Grafted Tendon Healing in Tibial Tunnel Is Inferior to Healing in Femoral Tunnel After Anterior Cruciate Ligament Reconstruction: A Histomorphometric Study in Rabbits

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Purpose: This study aimed to test whether graft healing in the tibial tunnel was inferior to that in the femoral tunnel after anterior cruciate ligament (ACL) reconstruction in rabbits. **Methods:** Surgical reconstruction by use of the digital extensor tendon in the bone tunnel was performed in 18 rabbits. The rabbits were killed at weeks 2, 6, and 12 postoperatively, with 6 at each time point, for histologic examination. **Results:** The transiently formed cartilaginous interface was gradually mineralized during re-establishment of direct tendon-to-bone integration, which was observed significantly less in the tibial tunnel than in the femoral tunnel ($P < .05$). The cell density of the graft was significantly lower in the tibial tunnel than that in the femoral tunnel at weeks 2 and 6 postoperatively ($P < .05$ for both). An increase in the immature type III collagen content was accompanied by a decrease in graft collagen fiber organization, with healing over time in both the femoral and tibial tunnels. The collagen fiber organization of the graft was significantly poorer in the tibial tunnel than that in the femoral tunnel at week 12 after surgery ($P < .05$). **Conclusions:** Grafted tendon healing in the tibial tunnel was inferior to that in the femoral tunnel at the tendon-to-bone interface and with regard to the grafted tendon within the bone tunnel after ACL reconstruction in rabbits. **Clinical Relevance:** Future biopsy study is desirable to test whether this observation was valid clinically, which might provide a scientific basis for therapeutic targets to improve the outcome of ACL surgery.

Arthroscopic reconstruction of grafted tendon in the femoral and tibial tunnels is a common surgical procedure to replace a torn anterior cruciate ligament (ACL) in patients. Yet 11% to 32% of patients showed an unsatisfactory prognosis, and up to 10% required surgical revision.[1-3] Unsecure or failed graft healing in the bone tunnel was one of the major causes of surgical revision.[4,5] Graft healing included graft incorporation to the surrounding bone and intraosseous graft remodeling.[4,5] Rodeo et al.[6] described that graft incorporation relied on bony ingrowth at the tendon-to-bone (T-B) healing interface for T-B collagen fiber reconnection. An immunohistochemical study showed that the process of such bony ingrowth during T-B healing resembled endochondral ossification.[7] Graft remodeling in the bone tunnel required host cell repopulation and subsequent matrix deposition,[8] which was slower and later than that for graft incorporation.[7]

It has commonly been considered that graft fixation is more problematic in the tibial tunnel than in the femoral tunnel, in association with a lower bone mass in the tibial tunnel.[9,10] However, the influence of such disparity in the osseous environment on graft healing

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has never been investigated. Theoretically, the osseous environment provided a contact surface for graft incorporation[11] and hosted healing cells for graft remodeling.[8] It was hypothesized that grafted tendon healing in the tibial tunnel was inferior to that in the femoral tunnel after ACL reconstruction.

To test the hypothesis, this study was designed to compare the healing quality of graft incorporation and remodeling between the femoral tunnel and tibial tunnel after ACL reconstruction in an established rabbit model.[12] The findings of this study might be helpful to locate the weakest site of the graft construct, where the target of enhancement should be to improve the outcome of ACL surgery.

METHODS

Study Design

This experiment was approved by the research ethics committee of the authors' institution. Eighteen skeletally mature female New Zealand white rabbits (age, 26 weeks; weight, 3.5 to 4.0 kg) were used in this study. ACL reconstruction with a long digital extensor tendon graft was performed. Rabbits were killed at weeks 2, 6, and 12 postoperatively, with 6 rabbits at each time point, before femur-graft-tibia complexes were harvested for histomorphometric analysis.

