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Original Article

Single-layer periosteum progenitor cell sheet significantly promotes tendon–bone healing in comparison with acellular collagen sheet in anterior cruciate ligament reconstruction

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ABSTRACT

Purpose: Successful tendon–bone healing in anterior cruciate ligament reconstruction can be achieved by introducing a suitable medium into the tendon–bone junction. Two new feasible media, periosteum progenitor cell sheet (PPCS) and acellular collagen sheet (ACS), were utilized to promote tendon–bone healing. This study aimed to compare the effects of these two media on tendon–bone healing in a rabbit model.

Methods: Periosteum progenitor cells were harvested from rabbit tibial periosteum, cultivated on polymerized fibrin-coated dishes, and obtained as PPCS. ACS was prepared from rabbit small intestinal submucosa. Twenty rabbits were used in this study. Tendon grafts wrapped in cell sheet were pulled into a bone tunnel in a model of anterior cruciate ligament reconstruction. Rabbits were sacrificed at 8 weeks postoperatively for histological, immunohistochemical, and biomechanical assay.

Results: The monolayer PPCS that had been wrapped around the tendon before surgery was analysed. Histological staining revealed that PPCS gave rise to higher collagen and glycosaminoglycan deposition with fibrocartilage formation than ACS at the tendon–bone junction at 8 weeks. In addition, PPCS produced significantly better mechanical results compared with ACS, including increased maximum load, stiffness, and maximum load per length of bone tunnel.

Conclusions: Single-layer PPCS significantly enhanced tendon–bone healing compared with ACS, with fibrocartilage formation at the tendon interface and superior biomechanical strength.

1. Introduction

Anterior cruciate ligament (ACL) reconstruction using hamstring tendon grafts has gained popularity in orthopedic sports medicine. Enhancement of tendon–bone healing has been the focus of successful ACL reconstruction, with an emphasis on the osteointegration of tendon grafts within the bone tunnel.[1] Increased strength of healing and faster tendon–bone healing allow early postoperative rehabilitation to achieve a full range of motion. With better healing strength to meet the mechanical loading required by rehabilitation, this may contribute to a higher success rate and prevent graft failure.

Numerous methods have been reported to achieve better and quicker osteointegration of tendon graft in the bone tunnel, such as the use of bone morphogenetic protein-2,[2] transforming growth factor (TGF) β1,[3,4] TGF-β3,[4,5] stem cells,[6–8] periosteum,[9–13] calcium phosphate,[14,15] gene therapy,[16] ultrasound stimulation,[17] and hyperbaric oxygen.[18] Application of these substances into the bone tunnel aims to enhance the integration of tendon grafts at the tendon–bone interface.[2–16] One of these media, auto-periosteum, has proved to be a valuable therapeutic material in promoting biological recovery after ACL reconstruction.[10–13] Therefore, preformed periosteum formatted by tissue engineering technology is worth investigating for ACL reconstruction.

The periosteum is composed of two layers. The inner cambium layer consists of chondroprogenitor and osteoprogenitor cells that

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have the ability to differentiate into different kinds of tissue such as cartilage and bone.[9,19–22] Previous studies have revealed that periosteum enhances healing of the tendon–bone interface by forming fibrocartilage and calcified fibrocartilage.[10,11] In clinical studies, periosteum-wrapped tendon graft can enhance tendon–bone healing in ACL reconstruction, with satisfactory clinical results.[12,13]

Bearing these previous encouraging results in mind, we extracted periosteum progenitor cells from periosteum, which has the ability to promote tendon–bone healing. Using bioengineering technology, a periosteum progenitor cell-rich material can be preformed and used to promote tendon–bone healing. This could eliminate morbidity at the donor site, minimize the thickness of the wrapping material, and shorten the tendon graft preparation time in ACL reconstruction.

Scaffold-free cell sheet engineering has been available since 1993.[23] This allows cultured cells to be noninvasively harvested as a single, continuous cell sheet with intact cell–cell junctions and deposited extracellular matrix.[24] Cell sheet transplantation has been used in many studies to transplant various kinds of cell.[25,26] In this study, we wrapped the periosteum progenitor cell sheet (PPCS) around the tendon graft to determine its effect on tendon–bone healing.

