

Original Article

Tendon-Derived Stem Cell Sheet Promotes Early-Stage Tendon-Bone Healing

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Abstract

Objectives: To investigate the role of a tendon-derived stem cell (TDSC) sheet in tendon-bone healing within an extra-articular bone tunnel rat model. **Methods:** Sprague-Dawley rats were randomly assigned to experimental and control groups. The superficial flexor tendon, with or without a TDSC sheet, was transplanted into a 1.0-mm diameter bone tunnel in the proximal tibia. The impact of the TDSC sheet on tendon-bone healing was assessed through radiological analysis, histological staining, and biomechanical testing. **Results:** The TDSC sheet significantly enhanced tendon-bone healing, as evidenced by higher tunnel bone mineral density and bone volume/total volume at 4 weeks post-operation. Hematoxylin-eosin staining revealed that the TDSC sheet promoted the alignment of perpendicular collagen fibers connecting the tendon to the bone, along with Sharpey's fibers and new bone formation at the tendon-bone junction at both 4 and 8 weeks. Additionally, Masson's staining demonstrated that the tendon-bone interface was filled with abundant collagen fibers, with a significantly higher proportion of collagen fiber area in the TDSC sheet group compared to the control group at both 4 and 8 weeks. **Conclusion:** The TDSC sheet may enhance tendon-bone healing in the early stages, providing a potential therapeutic approach to accelerate tendon-bone remodeling.

Keywords: Cell Sheet, TDSCs, Tendon-bone Healing

Introduction

Anterior cruciate ligament (ACL) injuries are common, particularly among athletes. Various stem cells have been applied in both animal and clinical studies to promote tendon-bone healing¹⁻³. However, the scaffold-based delivery of stem cells faces several limitations, including inadequate cell migration and retention within scaffolds, host inflammatory reactions, and reduced capacity to induce cell proliferation.

As a result, biologic enhancement of tendon-bone healing remains a significant clinical challenge requiring effective solutions.

The concept of biomimetic materials using scaffold-free cell sheets was first introduced by Okano et al.⁴. By preserving cellular junctions, endogenous extracellular matrix (ECM), and mimicking the cellular microenvironment, cell sheet techniques address many of the challenges associated with scaffold-based approaches. Moreover, these techniques can be applied to various cell types, making them a versatile tool in cell-based regenerative medicine⁵. Consequently, cell sheet engineering has emerged as a pivotal tissue engineering strategy in applications such as myocardial remodeling, hepatocyte preservation, and periodontal regeneration⁶⁻⁸.

Tendon-derived stem cells (TDSCs) are a newly identified cell type in tendons with stem cell properties. They exhibit high clonogenicity, robust proliferative capacity, and greater multi-differentiation potential compared to bone marrow-

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derived stem cells (BMSCs)⁹, making them a promising candidate for promoting tendon-bone healing in animal models¹⁰. Consequently, TDSCs could serve as an ideal cell source for enhancing tendon-bone healing. Our preliminary (unpublished) data indicate that L-ascorbic acid alone induces TDSCs to form cell sheets, characterized by upregulated stem cell marker expression and enhanced multi-differentiation potential. Based on these findings, we hypothesize that an L-ascorbic acid-induced TDSC sheet could effectively promote tendon graft healing in a rat tibia bone tunnel model.

Materials and methods

Animals

A total of 72 outbred male Sprague-Dawley rats, sourced from Charles River Laboratories, were selected for transplantation to assess the effect of the TDSC sheet on tendon-bone healing. The rats were group-housed under a 12-hour light–dark cycle at 22°C, with *ad libitum* access to food and water based on a standard laboratory diet. They were randomly assigned to either the experimental or control group, with 36 rats in each group.

