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Rotator cuff repair augmentation in a rat model that combines a multilayer xenograft tendon scaffold with bone marrow stromal cells

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Abstract

Hypothesis—A composite of multilayer tendon slices (COMTS) seeded with bone marrow stromal cells (BMSCs) may impart mechanical and biologic augmentation effects on supraspinatus tendon repair under tension, thereby improving the healing process after surgery in rats.

Methods—Adult female Lewis rats (n = 39) underwent transection of the supraspinatus tendon and a 2-mm tendon resection at the distal end, followed by immediate repair to its bony insertion site under tension. Animals received 1 of 3 treatments at the repair site: (1) no augmentation, (2) COMTS augmentation alone, or (3) BMSC-seeded COMTS augmentation. BMSCs were labeled with a fluorescent cell marker. Animals were euthanized 6 weeks after surgery, and the extent of healing of the repaired supraspinatus tendon was evaluated with biomechanical testing and histologic analysis.

Results—Histologic analysis showed gap formation between the repaired tendon and bone in all specimens, regardless of treatment. Robust fibrous tissue was observed in rats with BMSC-seeded COMTS augmentation; however, fibrous tissue was scarce within the gap in rats with no augmentation or COMTS-only augmentation. Labeled transplanted BMSCs were observed throughout the repair site. Biomechanical analysis showed that the repairs augmented with BMSC-seeded COMTS had significantly greater ultimate load to failure and stiffness compared with other treatments. However, baseline (time 0) data showed that COMTS-only augmentation did not increase mechanical strength of the repair site.

Conclusion—Although the COMTS scaffold did not increase the initial repair strength, the BMSC-seeded scaffold increased healing strength and stiffness 6 weeks after rotator cuff repair in a rat model.

Level of evidence—Basic Science Study, Animal Model.

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Keywords

Bone marrow stromal cell; composite of multilayer tendon slices; rotator cuff tear; scaffold; xenograft; tendon; biomechanics; animal model

Despite advances in repair techniques, the repair of large-to-massive rotator cuff tears is still challenging. Multiple factors are associated with low healing rates after these repairs, including increased age, tendon quality, muscle atrophy, size of the tear, and gap formation at the repair site shortly after surgery.²² The increased tension needed to hold the once-retracted tendon in the repaired position is a primary cause of gap formation and failure to heal after surgery.^{9,14}

Efforts to improve clinical outcomes have been reported on mechanical augmentation with graft materials, such as human dermal graft,⁵ porcine dermal graft,³ small intestine submucosa,²⁷ and autologous biceps tendon,³⁰ with promising results. Nevertheless, grafting materials used clinically may not provide sufficient mechanical support to promote sound healing, thereby resulting in high failure rates.^{18,27} Researchers are exploring biologic augmentation of the rotator cuff using bone marrow stromal cells (BMSCs), which have the potential to differentiate into various tissue types.⁸ Currently, the effectiveness of BMSC treatment remains controversial.^{15,34}

Omae et al²⁵ previously reported significant healing in a composite of multilayer tendon slices (COMTS) model to increase the surface area seeded with BMSCs in vitro. In a subsequent in vivo study, Omae et al²⁴ also demonstrated that a xenograft COMTS scaffold seeded with BMSCs survived for 2 weeks after transplantation and could be incorporated in a rabbit patellar tendon defect model, with the transplanted BMSCs expressing a tendon phenotype. On the basis of our experience in tendon engineering, we posited that the fundamental approach to repairing large rotator cuff tears potentially can shift to using BMSC-seeded COMTS xenograft scaffolds.

The purpose of the present study was to evaluate the mechanical and biologic effects of BMSC-seeded COMTS scaffolds on the repair of supraspinatus tendons under tension in rats. We hypothesized that BMSC-seeded COMTS would mechanically improve the initial repair strength to prevent gap formation and biologically augment the postoperative healing process.