Animal Surgery

The rabbit model for ACL reconstruction was established according to an established protocol.[12] In brief, the rabbits were operated on under general anesthesia with 10% ketamine/2% xylazine (Alfasan, Woerden, Holland; 1 mL/1 mL), and sedation was maintained with 2.5% sodium phenobarbital injected intravenously (Sigma-Aldrich, St Louis, MO). The long digital extensor tendon graft was harvested, and graft preparation was done by removing the attached muscle and passing the holding sutures through each end of the tendon graft in an interdigitating whipstitch fashion, which mimicked the suspensory fixation such as the EndoButton (Smith & Nephew Endoscopy, Andover, MA) used in the clinical setting. A medial parapatellar arthrotomy was performed to expose the knee joint. The patella was then dislocated, and the infrapatellar fat pad was removed to expose the joint cavity. After the ACL was excised, femoral and tibial tunnels were created through the footprint of the original ACL. The graft was then inserted and routed through the bone tunnels by use of holding sutures. The graft at the extra-articular exit of the tunnel was fixed with maximum tension to the neighboring soft tissue by secure knots. The wound was closed in layers and wrapped with dressing. The rabbits were allowed free cage movement after surgery. The femur-graft-tibia complexes were harvested for histologic evaluation at each time point.

Histomorphometric Analysis

The samples were fixed in 10% neutralized formalin, decalcified with 9% formic acid for 3 weeks, and then cut across the central longitudinal axis of the bone tunnel from the anteromedial side to the posterolateral side (Fig 1A). They were processed in a histocentre (Histokinette 2000; Reichert-Jung, Nussloch, Germany) and embedded in paraffin wax (Thermolyne Sybron, Dubuque, IA). A total of 15 to 20 consecutive sections of 7 μm were taken for histologic examination, and it was estimated that the sections were within a distance of 0.10 to 0.15 mm from the central axis of the bone tunnel. The sections were stained by H&E and safranin O and then analyzed with a microscopic imaging system (Leica Q500MC; Leica Cambridge, Cambridge, England) (Fig 1B).

T-B healing quality was graded morphologically regarding new bone formation, cartilaginous tissue formation, and T-B collagen fiber reconnection. A modification of the histologic scoring system of Yeh et al.[13] was done with three categories: none, presence, significant scored by 0, 1, 2, respectively, and the perfect score would be 6 (Table 1). New bone formation was evaluated after H&E staining.[14] The fibrocartilaginous tissue formation at the T-B healing interface was detected by safranin O staining,[14] and the area of positive safranin O staining was also measured with the MetaMorph Image Analysis System 6.3 (Universal Imaging [Molecular Devices], Downingtown, PA). T-B collagen fiber reconnection was examined under polarized microscopy. The sections were graded by 2 trained researchers, and the final results were determined by consensus to minimize sampling error and misinterpretation.

Intraosseous graft remodeling was evaluated by semi-quantification of cell density, cell shape, collagen fiber composition, and organization. The sampling microscopic fields were taken from the mid portions of intraosseous grafted tendon midsubstance. To measure cellularity, the cell nuclei in blue after H&E staining were observed under light microscopy. Cell density was then calculated by dividing the total number of cells by the measured tissue area. Morphologic