Tendon-derived stem cell isolation and culture

36 male Sprague-Dawley rats (age: 8 weeks; weight: 250–300 g) were selected for tissue extraction. The procedure for isolating and culturing Sprague-Dawley rat tendon-derived stem cells (TDSCs) was adapted from a previous study¹¹. Briefly, only the mid-substance of the tendon tissue was collected, minced, and then digested for 2.5 hours at 37°C with type I collagenase (SCR103, 3 mg/mL; Sigma, USA). The tissue was then passed through a 70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ) to yield a single-cell suspension. The released cells were washed in PBS by centrifugation at 300 g for 5 minutes and resuspended in low-glucose Dulbecco's Modified Eagle Medium (LG-DMEM) (Invitrogen Corporation, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (complete culture medium) (all from Invitrogen Corporation, Carlsbad, USA). On day 7, cells were trypsinized and pooled as passage 0 (PO). The culture medium was changed every 3 days throughout the experiments.

Preparation of the Cell Sheet

TDSCs were plated at a density of 5,000 cells/cm² in 35-mm dishes and cultured in complete culture medium until they reached 90–95% confluence. To induce cell sheet formation, TDSCs were treated with L-ascorbic acid (50 µg/mL) (AO278; Sigma, USA), a form of vitamin C, in complete culture medium at 37°C and 5% CO₂, as described previously¹². After 2 weeks of L-ascorbic acid treatment, abundant extracellular matrix (ECM) was produced, indicating that cell sheets had formed. The TDSC sheet was then detached from the culture plate by rinsing with saline for subsequent experiments.

Transplantation in SD rats

Seventy-two outbred Sprague-Dawley rats (age: 12 weeks) were selected for transplantation to assess the effect of the TDSC sheet on tendon-bone healing *in vivo*. Animals were anesthetized via intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg). Aseptic techniques were employed during the procedure. The superficial flexor tendon was harvested through a longitudinal incision. 4-0 Ethibond sutures (Ethicon, Somerville, NJ) were passed through each end of the harvested tendon graft, which was then wrapped in gauze moistened with physiological saline in preparation for the subsequent experiment.

A longitudinal incision of 1.5 cm was made from the distal polar of the patella to the distal tibial tubercle. The subcutaneous tissue was separated at both ends to expose the proximal tibia, and a 1.0-mm Kirschner wire was inserted perpendicular to the long bone axis to create a bone tunnel starting from Gerdy's tubercle. A 0.5-mm sewing needle was used to guide the tendon graft, which had been wrapped with the TDSC sheet, through the bone tunnel. Both ends of the tendon graft were sutured to the periosteum of the tibial spine. The skin was closed using 3-0 nylon sutures. The same procedure was then performed on another set of 36 Sprague-Dawley rats as the control group, without the administration of TDSC sheets.

Postoperatively, 3 mL of 0.5 mg/mL carprofen was injected subcutaneously for analgesia and to supplement the fluid volume. The animals were allowed free movement immediately after regaining consciousness. At weeks 2, 4, and 8 post-surgery, 6 animals from each group were sacrificed under anesthesia. After the soft tissue was removed from the tibia, except for the transplanted tendon, the bone-graft-bone complexes were collected for high-resolution radiography and computed tomography (CT) imaging to assess mineralized tissue inside the tunnels. Histological analysis was then performed. Additionally, 6 animals from each group were harvested at the same time for biomechanical testing (total n = 12 per time point per treatment group).

X-ray imaging and micro-CT imaging Analysis

The experimental samples were fixed in 4% paraformaldehyde. After all specimens were collected, X-ray and CT measurements were performed by the same investigator. High-resolution X-ray radiography (MX-20, Faxitron, USA) with a camera (MX-20, Faxitron, USA) was used to scan the bone-graft-bone complex, allowing for observation of bone remodeling around the tunnel. This method provided direct visual insight to evaluate mineral content and mineral density within the tunnel. Simultaneously, micro-CT imaging analysis (SkyScan 1176, Bruker, Germany) was performed, with vertical displacement of 18 µm per scan, covering the region from the entry to the exit with approximately 900 consecutive sections. A circular region of interest (ROI) with a diameter of 1.0 mm was applied for 3D reconstruction using the built-in software. Bone mineral density (BMD, measured