Materials and methods

Study design

Shoulder surgery was performed in 39 adult female Lewis rats. Lewis rats were chosen because they are inbred to the point of being essentially syngeneic. Therefore, transplantation of cells from one rat to another is analogous to an autograft transplantation, which limits the risk of graft rejection.¹⁶ Rats underwent transection of the supraspinatus tendon and a 2-mm tendon resection at the distal end, followed by immediate repair to its bony insertion site under tension. The animals received 1 of 3 treatments at the repair site: (1) no augmentation, (2) COMTS augmentation alone (COMTS-only group), or (3) BMSC-

seeded COMTS augmentation (BMSC-COMTS group), with 13 animals per group. Six weeks after surgery, the animals were humanely killed with CO₂ asphyxiation (11 for biomechanical testing and 2 for histologic analysis). The same procedure was performed in 22 rat cadaveric shoulders, with or without COMTS augmentation, and served as baseline (time 0) controls for the no-augmentation and COMTS-only groups during biomechanical testing.

COMTS scaffold preparation

Deep digital flexor tendons were obtained from the hind limbs of 8 mixed-breed dogs (weight, 21–26 kg) that had been euthanized for other approved studies. Harvested tendons were trimmed into segments ~20 mm in length, immersed in liquid nitrogen for 2 minutes, and then thawed in saline at 37°C for 10 minutes. This procedure was repeated 5 times to kill residual cells in the tendon.²⁴ After washing in phosphate-buffered saline (3 × 30 minutes), tendon segments were incubated in 20 mL nuclease solution (RNase from bovine pancreas, 1.5 U/mL; Roche Diagnostics, Indianapolis, IN, USA) for 12 hours at 37°C. Finally, the tendon segments were rinsed in 50 mL phosphate-buffered saline (3 × 30 minutes) at room temperature with gentle agitation.

Each tendon segment was frozen at –20°C, fixed on a Leica CM1850 cryostat (Leica Biosystems, Buffalo Grove, IL, USA) with optimum Tissue-Tek cutting temperature compound (Sakura Finetek, Torrance, CA, USA), and sliced longitudinally into 5 layers (each 100-µm thick), leaving a ~5 mm portion intact on one end of the tendon segment. This specific slicing method was termed the *tendon-book technique*. Sliced COMTS were rinsed twice by immersing in saline to remove cutting compound. COMTS were then dried in a lyophilizer (Benchtop Manifold Freeze Dryer [BT48]; Millrock Technology Inc, Kingston, NY, USA) for 24 hours. Each dried COMTS was trimmed to a 4-mm × 10-mm rectangle, leaving a 2-mm attachment site (ie, the tendon book “spine”; Fig. 1). Finally, COMTS were sterilized with ethylene oxide gas.

BMSC harvesting

BMSCs were collected from 6 adult Lewis rats. Bilateral femora and tibiae were harvested under sterile conditions. The intramedullary canals of the long bones were washed with 10 mL of cell culture medium with 20% heparin. Culture medium consisted of the minimal essential medium with Earle’s salts (Thermo Fischer Scientific, GIBCO, Waltham, MA, USA), 10% fetal bovine serum, and 1% antibiotics (antibiotic-antimycotic; GIBCO). Harvested bone marrow cells were transferred to a 50-mL centrifuge tube and filtered through a Falcon 70-µm Cell Strainer (Corning Life Sciences DL, Corning, NY, USA). Cells were centrifuged at 380g (1500 rpm) for 5 minutes at room temperature, heparin was removed, and the cell pellet was resuspended in 20 mL of cell culture medium and divided into two 100-mm dishes. Bone marrow cells were incubated at 37°C with 5% CO₂ at 100% humidity. After 3 days, the medium containing floating cells was removed, and fresh medium was added to the adherent cells. These adherent cells were defined as BMSCs.²⁸ Culture medium was changed every third day. After BMSCs reached confluence, they were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA) 0.25% with phenol red (GIBCO) and subcultured. Cells from passage 2 or 3 were used for the experiments.

Engineered tendon preparation, with or without BMSC seeding

On the day of surgery, adherent BMSCs were trypsinized and centrifuged at 380g (1500 rpm) for 5 minutes to remove the trypsin-EDTA solution. Cells were counted using a hemocytometer and mixed with 0.5 mg/mL bovine collagen gel (PureCol; Advanced BioMatrix, Carlsbad, CA, USA), following an established method,²⁶ to a final concentration of 10.0×10^6 cells/mL. Each tendon layer of the COMTS was applied with 20 μ L of the cell-gel mixture. The scaffold was then secured with a sterilized TKLV-2 microvascular clamp (Synovis, St. Paul, MN, USA) and placed in culture medium until surgical implantation. For the COMTS-only group, collagen gel solution without BMSCs was pasted onto the COMTS scaffold.