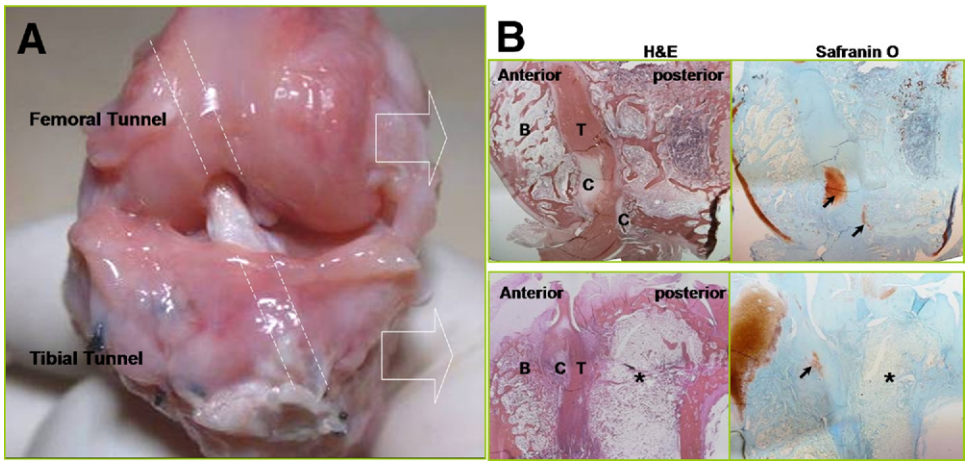

FIGURE 1. The femur–ACL graft–tibia complex was harvested at a specific time point for histologic examination, that is, week 6 after surgery. (A) The samples were cut longitudinally across the central axis of the bone tunnel (dotted line) from the anteromedial side to the posterolateral side. A total of 15 to 20 consecutive sections of 7 μm were taken for histologic examination, and it was estimated that the sections were within a distance of 0.10 to 0.15 mm from the central axis of the bone tunnel. (B) The longitudinal sections were stained with H&E and safranin O: the representative images show that the presence of cartilaginous interface tissue at the T-B interface was significantly less in the tibial tunnel than that in the femoral tunnel (arrows); it was also noted that there were fewer trabeculae in the tibial tunnel particularly on the posterolateral side than in the femoral tunnel (stars) (original magnification ×16). (B, bone; C, cartilage; T, tendon.)

changes of the nucleus were measured by use of the shape factor calculation: Shape factor = $4\pi A/P^2$ (where P indicates perimeter and A indicates area). A shape factor of 1 represents a circular object, and a shape factor of 0 represents a straight line. Shape data for each object were recorded and averaged by use of the aforementioned MetaMorph Image Analysis System 6.3.

Sirius red staining was used for the semi-quantification of mature and immature collagen content of intraosseous graft during remodeling: mature type I collagen fibers appear in yellow, orange, and red; whereas immature type III collagen fibers appear in green.[15] The composition of immature type III collagen was quantified through calculation of the fraction of green area over total area of the sampling field after color segmentation. The collagen organization of intraosseous graft was also measured after Sirius red staining by quantifying the birefringence of collagen under polarized light (based on brightness) according to a published method.[16] In brief, measurements were taken by rotating the polarization plane for maximum brightness to control the variations of the specimen orientation on the slides. The microscope fields were digitized, yielding an image in which noncollagenous material was dark (gray level 0) and collagenous material was depicted by gray scale from 1 to 255. The gray-scale measurement of digitized images for collagenous tissue of the intraosseous graft was performed on each section with MetaMorph Image Analysis System 6.3.

TABLE 1. *Histological Scoring System for Tendon-to-Bone Healing*

Characteristics	Points
Fibrocartilage formation	
Massive	2
Present	1
No	0
New bone formation	
Massive	2
Present	1
No	0
Tendon-to-bone collagen fibers reconnection	
Direct collagen fiber reconnection	2
Indirect reconnection through fibrous tissue	1
No	0

NOTE. A perfect score would be 6 points.

Statistical Analysis

The comparisons for the area of transient chondral callus, graft cell density, collagen composition, and organization among the different time points and different host bone milieus (femoral and tibial tunnel) were performed with 2-way analysis of variance. The comparison for the score of T-B healing quality was performed with the Friedman test. When overall significance of main effects was indicated without interaction, the difference between individual time points

and sites was assessed by post hoc test. The level of significance was set at $P < .05$. All data analyses were performed with SPSS analysis software, version 15.0 (SPSS, Chicago, IL).

RESULTS

The disparity of T-B healing was observed in the femoral and tibial tunnels after surgery (Fig 1). The transient cartilaginous tissue at the T-B healing interface was much less extensive in the tibial tunnel than in the femoral tunnel at weeks 2 and 6 after surgery ($P < .05$ for both). Such cartilaginous interface tissue was present at the T-B interface at week 2 after surgery; its area decreased with healing over time with chondrocytes aligned along the penetrating collagen fibers from bone to tendon at week 6 postoperatively, and the cartilaginous interface tissue diminished and was replaced by bony ingrowth with re-establishment of the T-B collagen fiber reconnection at 12 weeks after surgery (Fig 2 and Table 2). Fibrous healing interface tissue was dominant in the tibial tunnel, and loose fibrovascular tissue was present between bone and tendon at week 2 postoperatively; fibrous interface tissue became organized and reoriented along the long axis of grafted tendon and bone tunnel with healing over time with the newly formed osteoid close to the grafted tendon, and the grafted tendon connected with the bone tunnel indirectly through the fibrous interface tissue at week 12 after surgery (Fig 3). The score of T-B healing quality was significantly higher in the femoral tunnel than that in the tibial

