# Title Page Template

**Title of the Manuscript**

**Early and Adequate Braking of Abnormal Tibial Translation Determines Spontaneous Healing Success of the Anterior Cruciate Ligament in a Murine Model.**

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**Declarations**

*(When not applicable, please indicate "N/A")*

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- **List of abbreviations in the text: List of Abbreviations**

ACL: Anterior Cruciate Ligament

ACL-R: Anterior Cruciate Ligament Rupture

ANOVA: Analysis of Variance

ART: Aligned Rank Transform

ATT: Anterior Tibial Translation

CATT: Controlled Anterior Tibial Translation

CBP: Cross Bracing Protocol

COL1A1: Type I Collagen

ECM: Extra-Cellular Matrix

HE: Hematoxylin and Eosin

MCL: Medial Collateral Ligament

PsR: Picrosirius Red

*Abstract*

Purpose: The purpose of this study was to elucidate the acceptable range of mechanical stresses during the early phase of spontaneous anterior cruciate ligament (ACL) healing. We developed models with varying degrees of controlled anterior tibial translation (CATT) using nylon sutures of two diameters.

Methods: Male and female C57BL/6 mice were ruptured ACL and randomly classified into three groups: Tight-CATT; controlled anterior tibial translation (ATT) with 3-0 nylon suture, Loose-CATT; controlled ATT with 4-0 nylon suture, 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 braking force to control the ATT of each knee by anterior drawer testing, the success rate of the ACL healing, collagen maturation and COL1A1 expression with immunofluorescence in the healed ACL, and the mechanical strength 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 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. On the other hand, tightly ATT in the early

healing phase was insufficient to improve mechanical strength and collagen maturation. Therefore, our results suggest that tightly controlling abnormal tibial translation in the early phase promotes spontaneous ACL healing, and that it may be necessary to apply higher mechanical stress in the later stages to achieve greater healing.

*Introduction*

Anterior cruciate ligament (ACL) rupture is one of the most common knee injuries across all generations. In the United States, it is estimated to occur in more than 100,000 cases per year [1, 2] and impose a substantial economic burden, with annual treatment and related societal costs estimated in the multi-billion-dollar range [3,4]. For a long time, researchers considered that injured ACLs had a low capacity for spontaneous healing due to the unique environment within the joint capsule [5–7]. Therefore, ACL reconstruction is the current gold standard treatment. ACL reconstruction can restore static and dynamic knee stability following ACL injury and facilitate return to pre-injury levels of activity [8]. However, several reviews have reported that ACL reconstruction does not yield superior patient-reported outcomes compared with rehabilitation alone and provides little benefit in preventing the development of knee osteoarthritis [9,10]. Recently, the proportion of older patients sustaining ACL injuries has increased relative to that of younger patients [11–13]. ACL reconstruction in older individuals has been reported to be associated with an increased risk of developing knee osteoarthritis, and the appropriateness of surgical treatment in this population therefore remains controversial [14,15].

Over the past several decades, growing evidence has suggested that the ACL has the potential to heal spontaneously without surgical intervention [16–20]. Ihara et al. reported that ACLs were healed spontaneously by minimizing abnormal sagittal deviation between the femur and tibia

using a knee brace following ACL injury [16,17]. Subsequently, Filbay et al. reported clinical outcomes of spontaneous ACL healing using the Cross Bracing Protocol (CBP), which maintained the knee at 90 degrees in patients with ACL injuries [21]. They showed that 90% of patients had evidence of healing on MRI after a three-month post-injury period. In addition, we previously showed that controlling anterior tibial translation following ACL injury enables spontaneous ACL healing in a rodent model [22]. Therefore, non-surgical treatment will probably be a valuable therapeutic option, particularly for patients who prefer to avoid surgical treatment. However, spontaneously healed ACLs that had undergone anterior tibial displacement formed scar tissue and exhibited lower mechanical strength than normal tissue. The next step is to identify the conditions that promote healing of the spontaneously healed ACL. It is well established that appropriate mechanical stress during ligament and tendon healing promotes tissue maturation and recovery of mechanical strength [23–25]. 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 for ACL-injured patients.

Therefore, the purpose of this study was to elucidate the acceptable braking condition after ACL injury. To achieve this goal, we developed two types of controlled anterior tibial translation (CATT) models using nylon sutures of two different diameters. Suture differences conferred varying degrees of anterior tibial translation (ATT) stability in murine knees. We hypothesized that a mild

125 increase in mechanical stress during the early phase of ACL healing does not inhibit healing but
126 rather contributes to improved tissue maturation and mechanical strength recovery of the healed
127 ACL.
128
129 *Methods*
130 *Experimental Design*
131 The University Animal Experiment Ethics Committee approved all experiments. We used
132 male (n=66) and female (n=66) 8-9 weeks old C57BL/6 mice (n=132). A total of 132 mice were
133 ruptured the left ACL and then were randomly classified into three groups: Tight-CATT group
134 (Figure 1B); controlled ATT with 3-0 nylon suture (n=57), Loose-CATT group (Figure 1C);
135 controlled ATT with 4-0 nylon suture (n=55), and ACL-R (ACL-Rupture) group (Figure 1D); not
136 controlled ATT (n=20). We used the right hindlimb as the intact group. All mice were housed in
137 plastic cages under a 12-h light/dark cycle. The mice were allowed unrestricted movement within the
138 cage and had free access to food and water. Three animals from both the Tight-CATT and Loose-
139 CATT groups were excluded from the experiment due to tibial fractures when the ACL ruptured,
140 and the abnormal findings during housing. Thus, a total of 126 mice were included in this study, and
141 were sacrificed immediately after injury for anterior drawer testing and histology (n=20); at 4 (n=52)

142 and 8 weeks (n=54), for anterior drawer testing, histology, immunofluorescence, and mechanical

143 testing.