To visualize implanted cells, BMSCs were labeled, after hemocytometer counting but before mixing with collagen gel, with the fluorescent cell marker DiI (Vybrant DiI Cell-Labeling Solution; Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. This fluorescent dye was used previously for cell tracking in a study of BMSCs.¹⁰

Surgical procedure

Each rat underwent general anesthesia with 2% isoflurane and oxygen. The deltoid muscle was partially detached from the posterolateral section of the acromion and then split distally from the anterolateral corner of the acromion. The supraspinatus tendon was identified and separated from the subscapularis tendon anteriorly and the infraspinatus tendon posteriorly. The supraspinatus tendon was then transected sharply at its insertion site on the greater tuberosity using a scalpel blade. The remaining tendon fiber at the insertion site was removed by scraping with the scalpel. To simulate a condition in which the tendon is repaired under increased tension, 2 mm of the supraspinatus tendon was resected at its distal end.^{7,11} To ensure dimensional accuracy, a forceps tip that has 2-mm width was inserted underneath the supraspinatus tendon at its distal end, then the tendon was cut at the proximal edge of the tip so that the tendon was cut 2-mm medial to its insertion to the humerus. The remaining 2-mm tendon stump on the bone was removed subsequently.

The edge of the transected tendon was stabilized with a double-armed 5-0 Ethibond suture (Ethicon, Somerville, NJ, USA). One end of the suture was passed through the tendon transversely, and then small loops were made on both sides of the tendon. The other end was passed through a 0.5-mm drill hole that was created transversely in an anterior-posterior direction through the proximal part of the humerus. The suture was then tied to advance and repair the shortened tendon to its insertion point on the greater tuberosity. The detached deltoid muscle was repaired with a 4-0 polyglactin 910 suture (Vicryl; Ethicon). No further procedures were performed for the nonaugmented control group (Fig. 2, A).

For the groups with COMTS augmentation, a COMTS scaffold, with or without seeded BMSCs (depending on the treatment group), was placed on top of the repair site. The COMTS was stabilized with another double-armed suture that was passed through the supraspinatus tendon, both long sides of the COMTS, and a second drill hole in the humerus that was created parallel to the first one (Fig. 2, B). Schematics of the 3 treatments are

illustrated in Figure 2, C–E. After surgery, the rats were allowed to perform normal cage activities without immobilization.

Biomechanical testing of rotator cuff repair

The supraspinatus tendon and the humerus were isolated, and the peritendinous tissue was visualized with surgical loupes and removed completely. The humerus was embedded in polymethylmethacrylate in a custom-designed fixture, and the proximal end of the tendon was held in a spring-loaded clamp of a custom-built testing system in our laboratory (Fig. 3). Specimens were subjected to a preload of 0.2 N and preconditioned for 5 cycles of 0.1-mm displacement at a rate of 0.1 mm/s. Specimens were then tested to failure under uniaxial tension at a rate of 0.1 mm/s. The ultimate load to failure was obtained, and the stiffness of the repair was calculated from the force-displacement curve generated by a custom MATLAB program (MathWorks, Natick, MA, USA).

Histologic analysis and evaluation of cell migration

The shoulder was grossly harvested, leaving intact the scapula, humerus, and all muscles around the shoulder joint to preserve the whole structure of the specimen. Specimens were fixed overnight with 4% paraformaldehyde, decalcified with 15% EDTA, and embedded in Tissue-Tek. Coronal sections (10- μ m thick) were cut with a Leica CM1850 cryostat and stained with hematoxylin and eosin. The morphology of the repair site, alignment of the collagen fibers, distribution of cells, and degree of inflammatory infiltration response were examined under light microscopy.

Sections adjacent to the ones used for hematoxylin and eosin staining were examined with a LSM 510 confocal microscope (Carl Zeiss Microscopy, Jena, Germany) to determine the distribution of DiI-labeled transplanted BMSCs.

