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Effects of Trypsinization and Mineralization on Intrasynovial Tendon Allograft Healing to Bone

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Abstract

The purpose of the current study was to develop a novel technology to enhance tendon-to-bone interface healing by trypsinizing and mineralizing (TM) an intrasynovial tendon allograft in a rabbit bone tunnel model. Eight rabbit flexor digitorum profundus (FDP) tendons were used to optimize the trypsinization process. An additional 24 FDP tendons were stratified into control and TM groups; in each group, 4 tendons were used for in vitro evaluation of TM and 8 were transplanted into proximal tibial bone tunnels in rabbits. The samples were evaluated histologically and with mechanical testing at postoperative week 8. Maximum failure strength and linear stiffness were not significantly different between the control and TM tendons. A thin fibrous band of scar tissue formed at the graft-to-bone interface in the control group. However, only the TM group showed obvious new bone formation inside the tendon graft and a visible fibrocartilage layer at the bone tunnel entrance. This study is the first to explore effects of TM on the intrasynovial allograft healing to a bone tunnel. TM showed beneficial effects on chondrogenesis, osteogenesis, and integration of the intrasynovial tendon graft, but mechanical strength was the same as the control tendons in this short-term in vivo study.

Keywords

intrasynovial tendon; mineralization; tendon-to-bone healing; trypsinization

Introduction

Fibrocartilaginous entheses such as the anterior cruciate ligament (ACL), the flexor digitorum profundus (FDP) tendon insertion, or rotator cuff classically include 4 histologic zones: a tendinous zone, an unmineralized fibrocartilage zone, a mineralized fibrocartilage

zone, and a bone zone (1). However, several studies have shown that the bone-tendon junction consists of a gradual change in mineral content and tissue composition (2). The graded structure appears to allow more efficient force transmission without concentrating stress. Unfortunately, the graded tendon-bone interface is not regenerated after injury, usually leading to poor outcomes (3).

Extrasynovial tendon grafts are widely used in musculoskeletal reconstructive procedures, including ACL or posterior cruciate ligament repair, rotator cuff repair, and FDP tendon reconstruction (4–6). However, intrasynovial tendon grafts show better clinical outcomes when transferred to an intrasynovial environment (7). Several studies suggest that this improvement is mainly attributable to structural and metabolic differences in intrasynovial and extrasynovial tendons (8,9). Intrasynovial tendons have a low-friction surface consisting of lubricin, hyaluronic acid, and phospholipids, whereas extrasynovial tendons lack these components (10). Lubricin has been shown in vitro to inhibit apposed articular cartilage repair mechanically and histologically (11). Another in vitro study showed that digesting the flexor tendon with trypsin could remove surface lubricin, producing a mesh-like surface structure that enhances cell adherence (12). A recent in vivo study showed that lubricin might affect tendon healing to bone (6). Together, the evidence suggests that removing lubricin with trypsin may improve intrasynovial tendon-to-bone healing in vivo.

A recent in vitro study demonstrated that the outer layer of an intrasynovial tendon could be successfully mineralized; by combining Na_2HPO_4 extraction and fetuin enhancement, a graded mineral zone could be formed within the tendon tissue (13). However, this concept has not been investigated in an in vivo model. Therefore, the objective of the current study was to develop a novel tendon graft by combining trypsinization and mineralization (TM) treatments and transplanting the treated graft into a rabbit tibia bone tunnel model to determine whether tendon-to-bone interface healing improved.

Methods

The study protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Tendon Samples

Sixteen hind paws were obtained from 8 adult New Zealand white rabbits (weight, 3.3–3.5 kg) that were euthanized for other IACUC-approved studies. Immediately after sacrifice, the hind paws were harvested and stored in a -80°C freezer. After thawing at room temperature, 32 FDP tendons with the distal phalanx were obtained from the third and fourth digits.