144

145 *Nylon Suture Experiments*

146 Before creating the CATT models, we measured the mechanical properties of each nylon

147 suture using a mechanical testing machine (Univert. CellScale Biomaterial Testing, Canada). The

148 purpose of this experiment was to investigate the differences in mechanical properties between 3-0

149 and 4-0 nylon sutures (Akiyama Medical MFG, Co., Ltd., Tokyo, Japan). Each nylon suture was

150 measured in a double layer since a nylon suture was looped outside the joint to control ATT in the

151 CATT model. In the failure testing, we stretched the nylon sutures at a preload of 0.1 N and a rate of

152 20 mm/min until they ruptured. During the test, load (N) and displacement (mm) were recorded, and

153 we measured the load at failure (N) and the stiffness (N/mm). Then, we performed the cyclic test to

154 evaluate viscoelasticity. Nylon sutures were repeated with 5 seconds of stretching up to 90%, 80%,

155 70%, and 60% of the failure load of each nylon suture, followed by 5 seconds of relaxation (1 cycle:

156 10 seconds). We recorded the number of cycles at the point of rupture.

157

158 *Procedure for Creating the Model*

159 All mice were anesthetized with a combination of anesthetics (medetomidine, 0.375 mg/kg;
160 midazolam, 2.0 mg/kg; and butorphanol, 2.5 mg/kg) for sedation and pain control, and then we
161 ruptured the ACL non-invasively according to a previous study [26]. We fixed the hindlimb with the
162 knee joint at 90 degrees and ruptured the ACL by pushing the femur backward against the tibia. In
163 non-invasive ACL ruptures, the ligament is generally ruptured in the mid-substance region (Figure
164 1E). Then, we created bone tunnels at the femoral condyle and proximal tibia using a 26G needle
165 (Terumo Co., Ltd., Tokyo, Japan). In the Tight- and Loose-CATT groups, we tightly looped a single
166 3-0 or 4-0 nylon suture through the two tunnels to control the ATT. In the ACL-R group, we loosely
167 looped a 3-0 nylon suture through the two tunnels not to control the ATT.
168
169 *Anterior Drawer Testing*
170 We performed anterior drawer testing to evaluate the braking force to control the ATT of
171 each knee, carefully removing the muscle from the knee while retaining the suture. Then, we fixed
172 the knee joint at 90 degrees on the self-made anterior drawer testing device. The tibia was pulled
173 forward with a 0.05 kgf constant force spring (Sanko Spring Co., Ltd., Fukuoka, Japan), and X-ray
174 images of the sagittal plane of the knee joint were taken. We measured ATT from X-ray images
175 using ImageJ (National Institutes of Health, Bethesda, MD, USA) to evaluate the braking force
176 exerted by nylon sutures. The ATT was defined as the perpendicular line from the most prominent

177 point of the femoral condyle to the long axis of the tibia. We then removed the nylon suture and
178 again measured the ATT to evaluate the potential characteristics of the knee stability with healed
179 ACL. The knees in which the ACL was not healed in histology were excluded from the latter
180 analysis.
181
182 *Histology*
183 After anterior drawer testing, knee samples were immediately fixed (24-48h) in 4%
184 paraformaldehyde and decalcified with 10% ethylenediaminetetraacetic acid (Sigma-Aldrich Japan,
185 Tokyo, Japan) for 2 weeks. Finally, they were immersed in 5% and 30% sucrose for 4 and 12 hours,
186 respectively, and then freeze-embedded using OCT compound (Sakura Finetek Japan Co., Ltd.,
187 Tokyo, Japan). Each sample was sectioned at 12μm using a Cryostar NX70 (Thermo Fisher
188 Scientific, Kanagawa, Japan). We performed Hematoxylin & Eosin (HE) staining to observe ACL
189 continuity and morphological features, and Picrosirius Red (PsR) staining to evaluate the collagen
190 maturity of the healed ACL. We observed the stained sections with the BZ-X710 microscope
191 (Keyence, Osaka, Japan). We determined whether the ACL was healed from HE images obtained at
192 4 or 8 weeks post-injury. We defined ACLs with observed continuity as “healed” (Figure 1F), and
193 those with lost continuity as “non-healed” (Figure 1G). The success rate of ACL healing in each
194 group was calculated based on the HE images. PsR images converted to grayscale images (256

195 degrees) showed collagenous tissue as 1-255 on the grayscale and non-collagenous tissue as 0 on the
196 grayscale. A higher gray scale indicates higher ACL collagen maturation [27,28]. Using the BZ-
197 X710 analysis software, the entire ACL areas in the PsR polarization images were defined as
198 Regions of Interest (ROIs) and then converted to grayscale. We used the average grayscale value in
199 the ROI as the representative value of the healed ACL.
200
201 *Immunofluorescence*
202 We performed immunofluorescence to evaluate the expression of type I collagen
203 (COL1A1) in the healed ACL. ACL cryosections were blocked at room temperature for 1 hour using
204 DAKO Antibody Diluent with Background Reducing Components (Agilent Technologies, Inc., CA,
205 USA). The anti-COL1A1 primary antibody was incubated with a Goat polyclonal antibody (1:200;
206 AB758, Merck, Sigma-Aldrich, Taufkirchen, Germany) at 4°C overnight. The secondary antibody
207 was incubated with anti-Goat Rabbit antibody Alexa Fluor 488 (1:500, ab150141, Abcam,
208 Cambridge, UK) for 4 hours at room temperature. All sections were examined macroscopically
209 using a BZ-X710 microscope under controlled imaging conditions.
210
211 *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) [29,30] 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 $p < 0.05$.

230

231 *Results*

232 *Mechanical Properties of Two Nylon Sutures*

233 The results showed that the 3-0 nylon sutures had better mechanical properties. The load to
234 failure and stiffness were significantly higher for 3-0 nylon sutures ($p < 0.05$) (Figure 2A, B). In
235 cyclic testing, the 3-0 nylon sutures exhibited higher viscoelasticity and greater resistance to
236 elongation. The 4-0 nylon sutures ruptured in 3 cycles at 90% of the failure load, 12 cycles at 80%,
237 107 cycles at 70%, and 791 cycles at 60%. The 3-0 nylon sutures ruptured in 6 cycles at 90% and in
238 197 cycles at 80%. 3-0 nylon sutures did not rupture even after 2,000 cycles at loads lower than 70%
239 (Figure 2C).