Natural biological materials and acellular matrix produce differrent response in the body. Collagen is a very common and important human protein that is easily harvested from animal or human tissue. Currently, collagen is also widely used in clinical treatment. Small intestinal submucosa (SIS) from pigs is 90% composed of types I and III collagen, and contains a large number of cytokines, including basic fibroblast growth factor, TGF-β, epidermal growth factor, vascular endothelial growth factor, and insulin growth factor-1.[27,28] It acts as an extracellular matrix scaffold incorporating many kinds of growth factor to enhance the interaction among cells and promote tissue growth with a minimum immune response. SIS has been used in the repair of various anatomical structures such as tendon,[29] fascia,[30] and ligament.[31]

The acellular collagen sheet (ACS) harvested from rabbit SIS contains growth factors and cytokines, which are able to promote tissue healing, whereas the PPCS contains the periosteum progenitor cells that have the ability to differentiate into numerous connective tissues including fibrocartilage. We hypothesized that PPCS would be better than ACS at promoting tendon–bone healing. This study aimed to compare ACS and PPCS biomechanically and histologically in tendon–bone healing in a rabbit model of intra-articular ACL reconstruction.

2. Materials and methods

2.1. Isolation of periosteum progenitor cells

Periosteum progenitor cells were obtained according to the method previously described.[32] The periosteum was stripped from the tibia of mature New Zealand White rabbits. The harvested periosteum was then placed in a 0.25% trypsin solution and 0.1% ethylenediaminetetraacetic acid (EDTA) for 30 minutes at 37 °C and shaken in 1 mg/mL type I collagenase digestive solution for 90 minutes at 37 °C. After washing and centrifugation, the pellets were resuspended in high-glucose Dulbecco's modified Eagle's medium (Gibco BRL; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillin–streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). Periosteum progenitor cells were finely plated in a T25 culture flask (Corning Inc., Acton, MA, USA). Nonadherent cells were removed by changing the fresh medium after 5 days of culture. The culture medium was replenished every 3 days.

2.2. Preparation of PPCS

PPCS was prepared as previously described.[33] Thrombin (Sigma-Aldrich, St. Louis, MI, USA) was added into 100 mM $CaCl_2$ solution (Sigma-Aldrich) to a final concentration of 100 U/mL. Then solution was filtered through a 0.22 μm filter and mixed with phosphate buffer solution (PBS, pH = 7.4) a final concentration of 50 U/mL. Fibrinogen (Sigma-Aldrich) was added to the PBS to obtain a final concentration of 12.5 mg/mL. Thrombin solution was mixed with fibrinogen solution in equal volumes.

The mixture was placed into a 6 cm diameter culture dish containing a 4.7 cm diameter silicon O-ring. The culture dish was kept at room temperature for 2 hours. A total of 5×10^5 periosteum progenitor cells were seeded into a fibrin-coated dish, and then incubated under the same conditions for primary cell cultures as previously described. The culture medium containing aprotinin 350 KIU/mL (Protech Technology Enterprise Co. Ltd., Taiwan) was replaced every 3 days. After 10 days of culture, the silicon O-ring was removed, and the cell sheet was harvested using a scraper. A confocal microscopy assay and immunofluorescence staining were used to evaluate cell distribution and sheet continuity.

2.3. Preparation of ACS

Native intestinal submucosal membrane was harvested from the intestines of New Zealand White rabbits (weight approximately 3.5 kg). The intestines were cleaned with water for 1 hour and then rinsed in normal saline. They were then slipped over 10 mm diameter glass rods, the serosal and muscle layers being removed from the intestinal duct using gauze. After turning the intestinal duct over, the mucosal layer was also removed with gauze until only the submucosal layer remained. During this process, which lasted for 4 hours, the intestines were washed in water at 40°C, the end-product being SIS membrane.

The SIS was then treated with following chemical steps, according to a previously described method.[29] First, it was placed in 100 mM EDTA and 10 mM NaOH solution (pH 11–12) for 16 hours, before being washed with deionized water. It was then put into 1 M HCl and 1 M NaCl solution (pH 0–1) for 8 hours. It was again cleaned with deionized water, and then placed into 1 M NaCl and 10 mM PBS solution (pH 7–7.4) for 16 hours. The SIS was again cleaned with deionized water before being immersed in 10 mM PBS for 2 hours, transferred to sterile water (pH 5.8–7.0) for 2 hours, and freeze-dried for 24 hours. Next, the SIS was immersed in 2 mL 50 mM 2-(*N*-morpholino) ethanesulfonic acid in 40% ethanol (pH 5.0) with 30 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 6 mM *N*-hydroxysuccinimide at 4 °C for 24 hours. It was then cleaned with deionized water and again freeze-dried for 24 hours. Finally, the SIS, now in the form of an ACS, was sterilized with H_2O_2 treatment and stored at −20 °C.