Optimization of Trypsinization Time

To optimize the duration of trypsinization, 8 FDP tendons were divided into 4 groups: 0, 10, 30, and 60 minutes. Before trypsinization, the proximal part of the whole tendon was protected with an antimicrobial film (Ioban Incise drapes; 3M Health Care). Tendons then were suspended in an upright tube and digested in a 0.25% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich) at 37°C for the allotted time. After trypsinization, tendons were dipped into minimal essential medium (MEM) with

10% fetal bovine serum (FBS; Gibco-Life Technologies) for 10 minutes and then washed in saline solution (0.9% NaCl) for 30 minutes.

Samples were mounted and frozen in cryopreservation matrix (Tissue-Tek compound; Sakura Finetek) and longitudinally sectioned at 10 μ m with a cryostat (CM1850; Leica). The samples were mounted on charged glass slides (Fisher Scientific) and stained as previously described (10). Briefly, sections were rinsed 3 times with phosphate-buffered saline (PBS) and fixed with a 4% paraformaldehyde buffer for 5 minutes. Slides then were rinsed with PBS containing 3% Triton X-100 and blocked with PBS containing 3% Triton X-100, 0.25% bovine serum albumin, and 4% normal goat serum for 30 minutes. These slides were then rinsed with Tris buffer containing 0.1% bovine serum albumin (BSA) and 0.5% Triton X-100 (TBS) and incubated with 1 μ g/mL antilubricin monoclonal antibody S6.7925 for 2 hours at room temperature. After washing with TBS as above, slides were incubated with biotinylated horse antimouse immunoglobulin G (Vectastain Elite ABC kit, Vector Laboratories) for 30 minutes and Vectastain ABC reagent (Vectastain Elite ABC kit) for 30 minutes. ImmPACT AEC peroxidase substrate (Vector Laboratories) was applied to the slides for 10 minutes to detect lubricin. VECTOR hematoxylin (Vector Laboratories) was used for counterstaining. Aqueous mounting medium (Vector Laboratories) was applied to the slides. Slides were observed with a BH-2 microscope (Olympus).

Tendon Allograft Preparation

Tendons were divided into control and TM groups (12 per group). After removing the distal phalanx, control tendons were lyophilized, sterilized, and stored at -20°C until use.

Tendons in the TM group were trypsinized using the optimized protocol. After trypsinization, tendons were dipped into MEM with 10% FBS for 10 minutes and washed in saline solution (0.9% NaCl) for 30 minutes. After removing the distal phalanx, the proximal portion of the tendon was protected by wrapping with the antimicrobial film. The distal 1 cm of the tendon was treated by hanging the tendon upright in a 3% Na_2HPO_4 solution and shaking slowly and continuously for 24 hours at 4°C . Tendons were then lyophilized and stored at 4°C until further mineralization. The proximal portion of the lyophilized tendon was protected with antimicrobial film and additionally wrapped with rubber sleeves. The distal 1 cm of the tendon was mineralized by hanging the tendon upright in 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) with 100 mM NaCl, 5 mM CaCl_2 , 5 mM KH_2PO_4 , and 5 mg/mL fetuin (13). The mineralization process lasted 24 hours (incubation at 25°C with shaking). The tendons were lyophilized and sealed in a plastic bag for gas sterilization and stored at -20°C until use.

In Vitro Evaluation of the TM Tendon

Four tendons from each group were used for in vitro evaluations. To detect the presence of calcium phosphate-containing mineral, 2 tendons in each group were placed in 10 mL of 0.5% KOH containing 0.002% alizarin red S (Sigma-Aldrich) for 1 hour with end-over-end mixing. Tendons were then rinsed with 0.05% KOH for 1 hour and photographed. Two tendons in each group were used for histologic assessment (using the same methods as mentioned above). The sections were stained using the von Kossa kit (MasterTech)

according to the standard procedure. Immunohistochemical staining of lubricin was performed with the same methods mentioned above.

In Vivo Evaluation of the TM Tendon

Eight tendons from each group were used for in vivo evaluations. One hour before the operation, the FDP tendons were incubated in saline solution (0.9% NaCl) at 37°C for rehydration.