240

241 *Magnitude of Anterior Translation of Tibia*

242 First, in the knee joints with nylon sutures for controlling ATT, interactions between groups
243 and sex were observed at all timepoints ($p < 0.01$). Immediately after injury, the ATT was smallest
244 in the intact and Tight-CATT groups. The Loose-CATT group showed a higher ATT than the Tight-
245 CATT group, and the ACL-R group showed the highest ATT. However, there were no significant
246 differences among the groups or by sex (Figure 3C). In males at 4 and 8 weeks after injury, the ATT
247 was smallest in the intact group, followed by the Tight-CATT and Loose-CATT groups, which

248 showed similar ATT. The ACL-R group had the highest ATT. In contrast, in females, the ATT of
249 the Tight-CATT showed a trend to be smaller than that of the Loose-CATT. However, there were no
250 significant differences among the groups or by sex (Figures 3D, E).

251 Secondly, for anterior tibial instability of the knee with a healed ACL, fewer knees were
252 included in the Loose-CATT group because we excluded knees with non-healed ACLs from the
253 CATT group in this analysis. Interactions between groups and sex were observed at 4 weeks and 8
254 weeks ($p < 0.001$). In males at 4 and 8 weeks, intact had the smallest ATT, and the Tight-CATT and
255 Loose-CATT groups had similar ATT but higher ATT than intact. ACL-R showed the highest ATT.
256 In females, the ATT of the Tight-CATT showed a trend to be smaller than that of the Loose-CATT.
257 However, there were no significant differences among the groups or by sex (Figures 3F, G).

258

259 *ACL Healing Success Rate and Collagen Maturation of the ACL*

260 First, we reported the success rates of ACL healing in the Tight- and Loose-CATT groups at
261 4- and 8-week post-injury (Table 1). The Tight-CATT group showed a higher healing rate for both
262 males and females (42.9~100%), while the Loose-CATT group showed a lower healing rate
263 (17~57.1%) and no healed ACL in the ACL-R group (0%). Next, we showed HE-stained images of
264 the healed ACL. Compared to the intact ACL with parallel and regular nuclear arrangement, the
265 healed ACL showed heterogeneous nuclear aggregation not only in the healed region but also in the
266 ACL remnant region. It was also observed at 4- and 8-week post-injury (Figure 4A-H).

267 In collagen maturation of the healed ACL, there are no interactions observed at both 4- and
268 8-week post-injury. On the other hand, a significant main effect was observed in the group: the
269 Tight-CATT group had a significantly lower grayscale than intact ($p < 0.001$) at 4 weeks, and the
270 Tight- and Loose-CATT groups showed a significantly lower grayscale than intact ($p < 0.01$) at 8
271 weeks (Figure 5E, F).
272
273 *Type I Collagen Content of Healing ACL*
274 We performed immunofluorescence for COL1A1, a major ECM component, to assess ECM
275 maturity in the healed ACL. The green area indicates COL1A1, and the blue area indicates the
276 nucleus. In the intact ACL, we observed COL1A1 expression at the entire ACL region and nuclei
277 aligned longitudinally of the ACL (Figure 6A, B). On the other hand, the healed ACLs of the Tight-
278 and Loose-CATT groups showed limited expression outside the healed ACL and an irregular
279 orientation of COL1A1 compared with the intact ACL. In addition, the Tight- and Loose-CATT
280 groups showed higher and irregular nuclei aggregation throughout the ACL (Figure 6C-F). These
281 collagen features of the ACL were commonly observed at 4- and 8-weeks post-injury (Shown in
282 Figure 6 is at 8 weeks post-injury).
283
284 *Mechanical Properties of Healed ACL*

285 We fixed the femur and tibia in 1.5-mm tubes (Figure 7A) and performed mechanical testing
286 to evaluate the mechanical strength of the healed ACL. The number of ACLs for testing in the
287 Loose-CATT group was fewer because only the knees with confirmed ACL re-connections were
288 included when the knees were dissected. No interaction was observed at both 4- and 8-week post-
289 injury. On the other hand, a significant main effect was observed in the group: at 4 weeks, intact had
290 a significantly higher mechanical strength than the Tight-CATT ($p < 0.01$) and Loose-CATT group
291 ($p < 0.05$) (Figure 7B), and at 8 weeks, intact had a significantly higher mechanical strength than the
292 Tight-CATT group ($p < 0.01$) (Figure 7C). However, the healed ACLs in the Tight- and Loose-
293 CATT groups exhibited similar mechanical strength and were approximately 50% of normal ACL
294 strength.
295

296 *Discussion*

297 The most important finding of this study was that loosening of the controlling ATT
298 immediately after ACL injury is a negative factor for spontaneous healing of the ruptured ACL. The
299 results of the Loose-CATT group showed that increased mechanical stress immediately after injury
300 negatively affects ACL healing response. Conversely, the healed ACLs in both the CATT group
301 showed lower mechanical properties than those in the Intact group, and there were no significant
302 differences in mechanical properties or histological features between tight and loose conditions.
303 Overall, our results suggest that appropriate force control may be necessary to improve the success
304 rate of healing and the mechanical properties of the healed ACL.