Figure 2. Representative images showing T-B healing process with transient cartilaginous interface tissue formation in femoral tunnel. The interface tissue showing positive reactivity of safranin O (SO) (open arrows) was noted at week 2 after surgery. Such cartilaginous tissue became distinct with chondrocyte-like cells embedded in aligned collagen fibers (white arrow). Massive woven bone formation was accompanied by narrowing of the thickness of the cartilaginous interface. The residue of positive signals of safranin O could still be observed in the area of newly formed bone at week 6 postoperatively. Bone progressively grew into grafted tendon with a diminished cartilaginous interface and direct collagen fiber connection from bone to tendon at week 12 after surgery (original magnification ×100). (B, bone; C, cartilage; T, tendon; WB, woven bone.)

Table 2. *Spatiotemporal Changes of Tendon-to-Bone Healing Interface Tissue and Graft Remodeling After ACL Reconstruction*

Parameters	Week 2		Week 6		Week 12	
	Femoral Tunnel	Tibial Tunnel	Femoral Tunnel	Tibial Tunnel	Femoral Tunnel	Tibial Tunnel
Score of T-B healing quality [median (range)]	3 (3–4)	2 (2–4)	5 (4–5)*†	3 (2–4)*	6 (4–6)*†	4 (3–4)*†
Area of cartilaginous tissue (mm^2)	0.232 ± 0.069*	0.090 ± 0.031*	0.115 ± 0.042*†	0.027 ± 0.015*†	0†	0†
Graft cell density (per mm^2)	72 ± 18*	31 ± 5*	60 ± 10*	38 ± 7*	20 ± 11†	15 ± 3†
Shape factor of graft cell (0–1)	0.46 ± 0.18	0.55 ± 0.25	0.58 ± 0.12*	0.75 ± 0.17*†	0.70 ± 0.14†	0.78 ± 0.12†
Graft collagen composition (% with green color)	27 ± 11	35 ± 18	49 ± 14†	62 ± 15†	78 ± 12†	89 ± 10†
Graft collagen organization	153 ± 39	127 ± 28	106 ± 20†	91 ± 18†	85 ± 23*†	60 ± 12*†

*Significant difference between femoral and tibial tunnels at each specific time point by multiple comparisons after 2-way analysis of variance or Friedman test.

†Difference between the specific time points (week 6, week 12) and week 2 after surgery if the difference was statistically significant ($P < .05$) between the specific time points and week 2 by multiple comparisons after 2-way analysis of variance or Friedman test.

tunnel at weeks 6 and 12 postoperatively ($P < .05$ for both) (Table 2). Actually, as shown in Fig 1B, T-B healing was also not uniform along either the femoral or tibial tunnel. Cartilaginous interface tissue was present in the bone tunnel close to the intra-articular exit, whereas fibrous interface tissue occurred inside the bone tunnel. Accordingly, the collagen fiber orientation in the cartilaginous interface close to the intra-articular tunnel exit was oblique or perpendicular to the long axis of the bone tunnel, whereas the collagen fiber orientation in the fibrous interface inside the bone tunnel was almost parallel to the long axis of the bone tunnel (Figs 2 and 3).

The disparity of graft remodeling was also found in the femoral and tibial tunnels after surgery. As shown in Fig 4 and Table 2, the cell density of the graft in the tibial tunnel was significantly lower than that in the femoral tunnel at weeks 2 and 6 postoperatively. In both the femoral and tibial tunnels, there were no significant changes in the cell density of the graft between weeks 2 and 6. It dramatically dropped at week 12 by 67% and 61% in the femoral tunnel and tibial tunnel, respectively, in comparison with the cell density at week 6. The reduction in cell density was accompanied by a shift in the cell nucleus appearance with healing over time from round to rope-like, which was indicated by the shape factor. With respect to matrix deposition, the graft composition of immature type III collagen increased with a decrease in graft collagen fiber organization with healing over time in both the femoral and tibial tunnels. The birefringence of graft collagen fibers in the tibial tunnel was significantly lower than that in the femoral tunnel at week 12 after surgery ($P < .05$) (Fig 5 and Table 2).

