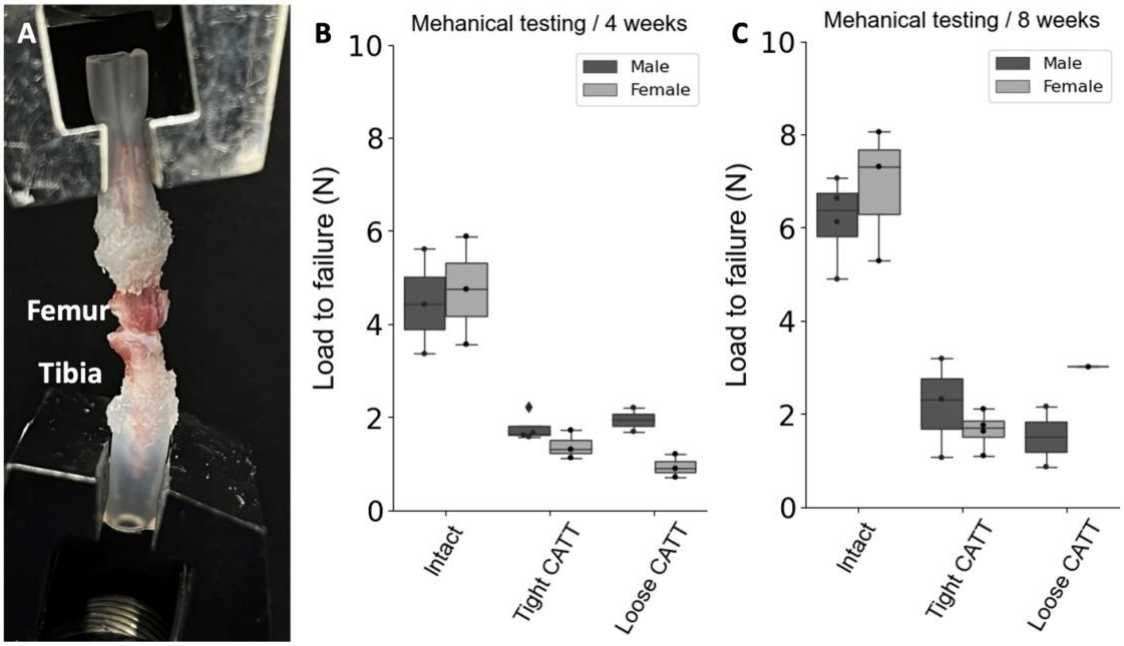

Figure 7. To perform biomechanical testing, the soft tissues of the knee, except the ACL, were dissected, and the femur and tibia were fixed in tubes (A). The load to failure (A) of the healed ACL at 4 (B) and 8 (C) weeks after injury was recorded.