Periosteal Augmentation of a Tendon Graft Improves Tendon Healing in the Bone Tunnel

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Abstract: Secure fixation of tendon or ligament to bone has been a challenging problem. The periosteum is an osteogenic organ that regulates bone growth and remodeling at the outer surface of cortical bone and also is known to play an important role in forming a tendon insertion site to bone. Therefore, we hypothesized that a freshly harvested periosteum can be used as a stimulative scaffold to biologically reinforce the attachment of tendon graft to bone. Using a rabbit hallucis longus tendon and calcaneus process model, we found that a periosteal augmentation of a tendon graft could enhance the structural integrity of the tendon-bone interface, when the periosteum is placed between the tendon and bone interface with the cambium layer facing toward the bone. Clinically, the use of an autogenous periosteum patch would be an optimal choice for biologic augmentation of the tendon graft in the bone tunnel, because the tissue is readily available for harvest from the patient's body.

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F ixation and appropriate integration of tendon or ligament to bone is a challenging problem. Rotator cuff repairs,^{15,20} collateral ligament reconstructions in the elbow,^{12,36} tendon transfers in the foot,^{17,31,39} and ligament reconstructions in the knee^{7,9,19} are a few examples where biomechanically secure soft tissue-to-bone fixation is required for successful outcomes. The success of these reconstructive surgeries depends on the ability of the graft to incorporate into the bone, which in turn, determines the fixation strength of the soft tissue-bone interface.^{9,42}

Various fixation methods have been used to secure soft tissue to bone, such as staples, sutures, endobuttons, or bioab-

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sorbable soft tissue screws in the bone tunnel.^{9,19,33} Although some experimental studies have reported the mechanical strength data of various fixation methods,^{7,15,21,25,41} the literature is sparse concerning the advantage of any method over another. Clinically, lack of rigid, biocompatible fixation of soft tissue to bone has caused complications at the reconstructive site.^{9,50} With anterior cruciate ligament reconstructions, there has been an incidence of bone tunnel widening, and some think this may be caused by relative motion of tendon graft in the bone tunnel.^{14,27,44,47}

In the literature, numerous studies have been published concerning the biomechanical or histologic characteristics of a tendon graft-bone interface using animal models^{6,18,22,28,32,42,48} and human samples.^{1,16,26,40} It has been observed that when a tendon-graft was implanted into a bone tunnel, the tendon-bone interface had a considerable inflammatory reaction followed by a gradual remodeling process driven by an invasion of fibroblasts, osteoclasts, and osteoblasts.^{3,18,48} The remodeling process of the tendon involved the turnover of existing collagen and the synthesis of new collagen at the tendon-bone interface.^{30,32} These newly formed collagen fibers gradually were incorporated into the surrounding trabecular bone, thereby forming Sharpey's fibers at the tendon-bone interface. With the progressive increase in Sharpey's fiber formation, the mechanical strength of the interface gradually increased with time.^{6,22,28,42} Rodeo et al⁴² reported that, during the course of healing, the failure mode of the tendon-bone construct progressed from a pull-out at the tendonbone interface to a midsubstance failure of the tendon. However, it was observed that the structural characteristics of Sharpey's fibers formed in the bone tunnel never were similar to those found at a normal tendon insertion site. Furthermore, the tensile strength of the experimental tendon-bone interfaces never reach those of normal insertion sites.^{6,22,28,29} Therefore. these findings suggest that the cause of the midsubstance failure of the tendon graft observed by Rodeo et al⁴² may be caused by atrophy of the graft rather than restoration of the normal tendon-bone interface.

It has been suggested that postoperative rehabilitation may play an important role for strengthening reconstructed tendons and ligaments.^{10,45} Early mobilization and controlled

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mechanical loading would accelerate healing and remodeling of repaired tendon and ligament by stimulating the DNA and protein synthesis⁴⁶ and thereby enhancing the matrix organization, size, and strength of the tissue.²⁴ However, the level of the force that can be applied to the tendon or ligament graft is limited by the weakness at the soft tissue-bone interface, and the failures of reconstructive surgical treatments often are attributed to premature failure of the soft tissue-bone interface during postoperative treatment. Therefore, early secure fixation at the soft tissue-bone interface could be considered a critically important factor for proper postoperative rehabilitation to succeed in reconstructive surgeries.