Eight adult New Zealand White rabbits (weight, 3.1–3.3 kg) were used for the in vivo study. After anesthesia, a longitudinal small incision was made at the medial side of the proximal tibia. A 1.7-mm bone tunnel was drilled bilaterally into the proximal tibial metaphysis, and a 1.2-mm bone tunnel was drilled bilaterally into the proximal tibia, 7 mm lower than the first tunnel and toward the knee joint. Two Kirschner wires were used to prove that the 2 bone tunnels were connected (Figure 1). The lower oblique bone tunnel passed through the tendon insertion tunnel and also the tibial plateau into the knee joint to create an intrasynovial environment for the tendon-bone tunnel. The entrance of the lower bone tunnel was blocked with wax (Lukens bone wax; Angiotech). The mineralized graft was randomly transplanted into one tibia and the control graft was transplanted into the contralateral side. The tendon graft was marked at 10 mm proximal to the distal end and guided into the upper bone tunnel from the medial side to the lateral side. To fix the tendon graft, a Bunnell suture (5-0 Ethibond polyester suture; Ethicon) was secured to the biceps femoris at the lateral aspect of the proximal tibia. The medial side of tendon graft was sutured to the semitendinosus tendon. Subcutaneous tissues and skin were closed with interrupted sutures (4-0 Vicryl suture; Ethicon). Postoperatively, rabbits were allowed free activity in individual cages. The animals were sacrificed 8 weeks later for testing.

Mechanical Testing

Six samples in each group were used for mechanical evaluation. After sacrifice, the tibia was isolated and the tendon graft was exposed through careful dissection. The suture used to fix the tendon on the lateral side was removed to eliminate the suture's holding strength on the measurement. The tibia was mounted onto a servohydraulic testing machine (MTS Systems Corp). The tendon graft outside the bone tunnel was gripped longitudinally along the bone tunnel. The distance from the tendon clamp to the tendon insertion point was set at 10 mm. To avoid tendon slipping, the tendon inside the clamp was sutured with a 4-0 Vicryl suture (Ethicon) before clamping. The tendon was pulled out at a rate of 20 mm/min until failure. The ultimate force and stiffness (the slope of force vs displacement) at the bone insertion point was measured to assess the mechanical quality of healing of the graft-bone juncture. The failure mode (where the tendon breaks) was recorded and further confirmed with histology.

Histology

The proximal tibia, including the bone tunnel, was harvested from the TM and control groups (2 per group). Specimens were fixed in 4% paraformaldehyde for 1 day and then decalcified with 15% EDTA for 5 weeks. Slides were prepared as described above. Sections

were stained with hematoxylin and eosin and the Masson trichrome kit (Sigma-Aldrich) for histologic and structural evaluation.

Data Analysis

Quantitative data (ultimate failure force and stiffness) are represented as the mean (SD) and analyzed statistically using the paired Student *t* test. The statistical significance level was set at 0.05.

Results

Optimizing Trypsinization Time

Lubricin was found on the surface and extracellular matrix of the control (untreated) FDP tendons (Figure 2A–B). After 10 minutes of trypsinization, only very limited staining of lubricin was seen on the extracellular matrix inside FDP tendons, and no staining was observed on the tendon surface (Figure 2A and C). No lubricin was evident on the surface and extracellular matrix after 30 or 60 minutes of trypsinization (Figure 2D–E). Therefore, we used the 10-minute trypsinization time for the study.

In Vitro Evaluation of the TM Tendon

After alizarin red S staining, tendons in the TM groups showed an obvious boundary between the mineralized (stained) and unmineralized portions. No red staining was observed on the control tendon (Figure 3A). After von Kossa staining, mineral deposition was visible inside the tendon in a graded manner. No mineral was deposited outside the mineral-treated portion of the tendon (Figure 3B–D). After immunohistochemical staining of lubricin (Figure 3E–H), only very limited staining was observed on the extracellular matrix of the FDP tendons, and no staining was seen on the tendon surface. Lubricin staining was evident on the proximal portion of tendon, where it was not trypsinized.