2.4. Animal study design and surgery

Twenty skeletally mature New Zealand White rabbits (each weight approximately 3.5 kg) were used in this study. Animal were randomly assigned to the PPCS and ACS groups, each group consisting of 10 rabbits undergoing ACL reconstruction. Five rabbits (10 limbs) randomly selected from each group were prepared for histological and immunohistochemical evaluation. The remaining five rabbits (10 limbs) from each group were utilized for biomechanical testing. Animal treatment conformed to the Guidelines for Care and Use of Laboratory Animals and was approved by the Committee of Experimental Animal Sciences.

Ketamine (40 mg/kg) with xylazine (Rompun, 5 mg/kg; Bayer Healthcare, Leverkusen, Germany) was injected intramuscularly to

induce general anesthesia. The rabbits underwent an operative procedure modeling ACL reconstruction. The long digital extensor tendon was identified and detached. The free end of the tendon was then sutured using a 3-0 Vicryl suture (Ethicon, Somerville, NJ, USA). The knee joint was explored via a medial parapatellar tendon approach, and the normal ACL was excised. Femoral tunnel and tibial tunnel were created with a 2.0 mm diameter drill through the footprint at 45° to the long axis of the bones. The length of the tunnel was measured with a depth gauge.

In the PPCS group, a 30 mm diameter semi-circular section of PPCS was wrapped around the tendon at each end in one layer. The middle part of the tendon graft, approximately 5 mm in length, was free of wrapping. The tendon graft was pulled manually through the drill hole using holding sutures, and the extraarticular graft was fixed to the nearby soft tissue under maximum tension with a 3-0 nylon suture, mimicking suspensory fixation. The joint capsule, fascia, and subcutaneous tissues were closed with interrupted 3-0 Vicryl sutures, and the skin was closed using interrupted 3-0 nylon sutures. The same procedure was then performed on the contralateral knee.

In the ACS group, the wrapping material was SIS. The tendon graft, drill diameter, drill length, drill axis, and fixation method were the same as for the PPCS group.

The limbs were immobilized postoperatively, and the rabbits were allowed to exercise as desired in individual cages. The animals were sacrificed at 8 weeks after surgery under anesthesia.

2.5. Histological examination

Five rabbits (10 limbs) randomly selected from each group were prepared for histological and immunohistochemical evaluation of the tendon–bone healing. The specimens were harvested and fixed with 10% formalin for 24 hours. After decalcification and dehydration in graded alcohols, the proximal tibia was embedded in paraffin, sectioned to a thickness of 5 μm, and processed for hematoxylin and eosin (H&E), Masson's trichrome (MT), and Safranin-O staining. The observer was blinded to the group the sample was taken from. Histological sections were observed and photographed using a light microscope.

2.6. Immunohistochemical assay

Tissue sections were deparaffinized, rinsed with PBS containing surfactant Tween-20 (Sigma-Aldrich), and treated with PBS containing 3% bovine serum albumin (BSA) for 30 minutes. To assess protein expression, the sections were conjugated primary antibodies for 1 hour, including polyclonal anti-collagen type I (Abcam, Cambridge, UK; 1:800 dilution) and polyclonal anti-collagen type II (Calbiochem, Darmstadt, Germany; 1:1000 dilution). The samples were rinsed three times with PBS, and conjugated secondary antibody for 1 hour. All proteins were marked with diaminobenzidine (DAB; Dako, Glostrup, Denmark), and the nuclei were stained with H&E. Specimen sections were examined under a light microscope to assess protein expression at the tendon–bone interface.

2.7. Biomechanical testing

The five remaining animals (10 limbs) from each group were used for mechanical testing 8 weeks after surgery. After sacrificing the animals, the limbs were wrapped in saline-soaked gauze and stored at −20 °C until testing. For failure testing, the frozen, saline-soaked specimens were thawed overnight at 4 °C. All the soft tissues except for the ACL were removed from the limbs (Fig. 1A). The limb with the affixed tendon was clamped using a specially designed fixture, and placed in a material-testing system (MTS Systems, Eden Prairie, MN, USA) for measuring the maximal load, stiffness, and maximum load per length of bone tunnel. The limb was positioned at the lower clamp to allow the tensile loading and the direction of the ACL tunnel to make an angle of 45° with the tensile axis (Fig. 1B). Tension was then applied continuously at a rate of 10 mm/min until failure.