Statistical analysis

Statistical analysis was performed using JMP 10.0.0 software (SAS Institute Inc, Cary NC, USA). For results of biomechanical testing (ultimate load to failure and stiffness), differences among the 5 groups (2 control groups and 3 treatment groups) were evaluated using 1-way analysis of variance, and subsequent comparisons were made with the Tukey-Kramer method. The level of significance was set at $P = .05$.

Results

Biomechanical testing

All specimens failed at the tendon-to-bone interface. Figure 4 shows the ultimate load to failure and stiffness at baseline (time 0) and at week 6 for the nonaugmented, COMTS-only, and BMSC-COMTS treatment groups. At time 0, we observed no difference among treatment and control groups ($P = .729$ for ultimate load to failure and $P = .9997$ for stiffness). By week 6, all groups showed significant increases in the ultimate load to failure ($P < .0001$ for both groups) and stiffness ($P < .0001$ for the nonaugmented group, and $P = .0007$ for COMTS-only group). However, values for both parameters were significantly higher in the BMSC-COMTS group than in the nonaugmented ($P < .0001$ for ultimate load

to failure and $P=.0301$ for stiffness) or COMTS-only groups ($P<.0001$ for ultimate load to failure and $P=.0023$ for stiffness).

Histologic analysis and evaluation of cell migration

In nonaugmented specimens, we observed a gap between tendon and bone that was filled with loose, fibrous, granulation tissue. The original tendon-to-bone junction had no fibrous connection. The COMTS-only group showed a similar separation between tendon and bone, but a wider band of fibrous tissue filled the gap. The fibrous tissue, which included the partially integrated COMTS scaffold, showed a heterogeneous structure with well-aligned spindle-shaped fibroblastic cells in some areas and poorly organized cells in others. Thin, fibrous connections were visible at the original tendon-to-bone junction and seemed to originate from 1 layer of the COMTS scaffold. In contrast, although specimens in the BMSC-COMTS group had a similar gap between tendon and bone, they showed robust fibrous tissues developing in the gap. The connective tissue was homogeneous and organized, with the characteristic crimped pattern of collagenous fibers and spindle-shaped fibroblasts that aligned parallel to the tendon structure. Although we observed conspicuous spaces between tendinous fibers at the original tendon-to-bone junction, a fair amount of aligned collagenous fibers had developed. A tidemark also was observed at the end of the fibrous tissue. A minimal level of inflammatory cell infiltration was observed at the repair site (Fig. 5).

Figure 6 shows DiI-labeled fluorescent BMSCs, as viewed with confocal microscopy. BMSCs were widely distributed in the fibrous tissue, filling in the gap. Notably, cells also were observed in the original tendon-to-bone interface.

Discussion

This study showed that the BMSC-seeded COMTS scaffold improved tendon-to-bone healing in this rat model of rotator cuff injury. We simulated increased tension on the repair site by resecting 2 mm of the supraspinatus tendon and reattaching the shortened tendon to its original insertion site. Six weeks after surgery, we observed significant improvement in tendon-to-bone healing in biomechanical strength for repairs augmented with BMSC-COMTS. These results corresponded to histologic observations in the BMSC-COMTS group, which showed robust, mature, and organized fibrous tissue at the repair site.

In this study, BMSCs from rats were combined with an acellular tendon slices composite from dogs, and this composite was then transplanted into another rat with rotator cuff injury. The rationale for using dog tendon instead of rat tendon was supported by 2 reasons: (1) the larger dog tendon slices were technically easier to manipulate, and (2) a xenograft model potentially has greater relevance for possible clinical applications because animal tendons are far easier to obtain than tissue from human cadavers. In fact, acellular collagen xenografts are already manufactured commercially and used clinically.¹⁷ To ameliorate the rejection reaction to the xenograft, repeated deep freezing–thawing of the tendon was performed, which decellularizes tissue^{1,2} and minimizes antigenicity.²³ This method is easy and safe compared with other methods of tissue preparation that use chemical agents. In this

study, no major rejection reactions were observed during the 6 postoperative weeks or were noted during dissection.