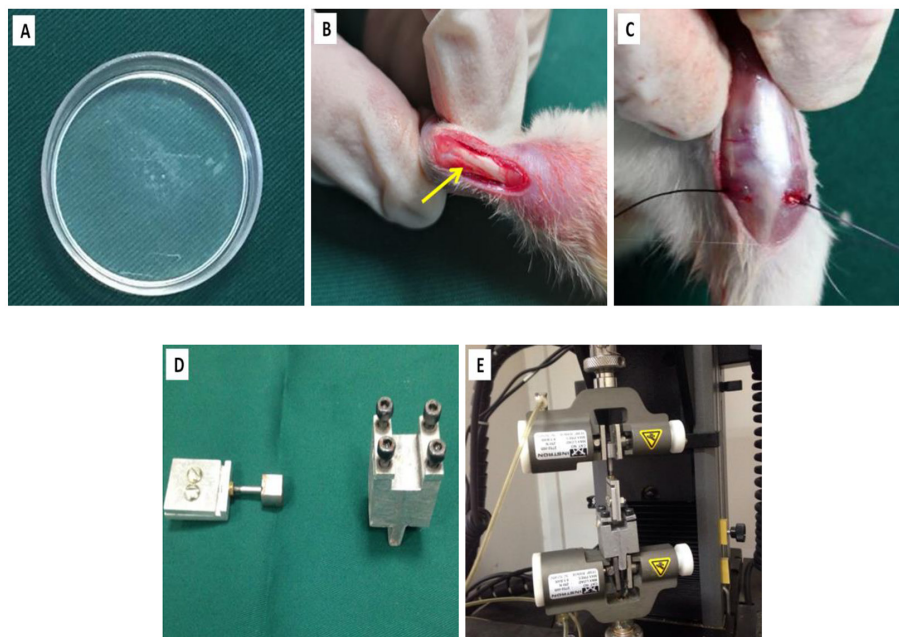


Figure 1. Experimental procedure for preparation of bone-graft-bone complex and biomechanical testing. (A) TDSC sheets were cultured for 14 days *in vitro*. (B) The superficial flexor tendon was harvested, with the yellow arrow indicating the tendon. (C) The tendon graft wrapped with the TDSC sheet was guided through the tibial tunnel. (D) A specially designed clamp was used for biomechanical testing. (E) The clamp with the bone-graft-bone complex was loaded onto a material testing machine, oriented along the direction of the tensile load, for biomechanical testing.

in milligrams of hydroxyapatite per cubic centimeter [mg HA/cm^3] and bone volume/total volume (BV/TV) within the bone tunnel were then measured.

HE and Masson's staining

After radiological analysis, all bone-graft-bone complexes were decalcified, dehydrated, and embedded in paraffin, as previously described¹³. The samples were then cut longitudinally into 5- μm thick sections parallel to the direction of the bone tunnel. These sections were processed for staining with hematoxylin and eosin (HE) and Masson's trichrome to identify new bone, Sharpey's fibers, and collagen fibers at the tendon-bone interface. The sections were observed under light microscopy (DMRXA2, Leica Microsystems Wetzlar GmbH, Germany) by two independent researchers.

Biomechanical Testing

Based on previous studies with slight modifications, we performed a biomechanical examination of the collected specimens¹⁴. Briefly, each specimen for biomechanical testing was wrapped in gauze moistened with physiological saline solution, then sealed in air-tight polychlorovinylidene film. The specimens were stored at -20°C until use. For testing,

the specimens were placed in a specially designed clamp. To assess the biomechanical properties of the healing interface, the clamp with the bone-graft-bone complex was loaded onto a material testing machine (Instron 5940, Instron Co., Ltd., USA) oriented along the direction of tensile load. The composite was subjected to a displacement speed of 0.5 mm/s until failure, generating a load-displacement curve, while 0.9% saline was used to maintain tissue moisture. The ultimate load to failure was recorded based on the maximum tensile stress obtained from the load-displacement curve. Ultimate stress (N/mm^2) was calculated by dividing the ultimate load by the length of the bone tunnel, which was measured using a vernier caliper after the tensile test.