DISCUSSION

To provide a better understanding of the biological healing process and develop better strategies to improve surgical outcome after ACL reconstruction, this study systemically characterized the healing process taking place in the T-B tunnel with an emphasis on cartilaginous and fibrous healing interface tissues. It was found that the transient cartilaginous interface was gradually replaced by bony ingrowth and re-establishment of the direct T-B collagen fiber reconnection postoperatively. The residue of proteoglycan was detected in the area of the newly formed bone at the T-B interface, which suggested that the process of bone formation resembled endochondral ossification. This observation was consistent with an immunohistochemical study after ACL reconstruction in rabbits.[7] The formation of transient cartilaginous tissue was possibly associated with compressive stress as well as tensile stress,[17] where grafted tendon was redirected at the intra-articular tunnel exit.[18] It has been established that the postnatal development of ACL-to-bone insertion resembles endochondral ossification.[19] The healing process with transient cartilaginous interface tissue formation would be favorable for regeneration of an ACL graft–to–bone tunnel insertion close to an intact one. However, there was less cartilaginous interface formation found in the tibial tunnel than in the femoral tunnel with healing over time.

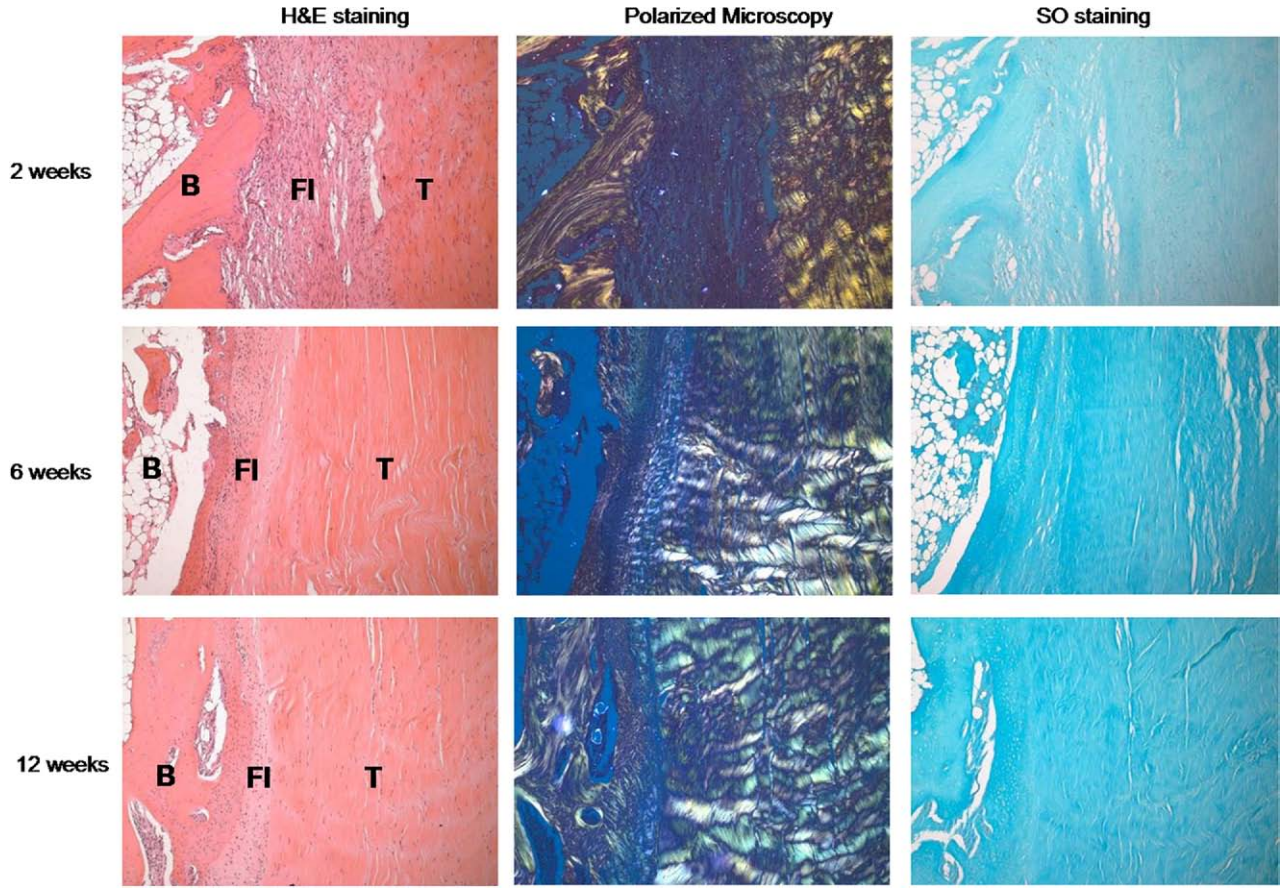

Figure 3. Representative images showing T-B healing process with transient fibrous interface tissue formation in tibial tunnel. The interface tissue was composed of loose organized fibrous tissue at 2 weeks after surgery. Closer bone apposition to grafted tendon was present with fibrous interface narrowing at weeks 6 and 12 after surgery. No direct T-B collagen fiber reconnection was found (original magnification ×100). (B, bone; FI, fibrous interface; T, tendon; SO, safranin O.)