305 In this study, we developed CATT models with varying levels of ATT braking force, using
306 two types of nylon suture, immediately after ACL injury. Results of the nylon suture experiment
307 showed that the 4-0 nylon suture used in the Loose-CATT group had higher suture elasticity
308 compared to the 3-0 nylon suture used in the Tight-CATT group. Subsequently, we confirmed in the
309 anterior drawer test that the Loose-CATT group exhibited a lower braking force than the Tight-
310 CATT group immediately after ACL injury. Therefore, the Loose-CATT group was defined as the
311 group with early exposure to the higher mechanical stress immediately after ACL injury. A clinical
312 study showed a lower rate of successful healing in younger ACL-injured patients because they have
313 higher activity levels compared with middle-aged and older patients[17], which supports our

314 findings. On the other hand, another study reported that 90% of ACL-injured patients had healed
315 ACLs with knee joints immobilized at 90 degrees using orthotics [21]. Furthermore, while proper
316 mechanical stress after ligament and tendon injury promotes healing [23,24,31–33], excessive
317 mechanical loading impairs biomechanical properties and tissue maturation [34–36]. In the ligament-
318 healing process, vascular-rich granulation tissue invades the ligament rupture site, initially sealing it
319 and then gradually replacing it with fibrous tissue [37]. The increase in ATT in Loose-CATT may
320 have increased mechanical stress on the ACL immediately after injury and physiologically inhibited
321 the formation of a fragile provisional matrix. In addition, loosening of the ATT would induce
322 abnormal biological modulation in the ACL healing. Excessive mechanical stress on ligaments and
323 tendons induces fibrous tissue to transition to an abnormal matrix, characterized by increased
324 proteoglycan content and type III collagen.[38,39]. In the wound-healing process, abnormal
325 mechanical stress prolongs inflammation, delays wound closure, and is a significant factor in
326 hypertrophic scar formation [40,41]. In our previous data, we reported that controlling abnormal
327 tibial motion after ACL injury can reduce MMPs that cause matrix degradation in the ACL
328 [22,42,43]. Therefore, increased ATT in the early post-injury period may have disrupted the fragile
329 ligaments physiologically or promoted the catabolic process of the injured ACL biochemically. Our
330 results suggested that loose braking by ATT inhibits early ACL healing.

331 Interestingly, we also found sex differences in ACL healing success rates. It is known that
332 there are sex differences in ACL injury mechanisms, and the ACL injury rate in women is 2~6 times
333 higher than that in men [3,44,45]. On the other hand, there is insufficient consensus regarding sex
334 differences in clinical outcomes after injury [46,47]. Surprisingly, our findings showed that the ACL
335 healing success rate was higher in females than in males. While the similar ATT braking force in the
336 male and female Tight-CATT group was observed immediately after injury, the ATT braking of the
337 male Tight-CATT group decreased after 4 weeks post-injury, compared with the female. These
338 results may be attributable to differences in cage activity between male and female mice. Previous
339 studies have reported that the amount of spontaneous exercise in wheel-running is higher in females
340 among C57BL/6 mice [48]. Additionally, a study examining home-cage activity levels found that
341 females exhibited significantly higher activity in the dark time [49]. In contrast, males exhibit more
342 aggressive behaviors, such as fighting and mounting, when kept with two or more animals [50]. In
343 this study, we housed 2-4 mice in one cage for both sexes. Based on this knowledge, male mice
344 might show more intense activity than females in a cage. Additionally, standing on two limbs in a
345 fighting or defending motion is more unstable than walking on four limbs [51,52], which would
346 increase stress on the knee joints, particularly the healing ACL. Thus, the aggressive behavioral
347 characteristics of male mice may have contributed to the reduced ACL healing rate. This finding
348 underscores the importance of balancing the quality and quantity of exercise during ACL healing.

349 Further study is needed to clarify the relationship between behavioral characteristics of the mice and
350 ACL healing.
351 We hypothesized that the increased mechanical stress on the healing ACL immediately after
352 injury would promote collagen maturation and mechanical strength, even if it led to a low success
353 rate in ACL healing. However, against our expectations, there was no difference in the quality of the
354 healed ACL between the Tight- and Loose-CATT groups. The healed ACLs in Tight- and Loose-
355 CATT groups showed similar collagen maturation and mechanical strength, and inferior properties
356 compared to the intact ACL. Accordingly, tight ATT braking is important for a higher success rate
357 of ACL healing in the early phase. On the other hand, it is suggested that an increase in mechanical
358 stress at the level of loosening does not promote ACL healing, because loosening occurs in both
359 Tight- and Loose-CATT and the quality of the healed ACL shows no difference. Previous studies
360 have shown that low-intensity exercise during the healing process of the supraspinatus tendon is
361 beneficial for maintaining structure and promoting repair in the early stages of healing, but is
362 insufficient to promote long-term healing [25]. In ACL-reconstructed mice, proper timing of
363 mechanical stress was more effective at promoting healing than immediate postoperative or delayed
364 mechanical stress [31]. This study revealed that the mild increase in mechanical force induced by
365 loosening is insufficient to improve ECM synthesis or the mechanical strength of the healed ACL.
366 Although our results suggest the importance of tight control of abnormal tibial translation in the

367 early phase after ACL injury, the optimal mechanical force for promoting ACL healing remains
368 unclear. Future study will focus on this point.
369 In this study, we have several limitations. First, mice are a commonly used model for
370 research on ligament and tendon healing. However, the structure and alignment of the mouse knee,
371 which is always flexed, differ from those of the human knee, which is typically extended. Therefore,
372 the mice are not a model that completely reconstructs the human condition. Second, the present data
373 were evaluated immediately after injury and at 4 and 8 weeks after injury. It was impossible to
374 evaluate differences in ATT and the healing process from 0 to 4 weeks. Therefore, we will analyze
375 at finer time points in the future to identify when CATT is no longer necessary. We will then be able
376 to determine the period during which orthotic management is necessary following ACL injury.
377 Finally, the current study examined, first, how increased mechanical stress affects ACL healing and
378 subsequent properties. Hence, it was unable to elucidate in detail the mechanisms by which
379 increased mechanical stress impaired ACL healing in the early stages of healing. In the future, we
380 will analyze biochemical data and intracellular changes up to 4 weeks after ACL injury to elucidate
381 the mechanism of mechanical transduction in ACL healing.
382 In this study, we investigated the effects of varying ATT braking forces on the ACL
383 spontaneous-healing success rate and the quality of the healed ACL in mice using CATT models
384 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 ACL healing mechanisms and informing conservative rehabilitation.