Statistical analysis

Statistical analysis was performed using SPSS software (version 25.0; IBM Corp., Armonk, NY, USA). All values are expressed as the mean \pm standard deviation. Independent samples t-tests were used to compare the means of two groups when the data were normally distributed and variances were equal. For non-parametric data or when the normality assumption was not met, Mann-Whitney U tests were used to compare medians between two groups. The Kruskal-Wallis H test was applied for comparisons involving more

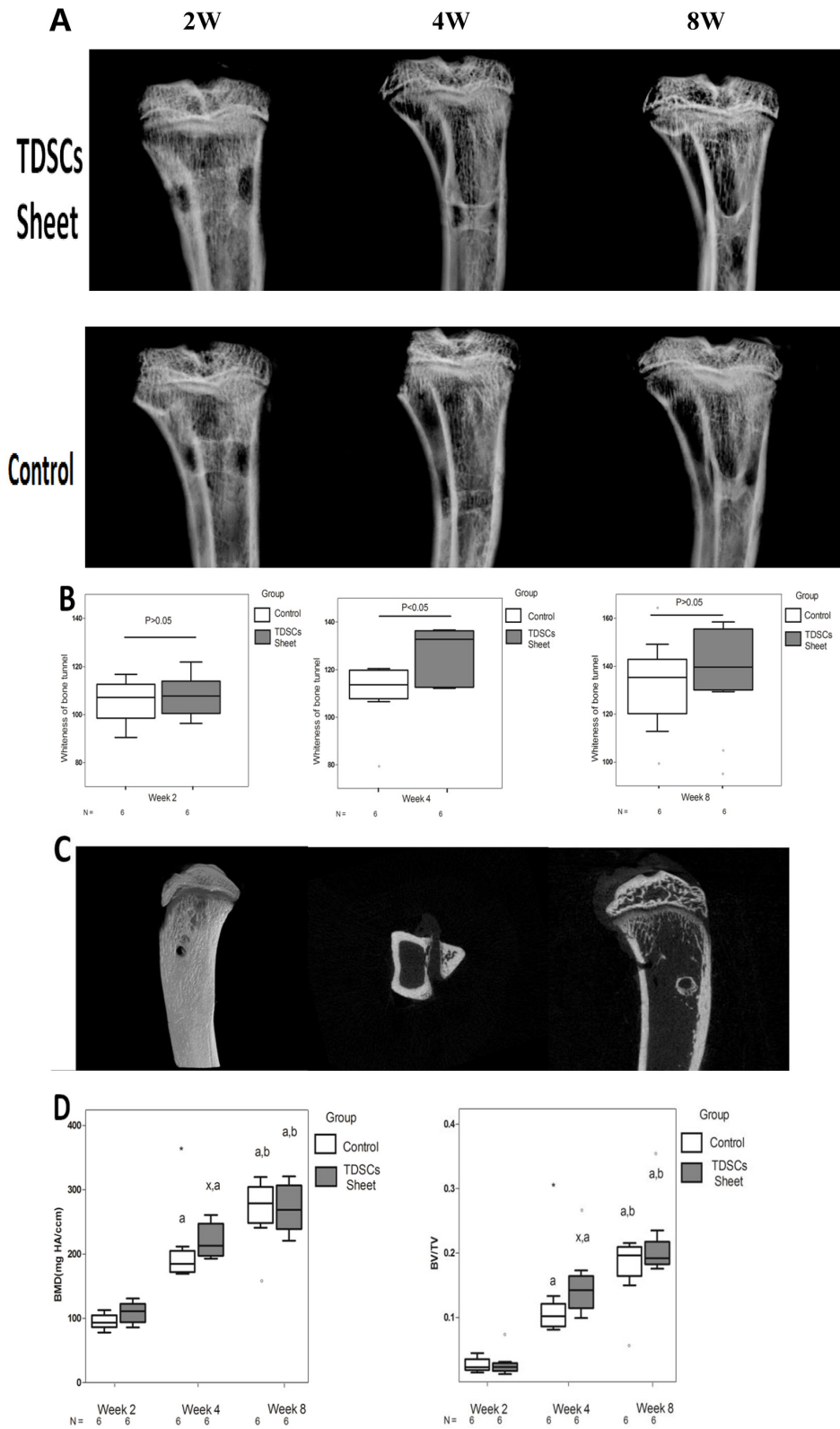


Figure 2. TDSC sheet advanced tendon-bone healing in imaging data. (A-B) The effect of TDSC sheet on tendon-bone healing in injured rats at week 2 after the operation. High-resolution X-ray images showed that the bone tunnel was clear, with low local density observed 2 weeks post-operation. $n = X$ animals/group. (C-D) The effect of TDSC sheet on tendon-bone healing in injured rats at week 4 after the operation. High-resolution X-ray images showed that the tunnel was blurred, with a large amount of new bone tissue formation. $xP \leq 0.05$ compared with the control group; apost hoc $P \leq 0.05$ compared with week 2; bpost hoc $P \leq 0.05$ compared with week 4.