Mechanical Testing of In Vivo Samples

No significant difference was observed in maximum failure force between the control (mean [SD], 5.76 [3.21] N) and TM (mean [SD], 5.21 [4.21] N) tendons ($P>.05$). Likewise, no significant difference was measured in linear stiffness between the control (mean [SD], 1.93 [1.18] N/m) and TM (mean [SD], 2.43 [2.16] N/m) tendons ($P>.05$). Each group had 3 samples that failed at the graft midsubstance (portion outside of the bone tunnel). The other 3 samples from each group failed with partial pullout of the graft from the tibial bone tunnel. By comparing the 3 samples from each group that failed because of a pullout from the bone tunnel, no significant difference was observed in the maximum failure force between the control (mean [SD], 6.77 [3.79] N) and TM (mean [SD], 7.65 [5.05] N) tendons ($P>.05$).

Histology of in Vivo Samples

Inside the bone tunnel, a thin fibrous band of scar tissue formed at the graft-to-bone interface in the control group (Figure 4A–C). However, the TM group had thicker fibrous scar tissue at the graft-to-bone interface, and obvious new bone formation was evident inside the tendon graft (Figure 4D–F). More gaps were observed at the tendon-bone interface in the

control group than the TM group. Intense mononuclear cell infiltration was present inside the tendon grafts in both control and TM groups. At the tunnel entrance, only thick fibrous scar tissue formed at the interface in the control group, whereas a visible fibrocartilage zone formed at the interface in the TM group (Figure 5). No cell infiltration was present inside the tendon grafts in either the control or TM groups at this location. Outside the bone tunnel, intense mononuclear cell infiltration was present in the outer layer of the tendon grafts in both control and TM groups. No cell infiltration was present in the center of the tendon grafts in either the control or TM groups at this location (Figure 6).

Discussion

Tendon-to-bone interface healing is the most clinically challenging issue when a reconstructive tendon or ligament needs to integrate into bone for healing. A clinical retrieval study showed tendon-bone integration of allograft tendons is not achieved even 2 years after reconstruction (3). Delayed interface healing may result in serious complications such as repeat tearing of a surgically repaired rotator cuff (14) and knee joint instability after ACL reconstruction (15). The motivation of the current study was to modify the tendon into a bone-like tissue by TM, thus transforming the tendon-to-bone interface into a mineralized interface to enhance healing.

Trypsin is used for numerous biotechnological processes. It is used for detaching cells from culture dishes (0.25% trypsin-EDTA) and decellularization (0.05% trypsin-EDTA) (16,17). In a previous study, digestion with 0.25% trypsin-EDTA for 2 hours at 37°C was used to remove lubricin from the surface of canine FDP tendons (10). For the current study, because the diameter of rabbit FDP tendon is much smaller, a 10-minute digestion with 0.25% trypsin-EDTA was sufficient. To remove lubricin only on the tendon portion within the bone tunnel, the portion outside the tunnel was protected with antimicrobial film and the trypsinization process was terminated with 10% FBS in MEM. This method successfully removed lubricin from the distal portion of the FDP tendon (within the bone tunnel) while preserving lubricin on the proximal tendon portion (outside the bone tunnel).

Although bone–patellar tendon–bone graft for ACL reconstruction produces a bone-to-bone healing interface (rather than a tendon-to-bone interface), this graft is not suitable for reconstruction of a long tendon (eg, flexor tendon). Hashimoto et al (18) generated a bone-tendon-bone graft within the semitendinosus tendon by intratendinous injections of recombinant human BMP-2. Reconstruction of the ACL with this novel graft resulted in better restoration of ACL morphology and function. However, this method can be used only for autograft transplantation and not FDP tendon reconstruction because of unavailability of intrasynovial autograft for calcification. Mutsuzaki et al (19,20) also developed a calcium phosphate–hybridized tendon graft and reported positive results after transplantation, but the graft did not show graded minerals inside the tendon. In the current study, a graded mineral deposition zone was generated inside the tendon by using a previous established method (13). Furthermore, after protecting the proximal portion of the tendon with antimicrobial film and rubber sleeves, only the distal portion was mineralized and an obvious boundary formed between the mineralized and unmineralized portions of the tendon.