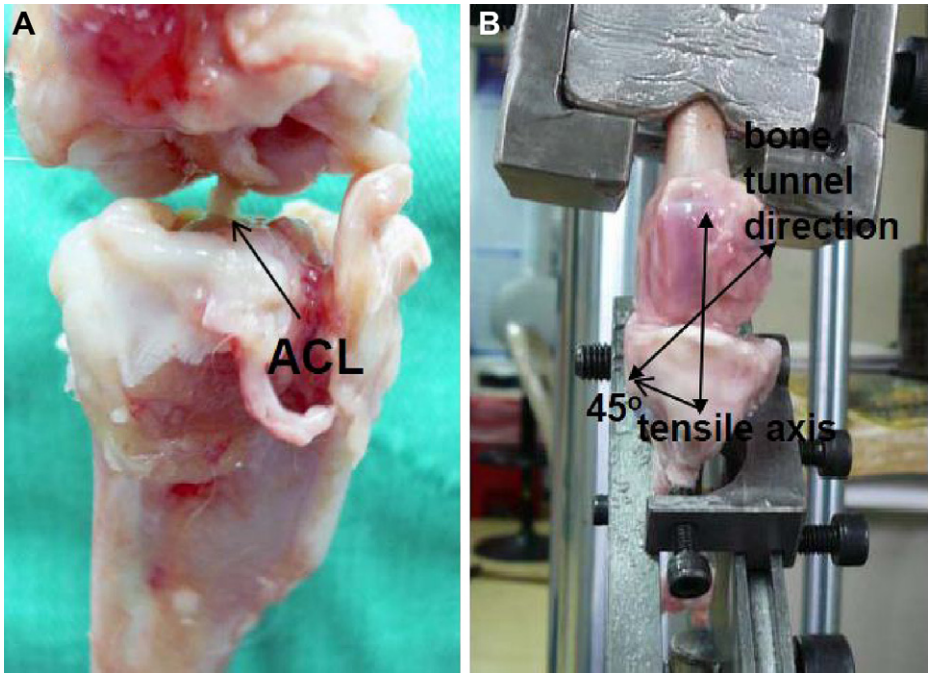

Fig. 1. Method for assaying mechanical properties. (A) Reconstructed anterior cruciate ligament (ACL) 8 weeks after surgery; (B) fixation of the femur with Wood's metal; the bone tunnel makes an angle of 45° with the tensile axis.

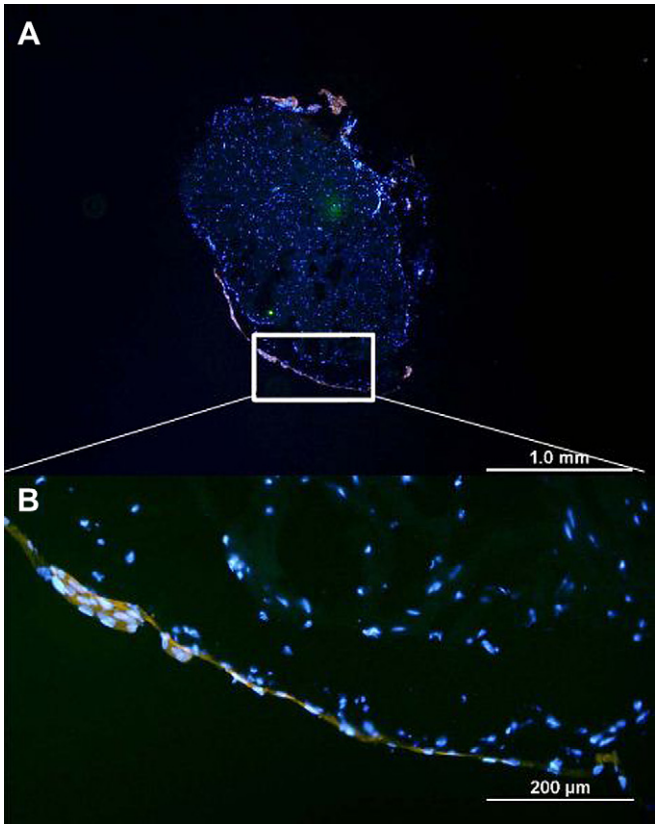

Fig. 2. The single-layer periosteal progenitor cell sheet wrapped around the tendon before surgery. The cell sheet was approximately 10 μm thick. Red = α-actin; blue = nucleus; green = fibrin.