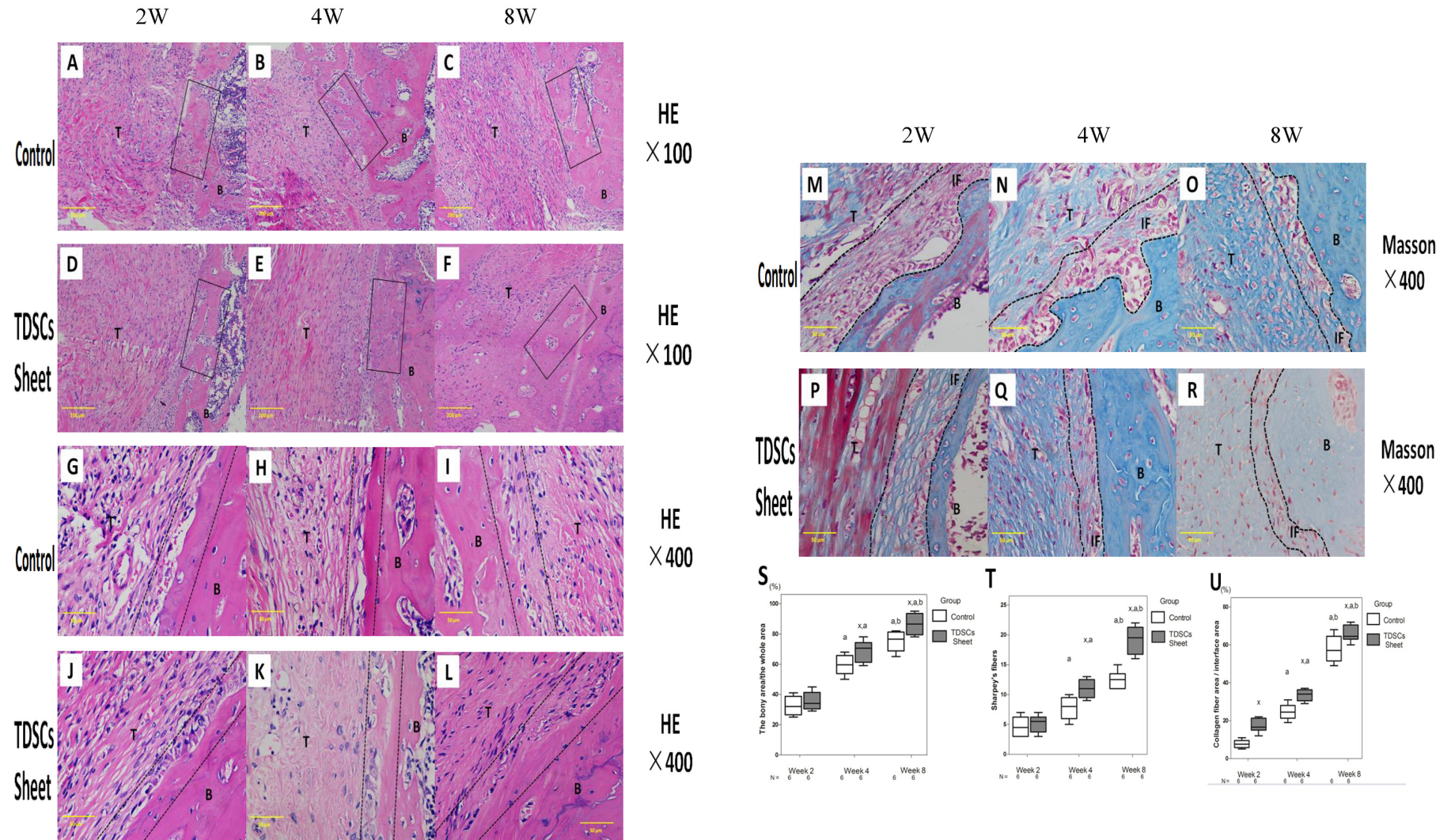


Figure 3. TDSC sheet advanced tendon-bone healing in pathology. HE Staining (Panels A-F and G-L): Control Group (A-C, G-I): Tissue sections at 2W, 4W, and 8W show changes in cellular density and morphology over time. TDSC Group (D-F, J-L): Similar time points are presented, highlighting differences in cellular response and organization compared to the control group. Masson Staining (Panels M-R): Control Group (M-O): Displays collagen fiber distribution and organization in the tissue. TDSC Group (P-R): Illustrates enhanced collagen formation and organization, suggesting improved tissue healing. Quantitative Data (Panels S-U): Panel S: Percentage of the area occupied by tissue over time for both groups. Panel T: “Sharpey’s index,” indicating the