To maximize the ability of BMSCs to provide biologic augmentation, we focused on developing a scaffold that could efficiently support a large number of BMSCs. A multilayered tendon construct to maximize the number of BMSCs being transplanted has been developed.^{24,25} The biggest challenge we faced in this study was the limited subacromial space available for the COMTS scaffold. To resolve this issue we developed a technique to make the multilayer tendon scaffold thin enough to graft in the available space, while ensuring that the fragile tendon slices remained securely layered in a flat patch. Specifically, we established the novel tendon-book technique reported here, in which the decellularized dog tendon was sliced to a 500- μ m thickness and partially cut longitudinally into 5 layers, similar to the leaves of a book. Scaffold handling during surgery also improved dramatically with this tendon-book technique. The finished COMTS had 5 layers (100 μ m per layer), which was twice as thick as the scaffold used in our previous study²⁴ but still was thinner than the scaffolds used in similar studies of rotator cuff repair in rats.^{4,33} The findings at dissection and the histologic analysis suggest that this scaffold was successfully grafted and did not cause impingement.

BMSCs potentially can differentiate into various cell types, including osteoblasts, chondrocytes, tenocytes, and adipocytes.^{6,8} They have been used to accelerate tissue healing, including tendon healing and tendon-to-bone healing in animal models.^{15,34} In the current study, the mechanical augmentation effect of the COMTS scaffold was minimal, as shown by the biomechanical test results of the time-0 specimens. Therefore, the positive changes in biomechanical properties at 6 weeks were largely attributable to the biologic augmentation effects of the BMSC-COMTS. Histologic analysis showed a more robust fibrous tissue at the repair sites with BMSC-COMTS augmentation, which corresponds with the greater mechanical strength. In addition, the BMSC cell tracking showed that transplanted BMSCs migrated widely in the fibrous tissue and also to the tendon-to-bone insertion site. Therefore, transplanted BMSCs can be reasonably assumed to have an important role in the healing process, including forming fibrous tissue through their proliferation and differentiation in this specific context and through trophic effects on intrinsic cells.

Although specimens from the BMSC-COMTS group showed better healing than the specimens from the other groups, the morphology still was far from normal. The histologic analysis showed conspicuous spaces between the tendinous fibers at the tendon-to-bone insertion site, which was considered to be a sign of an early stage of development. In a recent report, the tendon-to-bone interface was histologically observed after supraspinatus tendon repair with an acellular dermal matrix patch in rats.³³ The authors reported that the organization of collagenous tissue kept improving during the 12-week study. In our study, we had only 1 time point (postoperative week 6) to observe the specimens histologically. The regenerative process facilitated by transplanted BMSCs may have been incomplete at 6 weeks. We deduce that a later time point would have shown more organized and tighter collagenous tissue at the tendon-to-bone interface.

A high rate of gap formation between the repaired tendon and its bony insertion point after rotator cuff repair has been reported in experimental^{20,31} and clinical^{19,22} studies. Gap formation is thought to be associated with impaired rotator cuff tendon healing. In their study using a rat model, Killian et al²⁰ emphasized the importance of initial fixation strength on repair site integrity and healing. Our original goal in this study was to use the COMTS to prevent gap formation through mechanical augmentation of the initial fixation strength while using BMSCs to provide biologic augmentation of tendon-to-bone healing. Contrary to our expectations, biomechanical test data from the time-0 specimens showed that COMTS augmentation did not confer any significant mechanical advantage. In addition, the histologic analysis showed consistent gap formation in all specimens, regardless of the presence or absence of COMTS augmentation.

A prior report that described the mechanical testing various thicknesses of acellular canine Achilles tendons showed that tendon slices of 200 μ m or less in thickness had significantly inferior material properties.²⁹ Tendons in the current study were partially sliced into five 100- μ m layers to create a multilayered construct that fit in the narrow subacromial space of rats without causing impingement. Improving the augmentation needs to be addressed in a future studies in which we will focus on improving the initial strength of the scaffold.

In the current study, histologic observation indicated greater organization of tendinous fibers in the BMSC-COMTS specimens and was consistent with the greater tendon-to-bone healing strength observed during biomechanical testing.