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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.

**Figure Legend**

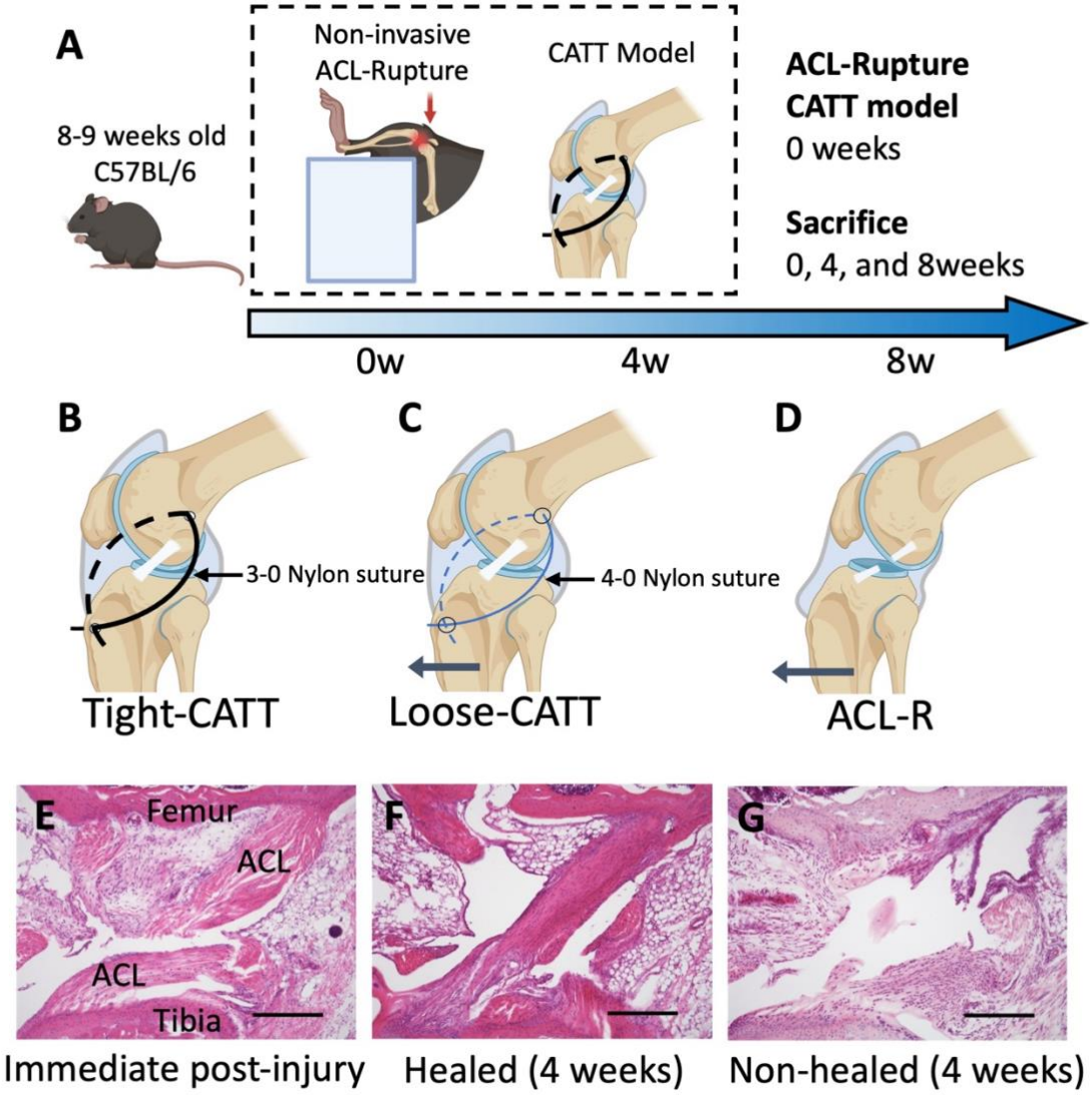

**Fig. 1** Study design: Mice were ruptured ACL non-invasively and created the CATT model immediately post-injury and sacrificed post-injury immediately, at 4- and 8-weeks post-injury (A). Mice were randomly classified into the Tight-CATT (B), Loose-CATT (C), and ACL-R (D) groups. The images of the ACL 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/

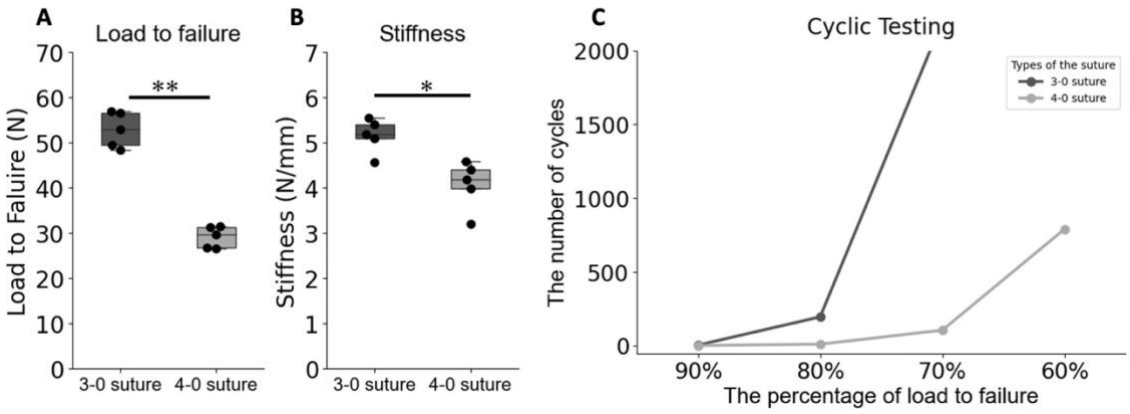


**Fig. 2** The load to failure (A) and stiffness (B) of the nylon suture in failure testing, and the number of cycles at the point of suture rupture in cyclic testing (C) were recorded (*$p < 0.05$, **$p < 0.01$).