This is the first experimental study to address the disparity of the histologic characteristics of T-B healing in the femoral and tibial tunnels postoperatively. As shown in histologic evaluations, T-B healing quality in the tibial tunnel was inferior to that in the femoral tunnel. The collagen fiber orientation at the healing interface was along the long axis of the grafted tendon and bone tunnel, suggesting their function in sustaining shear force across the T-B interface. It might be associated with the formation of fibrous interface tissue, which was predominantly found in the tibial tunnel. In contrast, the collagen fibers at the cartilaginous interface, which was extensive in the femoral tunnel, obliquely penetrated from bone to tendon. This might explain why the grafted tendon was prone to be pulled out from the tibial tunnel, which was reported in previous experimental studies.[12,20] Our findings might suggest that the tibial side was the weakest site of graft constructs where the target for enhancement should be to improve the outcome of ACL surgery. The limited clinical biopsy studies also showed the different healing interface tissue in patients (i.e., cartilaginous and fibrous tissue).[21-23] However, there were no clinical biopsy data available in both the femoral tunnel and tibial tunnel in the same patient for comparison. Thus future clinical biopsy study is desirable to test whether our observation in an animal model is relevant clinically, which might provide a scientific basis for therapeutic targets to improve the outcome of ACL reconstructive surgery.

Such disparity of T-B healing in the femoral and tibial tunnels was also reported after medial collateral ligament reconstruction in rabbits.[11] It was described that the transient cartilaginous interface tissue formation was much more extensive in the trabecula-filled