FDP tendon graft is usually indicated when the injured tendon cannot be repaired directly (21). A flexor tendon injury model therefore is more relevant than the proximal tibial bone tunnel model. In the present study, an extra-articular rabbit model was used to study the effect of TM on intrasynovial allograft tendon healing to bone (22). To increase clinical relevancy, another bone tunnel was made to lead synovial fluid into the bone tunnel from the knee joint. The lateral side of the tendon graft was sutured to the biceps femoris. The medial side of the tendon graft was sutured to the semitendinosus tendon with tension. However, the semitendinosus tendon was not detached from its bone insertion point because the diameter of FDP tendon is smaller than that of the semitendinosus tendon. Physiologic loading of the FDP tendon graft was not fully reestablished after reconstruction. Further study is needed to explore the effects of mechanical loading.

In the current study, maximum failure strength and linear stiffness were not significantly different between the 2 groups at week 8. Each group had 3 samples fail at the graft midsubstance and 3 samples fail via partial pullout from the tibial bone tunnel. No tendon was pulled completely free of the tibial tunnel. The results were similar to a study from Bhatia et al (23) that used a rabbit ACL reconstruction model and reported no significant differences in the maximum load and stiffness values among autograft, nonirradiated allograft, and irradiated allograft groups at 8 weeks, with most samples failing at the midsubstance. They also observed that irradiated samples were beginning to undergo severe remodeling and degeneration at 8 weeks (23). In the current study, both groups had intense mononuclear cell infiltration in the outer layer of the tendon grafts, and neither group had cell infiltration in the center of the tendon grafts. Severe tendon degeneration and remodeling occurred outside the bone tunnel, leading to mechanical weakening and subsequent failure at the tendon midsubstance during testing; thus, the tendon-to-bone interface healing strength could not be truly evaluated.

A cartilage-like layer was present at the entrance of the tendon-bone interface for the TM group but absent for the control group. This exciting finding indicates that it may be possible to establish a fibrocartilage transitional zone at the tendon-bone interface with the TM technique. Most importantly, obvious new bone formation was observed inside the tendon graft only in the TM group. The control group also had more gaps and less fibrous scar tissue at the tendon-bone interface. This suggests that TM of the intrasynovial allograft might have beneficial effects on chondrogenesis and osteogenesis, as well as on integration of the intrasynovial tendon graft.

We acknowledge some limitations of the study. First, the sample size was small (only 6 samples in each group were used for biomechanical testing and 2 samples were used for histologic analysis), which precluded a quantitative analysis. Future studies with a larger sample size and larger animal model should be performed to confirm our preliminary data. Second, this tendon-bone tunnel model was a simplified tendon-to-bone healing model. Although the bone tunnel was in a synovial environment, the tendon graft was not subject to efficient tensile loading. A future study will be translated to a clinically relevant model such as ACL or flexor tendon reconstruction. Third, for histologic analysis, we did not use staining methods such as Alcian Blue or Orange G to assess proteoglycans and keratin, which are important for fibrochondrogenesis (24). Finally, we studied the tendon-bone

interface healing at only one time point (8 weeks after graft transplantation) in the middle of the tissue regeneration stage for an allograft tendon. Future studies will investigate tendon-to-bone healing using this novel technique at an earlier or later stage of healing.

In summary, the current study is the first to explore the effects of TM on the intrasynovial allograft healing to a bone tunnel. TM showed some beneficial effects on chondrogenesis, osteogenesis, and integration of the intrasynovial tendon graft. However, mechanical failure strength of the graft was not different from that of control tendons. This unexpected result may be due to failure that occurred at the tendon midsubstance rather than at the tendon-to-bone healing interface.