quality of tissue repair. Panel U: Percentage of collagen fibers per area, emphasizing the differences between groups. T = Tendon; B = Bone; FC = Fibrocartilage.

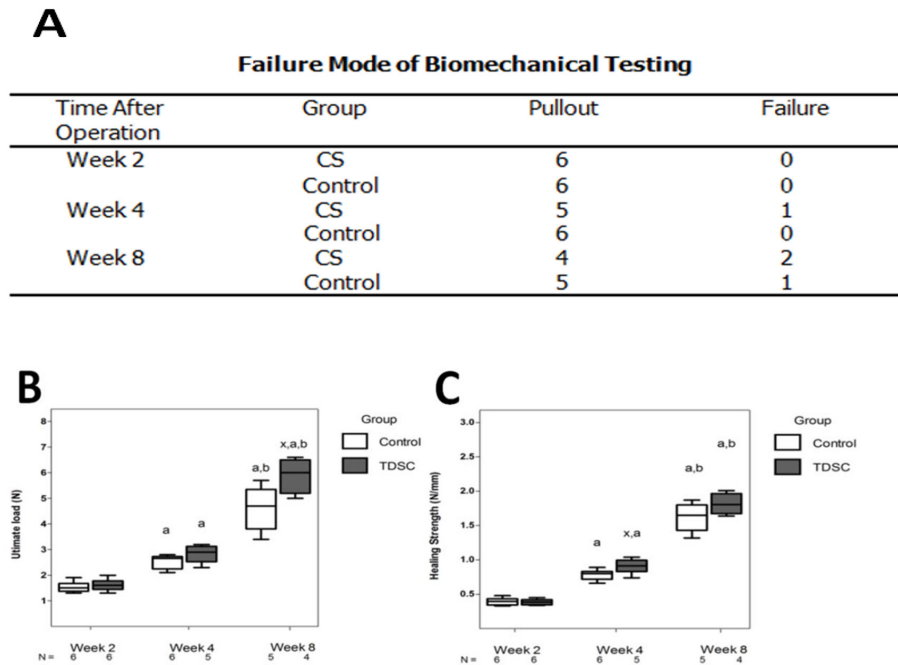


Figure 4. Biomechanical properties of the bone-graft-bone complex after reconstruction. The boxplots show significant improvement in the ultimate load at week 8 and ultimate stress at week 4 in the TDSC sheet group compared with the control group. $xP \leq .050$ compared with the control group; apost hoc $P \leq .050$ compared with week 2; bpost hoc $P \leq .050$ compared with week 4.

than two groups with non-parametric data. For comparisons between groups or within one group at different time points, one-way ANOVA was used. A p-value < 0.05 was considered statistically significant.