2.8. Statistical analysis

All statistical calculations were carried out using Excel 2003 (traditional Chinese version, Microsoft Corporation, Taipei, Taiwan). The differences between groups were established form a paired *t* test. Means (± standard deviations) were reported as appropriate. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Morphology of the PPCS wrapped around the tendon

After tendon graft was wrapped around by a single layer of PPCS, the morphology was seen under the confocal microscopy assay (Fig. 2A). Cells were seen to be spread continuously over fibrin and monolayer formation (Fig. 2B).

3.2. H&E staining

Attachment between the bone and collagen fibers at the tendon–bone healing interface was seen in all the limbs studied (Fig. 3). Cross-sections of the bone tunnel showed that fibrocartilage tissue had formed between the tendon and the bone at 8 weeks in the PPCS group (Fig. 3B). In the ACS group, new bone formation lining the bone tunnel had interdigitated only with the tissue of the fibrous interface. (Fig. 3A). There was no fibrocartilage formation in the ACS group.

3.3. MT staining

MT staining more clearly showed the collagen fiber and fibrocartilage formation at the tendon–bone interface (Fig. 4). In the PPCS group, fibrocartilage and loose collagen fiber formation was seen around the tendon (Fig. 4B), with mature fibrocartilage and dense collagen fibers at the tendon–bone interface (Fig. 4B). Only collagen fibers were seen to be lining the bone tunnel at 8 weeks in the ACS group (Fig. 4A).

3.4. Safranin-O staining

Chondrogenesis assays showed that most of the cartilage-like tissue resulting from PPCS treatment showed fibrocartilage deposits, with positive Safranin-O histochemical staining at the junction between the tendon and the bone (Fig. 5). The junctions between the tendon and the bone tunnel were filled with more glycosaminoglycans in dense cells (Fig. 5B). In the control group, fewer glycosaminoglycan deposits were found (Fig. 5A).

3.5. Collagen type I expression

Immunofluorescence for collagen type I showed positive staining, reflecting osteogenesis at the junction of the tendon and bone. Expression of type I collagen was observed around the fibrocartilage cells in both the ACS (Fig. 6A) and PPCS treatment (Fig. 6B) groups. Type I collagen was present in the new bone or between the tendon and fibrocartilage (Fig. 6).

3.6. Collagen type II expression

Immunofluorescence for type II collagen showed positive staining, reflecting chondrocyte differentiation. Type II collagen

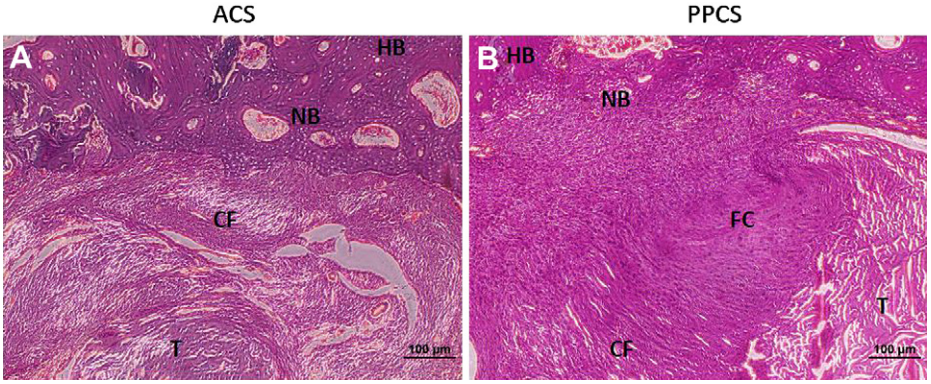

Fig. 3. Hematoxylin and eosin staining show fibrocartilage tissue formation in the bone tunnel at 8 weeks in the acellular collagen sheet (ACS) group (A) and the periosteum progenitor cell sheet (PPCS) group (B). CF = collagen fiber; FC = fibrocartilage; HB = host bone; NB = new bone; T = tendon.