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Abbreviations

ACL	anterior cruciate ligament
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FDP	flexor digitorum profundus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IACUC	Institutional Animal Care and Use Committee
MEM	minimal essential medium
PBS	phosphate-buffered saline
TBS	Tris buffer containing 0.1% BSA and 0.5% Triton X-100
TM	trypsinization and mineralization (trypsinized-mineralized)

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Figure 1.

Two tibial tunnels were created, with a horizontal tunnel for tendon transplantation and an oblique tunnel for synovialization of the tendon-transplant tunnel. A, After the horizontal tendon transplant tunnel was created with a horizontal Kirschner wire, a second Kirschner wire was inserted obliquely to meet the first wire. B, The horizontal Kirschner wire was withdrawn and the oblique Kirschner wire was further advanced into the knee joint. Thus, the horizontal tunnel was interconnected with the knee joint to form an intrasynovial environment.

Figure 2.

Optimizing duration of trypsinization for rabbit flexor digitorum profundus tendons. A, Samples were stained for lubricin. Boxes indicate lesions magnified in figure parts B–E. B, Lubricin was observed on the surface and substance of control (untreated) tendons. C–E, Lubricin expression decreased with trypsinization time (C, 10 minutes; D, 30 minutes; E, 60 minutes).

Figure 3.

In vitro evaluation of the trypsinized and mineralized (TM) tendon. A, Gross observation of tendon grafts (alizarin red S stain) shows obvious mineralization (dark purple staining) at the end of the TM tendon (arrow). No dark purple staining was observed on the control (CT) tendon. B, Von Kossa staining shows the junction between mineralized and unmineralized portions of a TM tendon. Boxes indicate regions magnified in figure parts C–D. C, Mineral deposition was visible inside the tendon in a graded manner. D, No mineral was deposited outside the mineral-treated portion of the tendon. E, Lubricin immunohistochemical staining. Boxes indicate regions magnified in figure parts F–H. F, No positive staining in the treated portion of the tendon. G, Positive staining in the tendon substance at the junction between treated and untreated regions. H, Obvious positive staining of the untreated tendon portion.

Figure 4.

Histology of the tendon graft inside the bone tunnel. All samples were stained with hematoxylin-eosin. A, Control tendon. Inside the bone tunnel, a clear gap is observed between the tendon graft (TG) and tibial bone (TB). B, Rectangular region from figure part A shows mononuclear cell infiltration (arrow) inside the graft. C, Circular region from figure part A shows interrupted fibrous bands of scar tissue at the interface (arrow). D, The trypsinized-mineralized tendon had thicker fibrous scar tissue at the graft-to-bone interface. E, Rectangular region from figure part D also shows mononuclear cell infiltration (arrow) inside the graft. F, Circular region from figure part D shows obvious new bone formation (arrow) inside the graft.

Figure 5.

Histology of the tendon graft at the entrance of the bone tunnel. Samples were stained with hematoxylin-eosin unless otherwise indicated. A, Control tendon graft. B, Rectangular region from figure part A shows the tendon graft at the bone tunnel entrance. C–D, Circular region from figure part A shows fibrous scar tissue forming at the interface in the control group (D, Masson trichrome stain). E, Trypsinized-mineralized graft. F, Rectangular region from figure part E shows the tendon graft. G–H, Circular region from figure part E shows fusion with the bone tunnel, with a visible fibrocartilage zone forming at the interface (H, Masson trichrome stain).

Figure 6. Histology of the tendon graft outside the bone tunnel. Samples were stained with hematoxylin-eosin. A and D, Control and trypsinized-mineralized tendon grafts, respectively. B and E, Rectangular regions from figure parts A and D, respectively, show no cell infiltration inside the tendon grafts. C and F, Circular regions from figure parts A and D, respectively, show a distinct layer of mononuclear cells on the graft surface.