Results

TDSC sheet advanced tendon-bone healing in imageology

To validate our hypothesis regarding the influence of the TDSC sheet on tendon-bone healing, we conducted an experimental procedure involving the preparation of the bone-graft-bone complex and biomechanical testing, as shown in Figure 1A-E. After culturing with L-ascorbic acid for 2 weeks *in vitro*, the resulting cell sheet was collected and transplanted into the tendon. High-resolution X-ray imaging revealed that, compared to the surrounding normal bone tissue, the mineral content and mineral density in the tunnel area were significantly lower, with no significant difference between the two groups at week 2 post-surgery. However, at week 4 post-surgery, a substantial amount of new bone formation and regenerative bone in the tunnel area was observed in the TDSC sheet group, with similar results recorded at week 8.

We then performed 3D reconstruction of the bone-graft-bone complex (Figure 2A-C), and micro-CT analysis revealed no significant differences between the two groups in BMD

or BV/TV at week 2 post-surgery. Further analysis at week 4 showed significantly higher tunnel BMD and BV/TV in the TDSC sheet group (BMD: 17.3% increase, $P = 0.048$; BV/TV: 35.2% increase, $P = 0.043$). However, there were no statistically significant differences between the control and TDSC sheet groups at week 8 (Figure 2D). The imaging data indicated that the TDSC sheet promoted tendon-bone healing.

TDSC sheet advanced tendon-bone healing in pathology

HE staining was performed to investigate the pathological improvement induced by the TDSC sheet. At weeks 4 and 8 post-surgery, the TDSC sheet treatment showed significant new bone regeneration between the tendon graft and the bone (tendon-bone gap) (Figure 3A-F). A rectangular area (100×200 μm) was drawn in the adjacent trabecular area near the tunnel wall to calculate the bone-occupation ratio using Scion Image Analysis software. The bone-occupation ratio was higher in the TDSC sheet group compared to the control group at both weeks 4 and 8.

Highly cellular granulation tissue was observed in the tendon-bone gap in both groups. At week 2 and 4, the tendon-bone gap in the control group appeared larger than in the TDSC sheet group, but no significant differences were noted at week 8 (Figure 3S). Quantitative analysis

showed that the number of Sharpey's fibers (counted in a randomly chosen view at HE×400 magnification) was significantly higher in the TDSC sheet group compared to the control group at weeks 4 and 8 (Figure 3G-L). Collagen fiber formation was clearly visible in the gap with Masson's staining (Figure 3M-R). Quantification using Scion Image Analysis software demonstrated that the total collagen fiber area was significantly greater in the TDSC sheet group than in the control group at weeks 2, 4, and 8 (Figure 3S-U).

The effect of TDSC sheet on biomechanical properties of the bone-graft-bone complex

Having validated the contribution of the TDSC sheet to tendon-bone healing through imaging and histopathology, we then conducted a biomechanical examination to assess its impact on the biomechanical properties of the bone-graft-bone complex. Significant improvements were observed in the ultimate load at week 8 (28.3% increase) and ultimate stress at week 4 (15.6% increase) in the TDSC sheet group compared to the control group. Both groups exhibited failure modes during biomechanical testing (Figure 4A-C).

Discussion

This study integrated stem cell biology with scaffold-free cell sheet engineering to enhance tendon-bone healing. We utilized TDSCs as a cell source to construct engineering cell sheets, leading to successful TDSC sheet implantation *in vivo*. The findings demonstrated that wrapping the tendon graft with the TDSC sheet significantly improved mineral content and mineral density, new bone formation, Sharpey's fibers, and collagen fiber formation, as well as the structural properties at the tendon-bone interface compared to the control.

Compared to synthetic scaffolds, the cell sheet technique effectively reduces issues related to biocompatibility, biodegradability, and immunogenicity¹⁵. Furthermore, cell sheets can eliminate the need for stem cell injection, which may lead to cell leakage and high rates of cell death. Due to the preservation of cellular junctions, endogenous extracellular matrix (ECM), and maintenance of the cellular microenvironment, cell sheet techniques avoid these problems. This approach has become a research hotspot in recent years within tissue engineering and has been widely applied to various tissues, including articular cartilage, cornea, esophagus, heart, periodontal ligament, and others^{16,17}. Compared to the traditional method using temperature-responsive culture dishes, our L-ascorbic acid-mediated technique provides a more novel, simple, and cost-effective method for obtaining cell sheets. Our procedural approach was similar to previously established protocols for mesenchymal stem cells (MSCs) in cell sheet formation¹⁸. In our observations, 50 µg/ml L-ascorbic acid was identified as the optimal concentration for TDSC sheet formation (unpublished data).