Several studies have suggested that biologic augmentation of the tendon-bone interface using a rhBMP would enhance the histologic and mechanical properties of the interface.^{4,37,43} However, the exogenous agent best suited for tendon-bone healing is currently uncertain. A similar paradigm incorporates the fact that periosteum is one of the natural, osteogenic organs existing in the body and is responsible for the lateral growth of cortical bone. Therefore, the objective of the current study was to assess whether structural integrity of the tendon in the bone tunnel can be improved by periosteal augmentation of the tendon-bone interface.

The periosteum has been reported to contain mesenchymal progenitor or stem cells capable of differentiating into either osteoblasts or chondrocytes depending on the culturing environment.^{34,35,38} The periosteum consists of two layers, the fibrous (outer) layer, which contains mostly fibroblasts with collagen fibers aligned parallel to the tissue surface and the cambium (inner) layer, which contains mostly undifferentiated precursor cells with granular matrix.²³ Therefore, when a periosteum patch containing fibrous and cambium layers is transplanted into the tendon-bone interface, it is expected that the net result will be different depending on whether the cambium side is placed toward the bone or toward the tendon. The current study will test the hypotheses that the periosteal cambium layer has a stronger osteogenic potential than the periosteal fibrous layer, and that the periosteal augmentation of a tendon graft yields improved structural properties of the tendon-bone interface when the periosteal cambium side is in contact with bone than when the periosteal fibrous side is in contact with bone.

MATERIALS AND METHODS

Histochemical Alkaline Phosphatase Assay

A fresh periosteum patch was harvested from the medial proximal tibia of a New Zealand White rabbit (6-months-old) and fixed with 10% formalin solution. Five-micrometer sections of the sample were cut and prepared using a standard protocol for paraffin embedding histologic evaluation.¹³ After the paraffin was removed with xylene, the tissue sections were dehydrated through graded ethyl alcohol. The prepared tissue

sections then were treated with an ALP substrate solution (86-R, Sigma Diagnostics, St Louis, MO) for 2 hours at room temperature, which contained p-nitrophenyl phosphate. A hydrolytic reaction of ALP released p-nitrophenol molecules from the ALP substrate solution, which turned the tissue section brown. After being washed with distilled water, the sections were counterstained with hematoxylin solution.

Animal Model and Surgical Procedures

Thirty male New Zealand White rabbits weighing 2.5 to 3.0 kg (6-months-old) were used as an animal model, in which a tendon-bone tunnel healing model was created using the hallucis longus tendon and the calcaneal posterior process of both hindlegs, similar to a model used by Liu et al.³² This model minimizes alterations of the biomechanics and load bearing in the ankle in rabbits, because the majority of the load in the hindlimb is supported by the Achilles tendon.

The rabbits were premedicated with 100 mg/kg ketamine HCl and 45 mg/kg xylazine intramuscularly for anesthesia induction followed by 1 mU/kg penicillin for infectious prophylaxis. During the surgery, isoflurane inhalational anesthesia, provided by a rebreathing mask and supplemented with oxygen, was used for sedation. After both hindlegs were shaved, the medial frontal aspect of the proximal tibia was exposed via a 10-mm longitudinal incision of the skin. A 5-mm \times 5-mm patch of periosteum was identified, the fibrous layer was marked with a surgical marker, and the periosteum patch was harvested using a surgical blade and a sharp elevator. Particular care was taken to minimize damage to the cambium layer of the periosteum patch. A 25-mm longitudinal incision then was made on the plantar aspect of the paw of the same leg and extended to the dorsal aspect of the Achilles tendon. The hallucis longus tendon then was identified and carefully separated from the dense plantar aponeurosis and subsequently transected at the distal end of the first metatarsal. Using a lowspeed hand drill and a 2.38-mm (³/₃₂ inch) diameter drill bit with continuous saline irrigation, a drill hole was made vertically at the middle of the calcaneal posterior process.

The portion of the hallucis longus tendon to be placed in the bone tunnel was first identified in the natural ankle position and then was treated with one of the four methods as described: Group A, periosteal graft was wrapped around the tendon with the fibrous layer facing toward the bone; Group B, periosteal graft was wrapped around the tendon with the cambium layer facing toward the bone; Group C, periosteal graft was subjected to three cycles of a freeze and thaw process to kill the cells, and then was wrapped around the tendon (positive control); and Group D, no periosteal graft was used with the tendon (negative control).