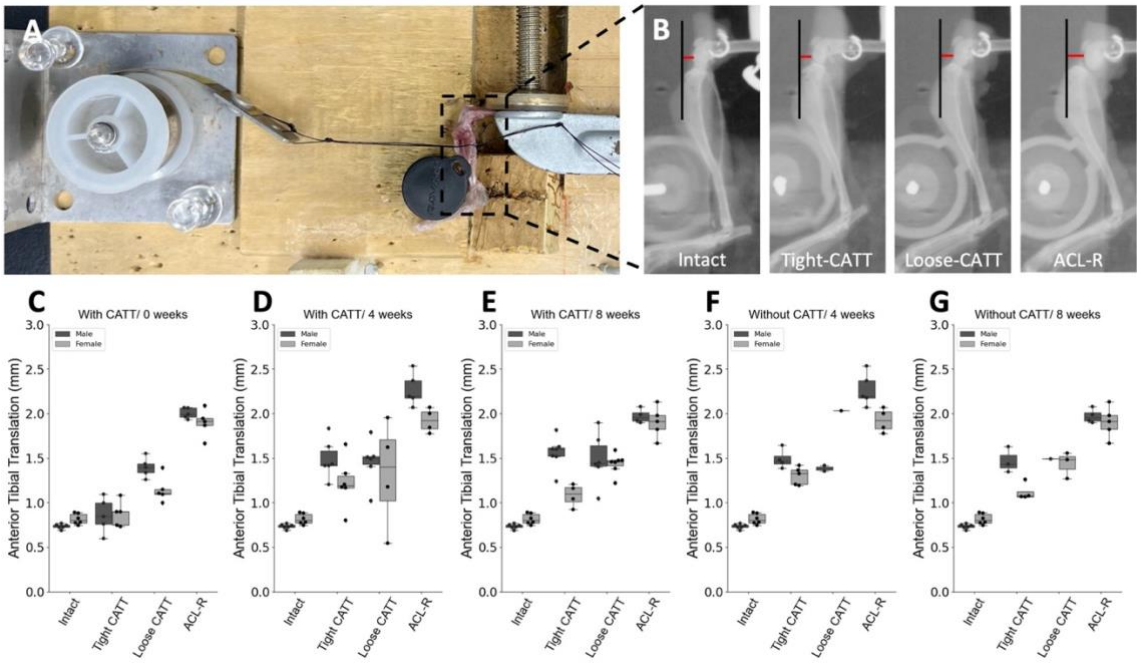


**Fig. 3** Anterior drawer testing was performed with the knee joint fixed at 90° via the self-made anterior drawer device (A). Representative X-ray images of each group (B). We evaluated the braking force of the ATT for each group immediately after injury (C), at 4 (D) and 8 weeks (E), and anterior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