The rat model itself has some substantial limitations.^{12,13,21,32} In addition, the rat model used in this study has several limitations. Our experimentally created acute supraspinatus tendon defect does not reflect human chronic rotator cuff tears, which are retracted because of tendon and muscle degeneration. In addition, the repair method was modified and considerably differed from typical clinical procedures. Our preliminary experiments indicated that preparing a cancellous bone trough and securely affixing the torn tendon was challenging primarily due to the small size of the rat humerus. Instead, a previously reported repair method was used that allowed us to use a consistent repair technique and reduce errors across specimens.

With regard to the histologic analysis, we did not perform a collagen-specific immunohistochemical analysis to identify the collagen type or polarized light microscopy to look at alignment of collagen fibers structure. We also did not perform any specific staining for cartilage to evaluate the transition zone of the tendon-to-bone junction (ie, the gradation of tendon, cartilage, and bone typically observed in an uninjured specimen).

The other limitation of this study was the lack of analysis of changes in gene expression between treatments. The gene expression during healing would have been an important addition.

Conclusion

Stronger tendon-to-bone healing was found in BMSCs treatment combined with a multilayer xenograft tendon scaffold, which resulted in increased formation of fibrous connective tissue

in the gap formed after supraspinatus tendon repair under tension after 6 weeks. Transplanted BMSCs migrated widely in the fibrous tissue, including to the tendon-to-bone interface, and contributed to the initial healing process after surgery. Because clinical studies of rotator cuff repair have shown a relatively high prevalence of gap formation and failure of complete healing, especially for large or massive tears, the biologic augmentation effect of BMSCs in combination with the multilayered xenograft tendon scaffold may provide a clinically important improvement in rotator cuff tear treatment.

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The protocol for this study was approved by the Mayo Clinic Institutional Animal Care and Use Committee (protocol number A26113).

Disclaimer

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Figure 1.

Tendon-book technique. A composite of multilayer tendon slices was made from a canine deep digital flexor tendon from the hind leg. The decellularized xenogenic tendon was partially sliced longitudinally into five 100- μ m-thick layers, leaving a portion of the tendon intact. The tendon was then trimmed to the following dimensions: length, 10 mm (including 2 mm of unsliced tendon); width, 4 mm; and thickness, 0.5 mm.

Figure 2. Surgical procedure. (A) Shortened supraspinatus tendon was reattached to the original bony insertion point at the greater tuberosity in the humerus immediately after the transection. (B) The repair site after augmentation with a composite of multilayer tendon slices (COMTS) scaffold. Schematic of the treatment groups: (C) without augmentation; (D) with COMTS only; and (E) with COMTS seeded with bone marrow stromal cells.

Figure 3. Biomechanical testing system shows the humerus embedded within a tube of polymethylmethacrylate in a custom fixture. The supraspinatus tendon is clamped by the material testing machine in the vertical plane.

Figure 4.

Biomechanical testing results. (A) Ultimate load to failure. (B) Stiffness. Results are shown as mean and standard deviation ($n = 11$ for each group). Both parameters increased significantly between baseline (time 0) and 6 weeks, regardless of treatment. The ultimate load to failure and stiffness at 6 weeks were significantly higher after augmentation with bone marrow stromal cells (BMSC)-composite of multilayer tendon slices (COMTS). Baseline measures of ultimate load to failure and stiffness were not different between treatment groups. $*P < .05$.

Figure 5. Representative sections used for histologic analysis (6 weeks after surgery). (A–C), Repaired supraspinatus tendon and the bony insertion site (original magnification $\times 20$). (D–F) Fibrous scar tissue in the gap (original magnification $\times 200$), as indicated by the * in panels A–C. HH, humeral head; T, tendon. (G–I) Tendon-to-bone insertion point (original magnification $\times 100$). All samples were stained with hematoxylin and eosin. BMSC, marrow stromal cells; COMTS, composite of multilayer tendon slices.

Figure 6.

Confocal laser microscopy images show (A and B) fibrous scar tissue in the gap and (C and D) the tendon-to-bone insertion point. *Red* areas indicate DiI-labeled bone marrow stromal cells labeled with DiI Cell-Labeling Solution (Life Technologies, Carlsbad, CA, USA).

BMSC, marrow stromal cells; *COMTS*, composite of multilayer tendon slices. *HH*, humeral head.