In Groups A, B, and C, the periosteal graft was secured to the tendon using a 6-0 Vicryl suture (Fig. 1A). To keep the diameter of the periosteally-augmented tendon graft similar to that of the negative group, the tendons in Groups A, B, and C



FIGURE 1A–B. (A) A rabbit's hallucis longus tendon was wrapped with a free periosteum patch. (B) A periosteum-wrapped hallucis longus tendon was passed through a 2.38-mm diameter bone tunnel in the calcaneus.

were trimmed approximately 0.5 mm around the tendon before the periosteal augmentation. The periosteum-wrapped tendon then was passed through the drill hole (Fig. 1B), and the distal end of the tendon was brought back around the bone and secured to the midsubstance of the tendon using an uninterrupted 3-0 proline suture. The tendon grafts in all groups had a snug fit within the bone tunnel. Finally, the incision was closed with 3-0 proline sutures and reinforced with staples. The contralateral side also was operated on in the same manner except that each limb was assigned randomly to one of the four treatment groups so that the 60 hindlegs from 30 rabbits were distributed equally among the groups (15 hindlegs per group). After surgery, all rabbits were returned to their normal cage activities ad libitum, and their daily behaviors were monitored closely. Animals were sacrificed at either 3 or 6 weeks after surgery, and the tendon-bone junctions were evaluated biomechanically and histologically as will be described. The animals used for the fluorescent histologic analyses received a series of fluorochrome treatments before sacrifice as will be described.

Biomechanical Testing

Twelve rabbits were sacrificed 6 weeks after the surgery. and the tendon-to-bone interfaces from these animals (24 hindlegs; six from Group A, seven from Group B, six from Group C, and five from Group D) were evaluated biomechanically using a load-to-failure pull-out test immediately after sacrifice. The calcaneus-tendon specimen was separated from the experimental leg of the animal, and all other soft tissues were removed except the hallucis longus tendon and the calcaneus. The distal portion of the hallucis longus tendon wrapped around the bone and fixed to the midsubstance of the tendon was removed before testing. The calcaneus bone then was fixed in a custom-built bone clamp, and the proximal free end of the tendon was fixed in a custom-built tendon clamp. The bone clamp consisted of two halo-rings and 10 set screws, which allowed a flexible adjustment of the bone specimen in a horizontal plane. The tendon clamp consisted of a pair of interlocking sinusoidal teeth and wedges, which prevented slippage of the tendon by a self-tightening mechanism (Fig. 2). The bone and tendon clamps were mounted in a materialtesting machine (Instron #1133, Canton, MA) with a 100-kg capacity tensile load-cell. After five cycles of preconditioning with 100 g, the tendon end was subjected to a load-to-failure pull-out test with a displacement rate of 10-inches per minute, and the load-displacement history was recorded continuously using a data acquisition board and Labview software (National Instrument[®], Austin, TX). All specimens were kept moisturized with a spray of a fine mist of saline buffer solution throughout the entire experiment.

A one-factor ANOVA was used to analyze the effect of the treatment group on the load-to-failure test. Subsequently, Fisher's protected least squares difference post hoc test was used to examine differences between groups. All statistics were done using Statview (SAS Institute, Cary, NC) at a significance level of 0.05.



FIGURE 2. A schematic of the mechanical testing setup shows sinusoidal self-tightening tendon clamp (A), isolated calcaneus bone (B), calcaneus bone clamps (C), load-cell (L), hallucis longus tendon (T). The tendon was pulled at 10 inches per minute.