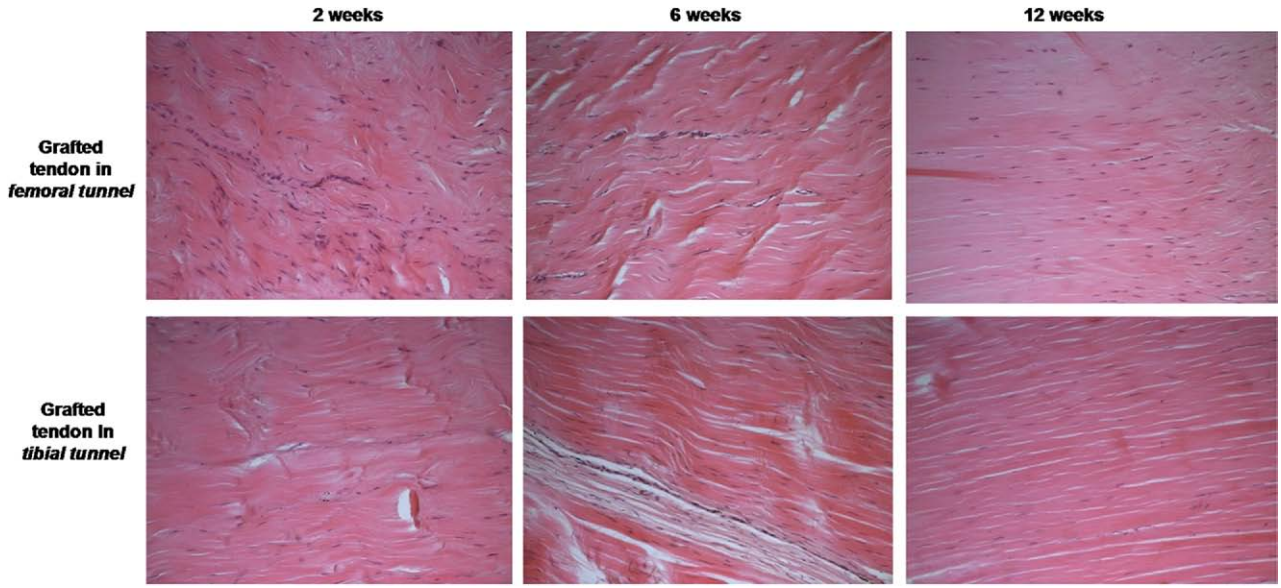

FIGURE 4. Representative images showing that the cell density of the grafted tendon in the tibial tunnel is significantly lower than that in the femoral tunnel at weeks 2 and 6 postoperatively (H&E staining, original magnification ×100).

femoral tunnel than in the marrow-filled tibial tunnel.[11] Grassman et al.[11] considered that the grafted tendon might depend on the trabecular bone architecture at the graft site. Actually, peri-graft bone provided the contact surface for graft anchorage; thus a large bone surface might be favorable for T-B healing theoretically. The relation between peri-graft bone mass and micro-architecture and T-B healing should be further investigated.