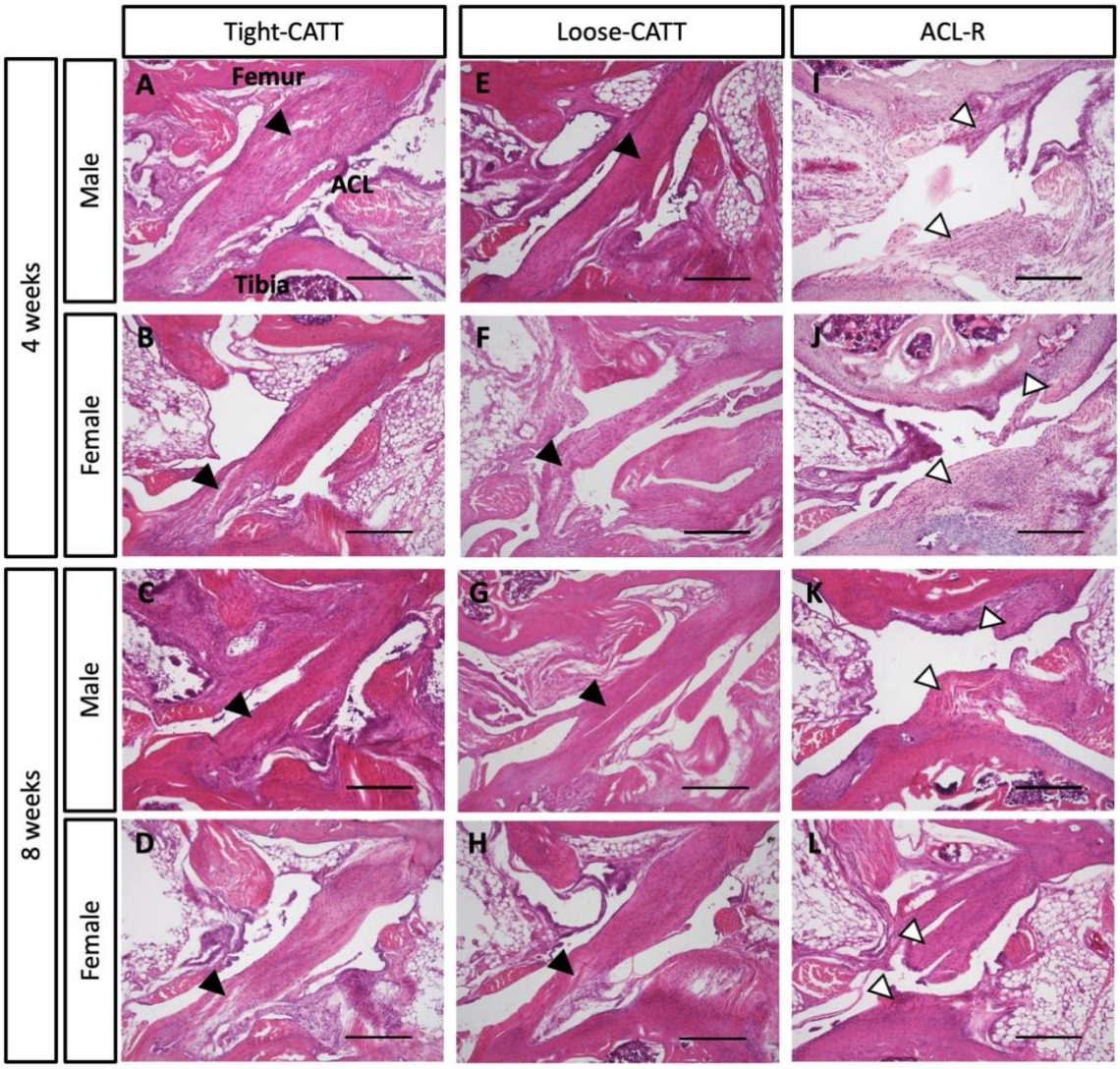


**Fig. 4** HE stained images of the Tight-CATT (A, B, C, and D), Loose-CATT (E, F, G, and H), ACL-R groups (I, J, K, and L) at 4 and 8 weeks (Scale bar = 300 μm). The black arrows point to the healing region of the healed ACL and the white arrows point to the non-healed ACL.

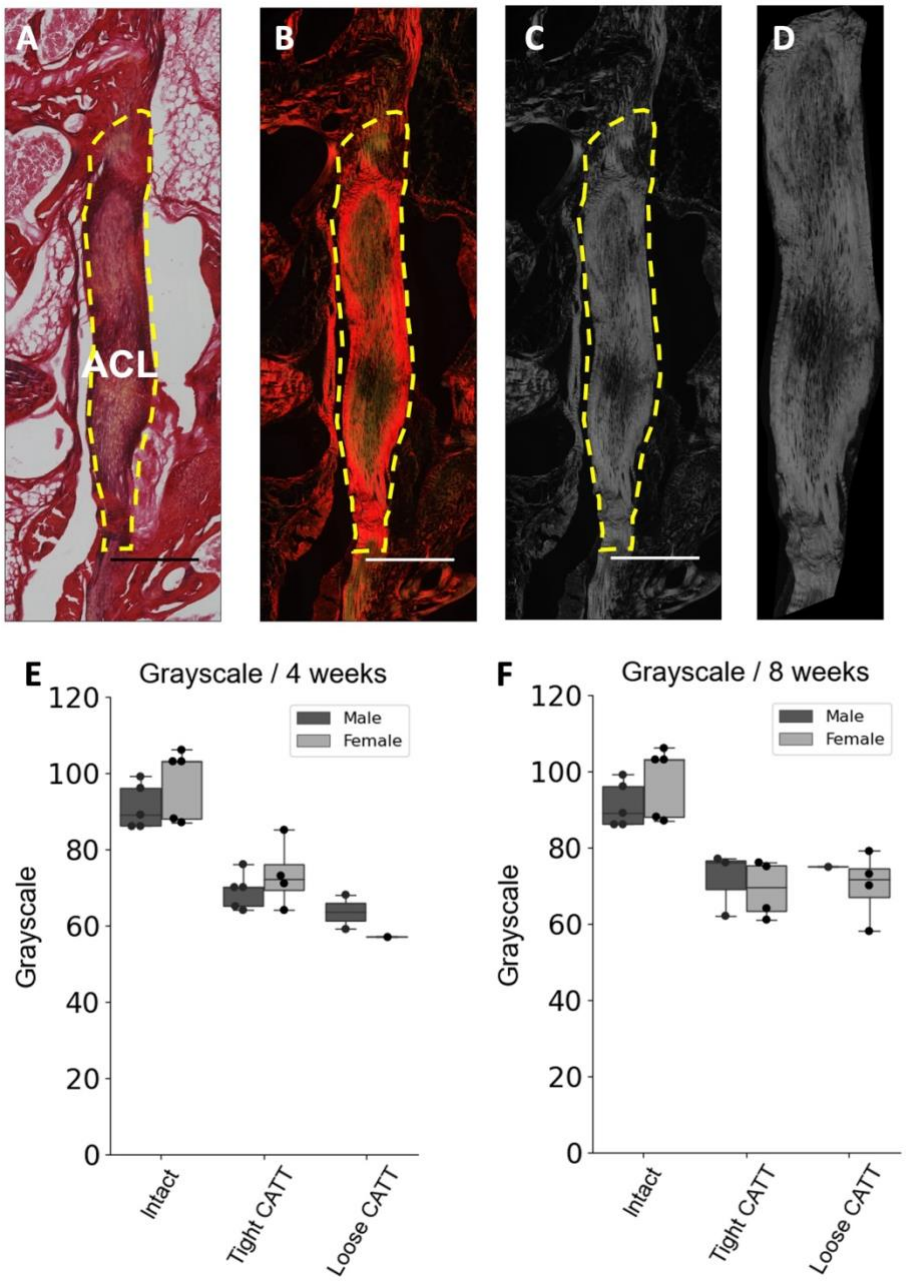


**Fig. 5** Picrosirius red stained (A) and polarized image (B) of the ACL. Picrosirius red images were converted to grayscale images (C) and the specified ACL area was specified as ROI (D). Collagen maturation of the ACL was evaluated at 4 (E) and 8 weeks (F) post-injury (Scale bar = 300 μm).