Fluorescent Histologic Analysis

To monitor the bone formation and remodeling process around the tendon in the bone tunnel, 10 of 30 rabbits (20 hindlegs, or five hindlegs per each group) received intramuscular injections of fluorochrome staining agents, 3% calcein (5 mg/kg) at Weeks 2 and 3, and 5% alizarin complexone (20 mg/kg) at Weeks 5 and 6, respectively. For the injection, all rabbits were sedated with a ketamine and xylazine cocktail (0.4 mL/kg), while fluorochrome agents were injected slowly to avoid causing adverse muscle, cardiac, and neural reactions. These rabbits were sacrificed 3 days after the final injection of alizarin complexone at the sixth week to allow sufficient time for the injected fluorochrome to be fully incorporated into the specimen. After the animals were sacrificed, the calcaneustendon specimens were isolated from the rabbit legs and placed in a 4% formaldehyde solution at 4° C for 2 days. After gradual dehydration using ethyl alcohol (50%, 60%, 70%, 80%, 90%, and 100%, each step for 2 hours) and acetone for 2 hours, the specimen was embedded in methylmethacrylate, sectioned at 0.8-mm thickness, and subsequently ground to approximately 50-um thickness. The sections were stained with toluidine blue and alizarin red (5%), and examined under a light microscope. Also, newly formed bone structure around a bone-tunnel was identified by the appearance of calcein and alizarin complexone under a fluorescent microscope.

Hematoxylin and Eosin Histologic Analysis

Eight additional rabbits (16 legs) had the same surgical protocol, were sacrificed at either 3 or 6 weeks after the surgery, and were used for histologic analyses with standard hematoxylin and eosin staining. Two hindlegs (one rabbit) were assigned to each period (3 or 6 weeks) per each surgical group, as described above. Two rabbits (3 and 6 weeks for Group C) were excluded from the study because of complications after the surgery. The tendon-bone specimen were harvested immediately after the sacrifice of the animal, fixed in 4% formaldehyde, and decalcified in Decal[®] (Decal Chemical Co, Congers, NY) overnight. Five-micrometer sections of the sample were prepared via paraffin embedding and stained with hematoxylin and eosin for histologic evaluation under regular and polarized light microscopes.

RESULTS

Histochemical Alkaline Phosphatase Assay

The heterogeneous osteogenic potential in periosteum was examined using histochemical localization of ALP activity. The cambium layer shows significant ALP activities (represented by dark stain in Fig. 3), suggesting a highly osteogenic nature of the layer, whereas the fibrous layer shows negligible ALP activities. In addition, the cambium layer in the periosteum had a higher cell density as compared with the fibrous layer.



FIGURE 3. The histochemical localization of ALP of a periosteum patch freshly harvested from a rabbit is shown. The cambium layer (C) shows a high level of ALP activities (represented by dark stain), whereas the fibrous layer (F) shows negligible ALP activities. The color version of this figure can be found on the journal website (www.corronline.com).

Biomechanical Data

In all biomechanical pull-out tests, the failure took place at the interface between the tendon and the bone tunnel. The ultimate failure load and the tensile stiffness were used as measures of the structural properties of the tendon-bone interface. The average and standard deviation of the ultimate failure load of each group is summarized in Figure 4. According to a onefactor ANOVA, the effect of the treatment was significant. The subsequent post hoc analysis revealed that Group B (fresh cambium layer facing toward bone) had higher ultimate failure load than any other groups and that it was statistically signifi-



FIGURE 4. The graph shows the ultimate failure load of the tendon-bone interface at 6 weeks after surgery. A = 6.9 ± 3.1 kg (n = 6), B = 11.1 ± 3.1 kg (n = 7), C = 4.4 ± 2.5 kg (n = 6), and D = 7.1 ± 3.2 kg (n = 5); *p < 0.05.



FIGURE 5A–D. Representative fluorescent histologies show calcein green (at 2 and 3 weeks) and alizarin red (at 5 and 6 weeks) staining from (A) Groups A, (B) Group B, (C) Group C, and (D) Group D. The white circle represents an approximated area of the bone tunnel created at the time of surgery. **T**-tendon. The color version of this figure can be found on the journal website (www.corronline.com).

cant (p < 0.05). There was no statistical difference in the ultimate failure load between Group A (fresh fibrous layer facing toward bone) and Group D (negative control). Group C (positive control) had the lowest ultimate failure load. The tensile stiffness was calculated from the linear region of the loaddisplacement curve. Although Group B (fresh cambium layer facing toward bone, 3.06 ± 1.36 kg/mm) showed the higher tensile stiffness than Group A (fresh fibrous layer facing toward bone, 2.25 ± 0.82 kg/mm) and Group D (negative control, 2.61 ± 1.20 kg/mm), there was no statistically significant difference between these groups. The average tensile stiffness of Group C (positive control, 1.31 ± 0.72 kg/mm) was significantly lower than any other groups (p < 0.05).