Furthermore, the TDSC sheet was non-invasively detached from the culture dish by rinsing with saline, preserving cell-to-cell junctions and the deposited extracellular matrix (ECM)¹⁹. This method offers an alternative approach for tissue regeneration, serving not only as an ideal scaffold-free technology but also opening up possibilities for the development of thick, stratified, and complex tissues²⁰.

Compared to BMSCs, TDSCs express higher levels of Oct4, exhibit greater clonogenicity and proliferative capacity, and show enhanced differentiation potential across tenogenic, osteogenic, chondrogenic, and adipogenic lineages²¹. A previous study demonstrated that TDSCs have superior regenerative potential in treating ruptured Achilles tendons in rats, compared to BMSCs²². Additionally, TDSCs can be isolated from residual tendon tissue after ligament reconstruction or total knee replacement, eliminating the need for additional procedures or associated pain. Therefore, TDSCs may serve as a more effective cell source than BMSCs for musculoskeletal tissue regeneration.

Upon completion of the operation, we assessed the therapeutic potential of the TDSC sheet in promoting tendon-bone healing at weeks 2, 4, and 8. Imaging results revealed that cell sheet transplantation had a more pronounced osteogenic effect at the bone tunnel sites compared to the control. Mifune et al. reported that osteogenic differentiation capacity plays a critical role in tendon-bone healing, out of all the differentiation potentials, in their study²³. Furthermore, our preliminary findings demonstrated that TDSC sheets have a stronger osteogenic differentiation ability than TDSCs alone. In the present study, the enhanced osteogenic differentiation of a large number of TDSCs likely contributed to the increased mineral content at the tendon-bone interface.

Additionally, it has been shown that TDSCs in cell sheets exhibit a higher proliferation rate and better preservation of stemness compared to TDSCs cultured on plastic dishes, which contributes to tendon graft remodeling (unpublished data). This is another important factor contributing to the observed increase in mineral content.

In the present study, greater numbers of Sharpey's fibers and collagen fibers were observed at the tendon-bone interface in the TDSC sheet group, accelerating the early remodeling of tendon-bone healing histologically. Previous studies have shown that the presence of new bone tissue and collagen fibers significantly improves tendon-to-bone healing at early time points²⁴. Additionally, these studies indicated that ultimate load and stress progressively increase with abundant production of new bone tissue and collagen fibers. In this study, biomechanical testing revealed that the ultimate load and stress in the TDSC sheet group were significantly higher than in the control group at both week 8 and week 4. These results align with imaging and histological observations, all supporting the notion that the TDSC sheet group has a more positive effect on tendon-bone healing. Furthermore, the higher integrity of the tunnel and graft tendon in the TDSC sheet group, compared to the control group, may explain

the superior biomechanical and imaging outcomes. Our investigations suggest that the TDSC sheet can enhance the healing response of tendon-bone interfaces during the early stages, making it a promising new approach for tendon-bone healing.

Conclusion

In this study, tendon grafts wrapped with TDSC sheets were successfully implanted into rat bone tunnels. At weeks 2, 4, and 8 post-reconstructions, the bone-graft-bone complexes were assessed using radiology, histology, and biomechanical testing, which demonstrated that the TDSC sheet promotes tendon-bone healing in the early stages. In conclusion, we propose that L-ascorbic acid-induced TDSC sheets offer an economical and practical engineering approach for enhancing tendon-bone healing and tissue regeneration.

Ethics approval

All experiments were approved by the Research Ethics Committee of Southeast University, China (Approval number: [2016]050601). All applicable institutional and national guidelines for the care and use of animals were followed.

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