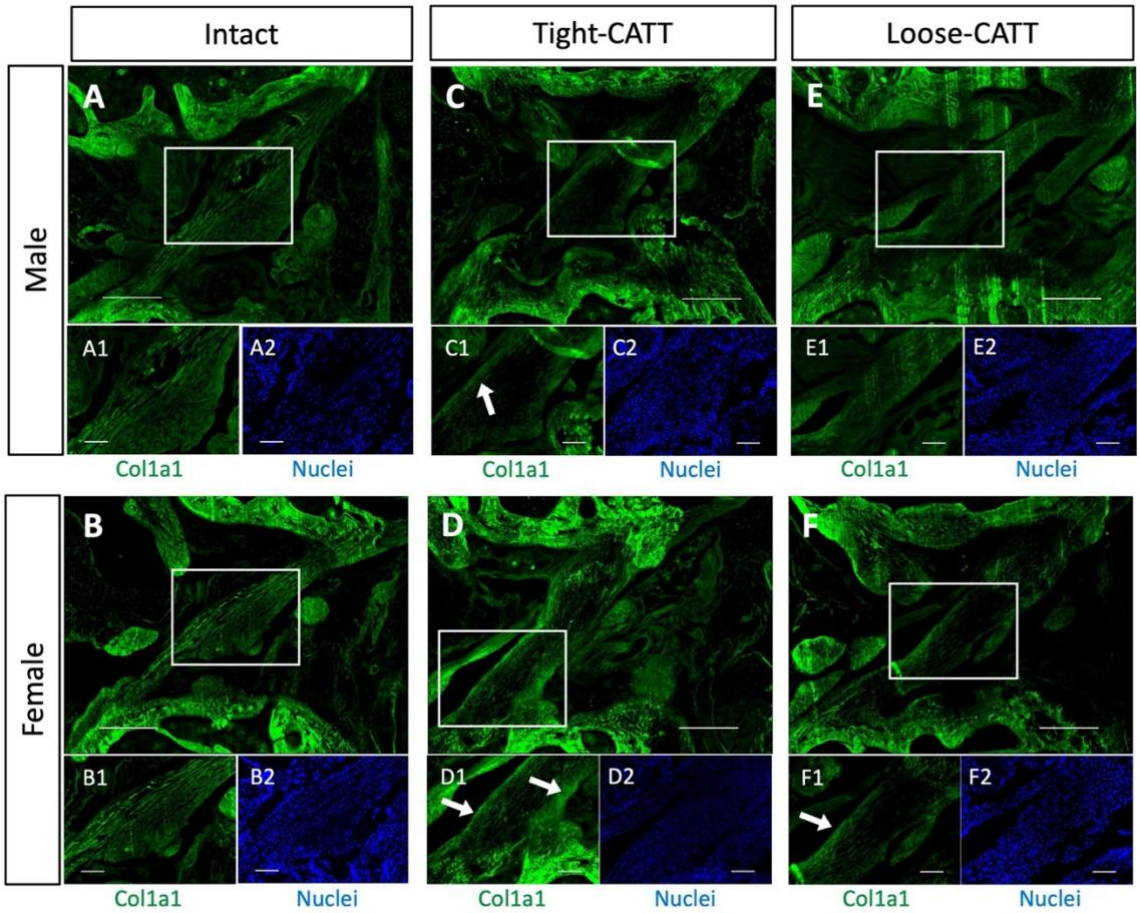


**Fig. 6** Immunofluorescence images targeted COL1A1 of the intact ACL (A, B), healed ACL of the Tight-CATT (C, D), and Loose-CATT (E, F) groups at 8 weeks (Scale bar = 300μm) and showing mid-substance of the ACL: COL1A1 (green) and DAPI (blue) (Scale bar = 100μm). Arrows pointed to areas of high COL1A1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

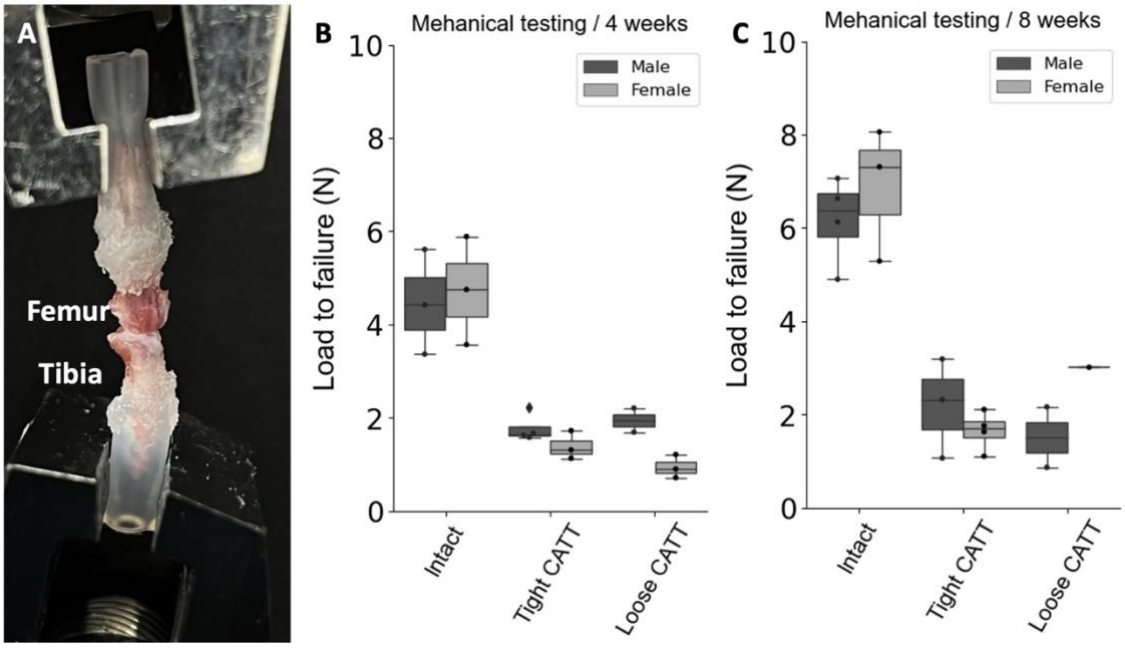


**Fig. 7** To perform biomechanical testing, the soft tissues of the knee, except the ACL, were dissected, and the femur and tibia were fixed in tubes (A). The load to failure (A) of the healed ACL at 4 (B) and 8 (C) weeks after injury was recorded.