HISTOLOGIC RESULTS

New bone formation around the tendon graft in the bone tunnel was evaluated qualitatively using two fluorescent colors (Fig. 5). Green (calcein at the first and second weeks after sur-



FIGURE 6A–C. (A) The histologic evaluation of a specimen from Group A at 3 weeks after surgery is shown (Stain, hematoxylin and eosin; magnification, $\times 200$). **B**-bone; **F**-fibrous layer; **C**-cambium layer; **T**-tendon (B) A magnified image ($\times 400$) is shown from the dotted rectangular area in (A). (C) The polarized light microscopic evaluation of the same area reveals a newly formed woven bone with unorganized structure (arrow). The color version of this figure can be found on the journal website (www.corronline.com).



FIGURE 7A–C. (A) The histologic evaluation of a specimen from Group B at 3 weeks after surgery is shown (Stain, hematoxylin and eosin; magnification, ×200). B-bone; F-fibrous layer; C-cambium layer; T-tendon (B) A magnified image (×400) is shown from the dotted rectangular area in (A). Chondrocytes developed in the cambium layer. (C) The polarized light microscopic evaluation of the same area reveals a newly formed woven bone with unorganized structure (arrow). The color version of this figure can be found on the journal website (www.corronline.com).

gery) represents the bone formation during the early stage of the healing process, and red (alizarin complexone at the fifth and sixth weeks after surgery) indicates that of the latter stage during the healing process. Fluorescent histologic evaluation revealed that Group B (fresh cambium layer facing toward bone) had the most organized and significant bone formation around the bone tunnel. In particular, the new bone formation was established very tightly around the grafted tendon in Group B, whereas a large amount of granular tissue was present in the interfacial zone in the other groups (A, C, and D). It also was observed that the diameter of the bone tunnel and the tendon graft had reduced during the 6-week period, indicating an active remodeling process of the tendon and bone during the healing period.



FIGURE 8A–C. (A) The histologic evaluation of a specimen from Group B at 3 weeks after surgery is shown (Stain, hematoxylin and eosin; magnification, $\times 200$). **B**-bone; I-interfacial tissue; **T**-tendon (B) A magnified image ($\times 400$) is shown from the dotted rectangular area in (A). (C) The polarized light microscopic evaluation of the same area shows that new bone formation is lacking in interfacial tissue. The color version of this figure can be found on the journal website (www.corronline.com).

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FIGURE 9A–C. (A) The histologic evaluation of a specimen from Group A at 6 weeks after surgery is shown (Stain, hematoxylin and eosin; magnification, \times 40). **B**-bone; **T**-tendon. The circle represents the approximated location of the initial bone tunnel of 2.38-mm in diameter. (B) A magnified image (\times 200) is shown from the dotted rectangular area in (A). (C) The polarized light microscopic evaluation of the same area reveals a newly formed trabecular bone with a well-organized structure. The color version of this figure can be found on the journal website (www.corronline. com).

Standard histologic evaluation with hematoxylin and eosin (Figs. 6-11) provided more detailed descriptions of the general healing process at the tendon-bone interface. Three weeks after the surgery, Groups A (Fig. 6) and B (Fig. 7) showed a presence of the interposed periosteum consisting of the fibrous (F) and cambium (C) layers of the bone tunnel. In Group A (Fig. 6B-C) and Group B (Fig. 7B-C), newly formed woven bone with an unorganized fibrous structure (arrows) was observed at the juxtaosseous region around the periosteum under regular light and polarized light microscopes. The bone formation in Group B (Fig. 7B) seemed to be driven by the osteochondral ossification of the periosteal cambium layer, which was evident by the abundant presence of chondrocytes at the cambium layer-bone interface. In Group A (Fig. 6B), however, chondrocytes were not found near the juxtaosseous region nor in the cambium layer. In Group D (Fig. 8), the ten-



don-bone interface was mostly an interfacial tissue without a woven bone formation.