Synovial fluid might be another potential factor

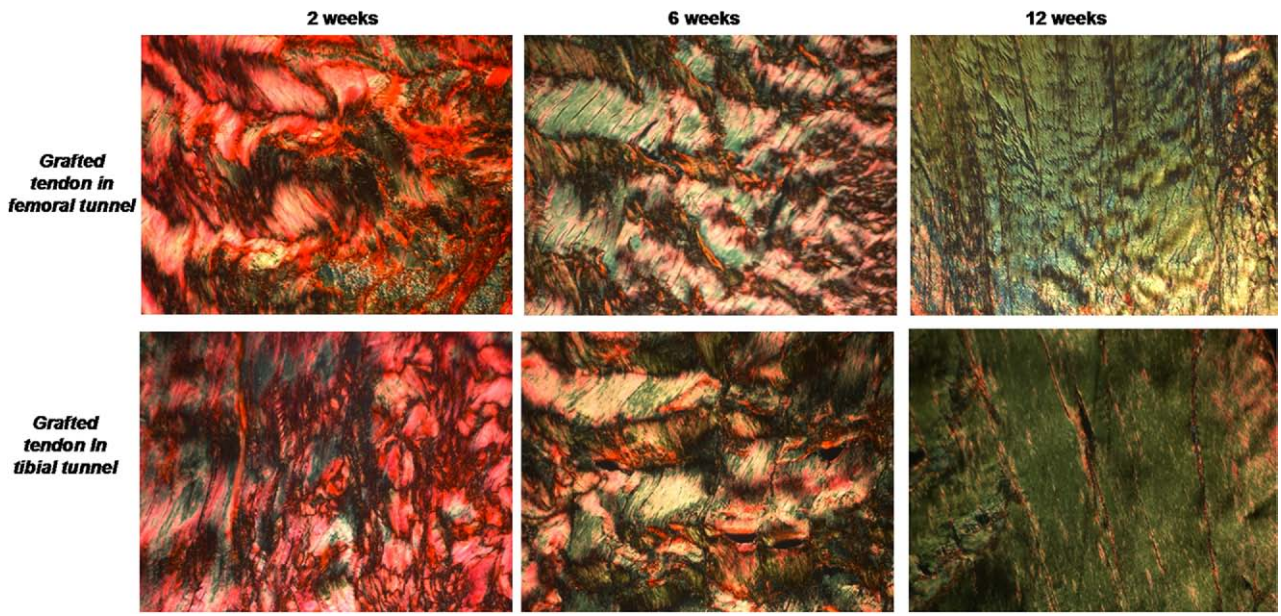

FIGURE 5. Representative images showing spatiotemporal changes of matrix composition of grafted tendon in femoral and tibial tunnels. It was found that type III collagen, which was visualized as a green color under polarized microscopy after Sirius red staining, increased with healing over time in both the femoral tunnel and tibial tunnel. The birefringence of grafted tendon collagen was lower in the tibial tunnel than that in the femoral tunnel (original magnification ×100).

affecting T-B healing in the intra-articular tibial bone tunnel. Several magnetic resonance imaging studies have shown that synovial fluid was prone to be tracked in the tibial tunnel.[24,25] It was reported that the level of the catalytic agents such as interleukin 1 in synovial fluid increased after ACL surgery.[26,27] The expression of interleukin 1 unregulated the expression level of chondrocyte matrix metalloproteinase 8 in synovial fluid.[28,29] Synovial fluid influx in the tibial tunnel might impair the formation of cartilaginous interface tissue and then T-B integration. This was supported by a recent experimental study that showed that inhibition of activities of matrix metalloproteinase 8 in synovial fluid by use of α2-macroglobulin could improve the mechanical properties of the T-B complex after ACL reconstruction in rabbits.[29]

The T-B healing interface was the weakest link of the T-B complex immediately after ACL reconstruction despite various fixations.[30] The previous experimental studies of other authors and our study have shown that the weakest point of the T-B complexes shifted to the grafted tendon midsubstance within the bone tunnel with re-establishment of the T-B connection.[12,31] Thus intraosseous graft remodeling was also important for the clinical outcome.[32] However, most previous experimental studies focused on the remodeling of intra-articular tendon graft, with a focus on the "ligamentization" process.[33] Few studies investigated the remodeling of intraosseous grafted tendon.

The grafted tendon remodeling required host cell repopulation and subsequent matrix remodeling.[8,34] In our study it was shown that the graft cellularity was higher at weeks 2 and 6 after surgery and then decreased with healing over time, accompanied by a shift in cell shape from round to rope-like. This might indicate the possibility of host cells turning into fibroblast-like cells for collagen synthesis. The newly formed collagen fibers were thin, were green in color after Sirius red staining, and increased with healing over time. The organization of such newly formed collagen fibers became poor with healing over time. It was shown that remodeling of the grafted tendon after ACL reconstruction was associated with an extensive decrease in the tensile strength of the graft in the first 12 weeks after surgery.[35] This was also the first study to show the disparity of graft cellularity, matrix composition, and organization observed in the femoral and tibial tunnels. The graft cellularity was obviously lower in the tibial tunnel than that in the femoral tunnel. The collagen fiber organization was poorer in the tibial tunnel than that in the femoral tunnel. This might explain why the grafted tendon ruptured in the midsubstance in the tibial tunnel at week 12 after surgery in this study. The remodeling process of the intraosseous graft was slower than the healing process at the T-B interface; thus it should also be the target for T-B healing enhancement.

A few limitations were present in our experimental study: (1) the findings were limited to a rabbit model and could not be extrapolated directly to patients because of the disparities in the anatomy, biomechanics, and kinetics of the knee joint between bipedal (i.e., human beings) and quadrupedal (i.e., rabbits) animals; (2) the graft choice used in our animal model was the extensor tendon instead of the hamstring tendon commonly used in clinical settings; (3) the grafted tendon was fixed by suture fixation in rabbits instead of with an interference screw, EndoButton, or cross-pin, which is currently used in patients; and (4) the histologic evaluations were not conducted in a blinded fashion, although consensus was reached between 2 trained researchers.

CONCLUSIONS

Grafted tendon healing in the tibial tunnel was inferior to that in the femoral tunnel at the T-B healing interface and the grafted tendon within the bone tunnel after ACL reconstruction in a rabbit model.

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