Six weeks after the surgery, Group A (Fig. 9) and Group B (Fig. 10) showed a significant new bone formation with a well-organized lamellar structure (Fig. 9C, 10C). In Group A, however, the tendon graft seemed to have had a rapid remodeling process. However, Group B showed a tight interdigitation between the tendon graft and the newly formed bone with abundant Sharpey's fibers (arrows in Fig. 10C). In Group D (Fig. 11), new bone formation around the tendon graft also was seen, but a thick interfacial tissue with an unorganized structure was evident around the tendon.

DISCUSSION

Using a rabbit hallucis longus tendon and calcaneus process model, we showed that a periosteal augmentation of the

FIGURE 10A–C. (A) The histologic evaluation of a specimen from Group B at 6 weeks after surgery is shown (stain, hematoxylin and eosin; magnification, \times 40). B-bone; T-tendon The circle represents the approximated location of the initial bone tunnel of 2.38-mm in diameter. (B) A magnified image (\times 200) is shown from the dotted rectangular area in (A). (C) The polarized light microscopic evaluation of the same area shows collagen fibers (arrows) interconnecting the newly formed bone and the tendon, which resemble the Sharpey's fibers. The color version of this figure can be found on the journal website (www.corronline. com).



FIGURE 11A–C. (A) The histologic evaluation of a specimen from Group B at 6 weeks after surgery is shown (Stain, hematoxylin and eosin; magnification, \times 40). B-bone; I-interfacial tissue; T-tendon The circle represents the approximated location of the initial bone tunnel of 2.38-mm in diameter. (B) A magnified image (\times 200) is shown from the dotted rectangular area in (A). (C) The polarized light microscopic evaluation of the same area shows lack of interconnecting collagen fibers between the bone and the interfacial tissue. The color version of this figure can be found on the journal website (www.corronline.com).

tendon graft could enhance the structural integrity of the tendon-bone interface. The periosteal cambium layer had to be placed facing toward the bone to obtain significant improvements in the mechanical strength and histologic appearance at the bone-tendon interface. When the periosteal fibrous layer was placed facing toward the bone tunnel, the interface strength was only as good as that of the negative control group (Group D). Biologic functions of the periosteal fibrous layer still are uncertain in the current study, but the mechanical and histologic findings support a hypothesis that the fibrous layer in periosteum may have similar biologic characteristics as the tendon in a bone tunnel. Use of an inert periosteal patch to augment the tendon graft (Group C) resulted in the lowest interface strength among groups, suggesting that the inert periosteum failed to provide osteogenic stimulation to the tendonbone interface. The fact that the interface strength of the inert periosteum group was lower than that of the negative control group (Group D) suggests that the inert periosteum might have blocked nutrient transport to the tendon graft and impeded biologic interactions between the tendon and the bone.

Periosteum plays an important role in providing an attachment between tendon and bone at indirect insertion sites. When a tendon graft was passed through a bone tunnel, Brooks⁸ observed that tendon healing only takes place between the tendon and periosteum, not directly between the tendon and bone. Therefore, it is reasonable to use the periosteum as an interface scaffold between the tendon graft and the host bone to enhance the tendon-bone attachment. The use of a periosteal autograft to enhance bone formation around a tendon graft is not a new idea. In 1930, Burman and Umansky¹¹ showed that a tibialis anticus tendon wrapped with a free periosteal graft develops significant ossification along the tendon in 3 weeks. However, it has not been studied whether a free periosteal graft can enhance tendon healing in a bone tunnel and whether the fibrous and cambium layers of the periosteum



produce different progress in the tendon-bone healing. The current study showed the use of periosteal augmentation placed between the tendon and the bone tunnel as a means to enhance the mechanical and biologic characteristics of tendonbone interface. Clinically, the use of an autogenous periosteum patch would be an optimal choice for biologic augmentation of the tendon graft in the bone tunnel, as the tissue is readily available for harvest from the patient's body.

The tendon-bone tunnel model used in the current study is an extraarticular model. There has been evidence that tendon healing in bone tunnel in the presence of synovial fluid may be dramatically different from that without synovial fluid.⁵ The inflammatory factors and cytokines in synovial fluid can impede the cell migration⁴⁹ and also cause increased collagenase activities in healing tissues.² It also was suggested that the proteolytic characteristics of synovial fluid could inhibit bone ingrowth in a bone tunnel.⁵ Therefore, the efficacy of periosteal augmentation in tendon-bone healing within an intraarticular environment requires additional investigation.

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