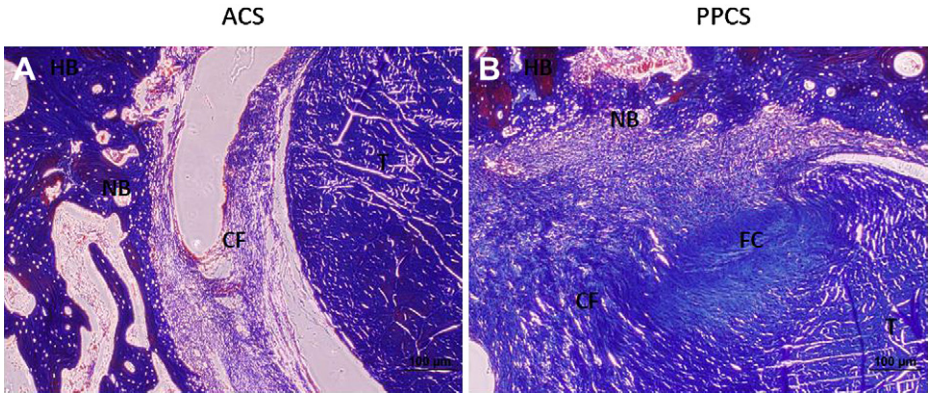

Fig. 4. Masson trichrome staining show fibrocartilage and collagen fiber formation around the tendon at 8 weeks in the acellular collagen sheet (ACS) group (A) and the periosteum progenitor cell sheet (PPCS) group (B). CF = collagen fiber; FC = fibrocartilage; HB = host bone; NB = new bone; T = tendon.

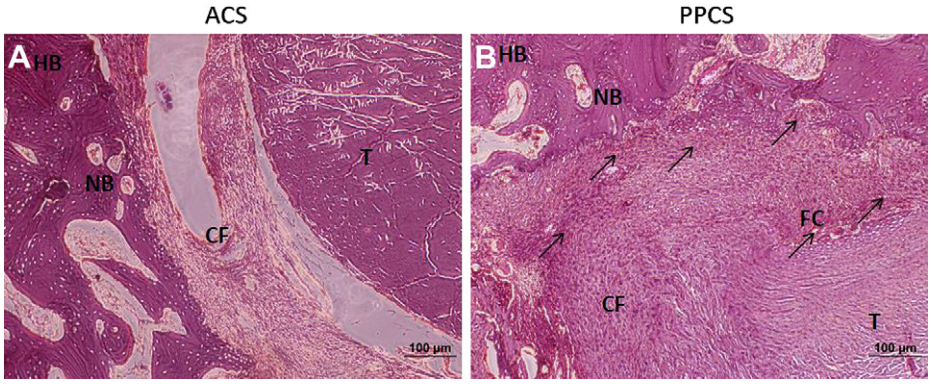

Fig. 5. Safranin-O staining showing glycosaminoglycans (black arrows) between the tendon and bone at 8 weeks in the acellular collagen sheet (ACS) group (A) and the periosteum progenitor cell sheet (PPCS) group (B). CF = collagen fiber; FC = fibrocartilage; HB = host bone; NB = new bone; T = tendon.

expression was observed around the fibrocartilage cells in the PPCS treatment group (Fig. 7B). Type II collagen expression had increased around the fibrocartilage, near the tendon, at 8 weeks (Fig. 7B). In addition, type II collagen expression was difficult to identify in the control group at 8 weeks (Fig. 7A).

3.7. Biomechanical testing

A significantly higher maximum load was found for the PPCS group (81.8 ± 54.3 N) than the ACS group (24.6 ± 9.5 N) at 8 weeks ($p = 0.038$, Student t test; Fig. 8A). Stiffness was also significantly greater in the PPCS group (28.5 ± 19.8 N/min) compared with the ACS group (8.1 ± 1.2 N/min; $p = 0.040$; Fig. 8B). The maximum load per total bone tunnel length was significantly higher in the PPCS group (3.7 ± 2.6 N/mm) than the ACS group (1.0 ± 0.5 N/mm; $p = 0.040$; Fig. 8C). According to these data, the values for the mechanical properties in PPCS group were more than three times greater than in the ACS group, and the difference was statistically significant at 8 weeks ($p < 0.05$; Fig. 8). The strength of the interface was compared with that between the shoulders for the PPCS and ACS groups, the former more closely approaching good healing of the tendon–bone junction at 8 weeks.

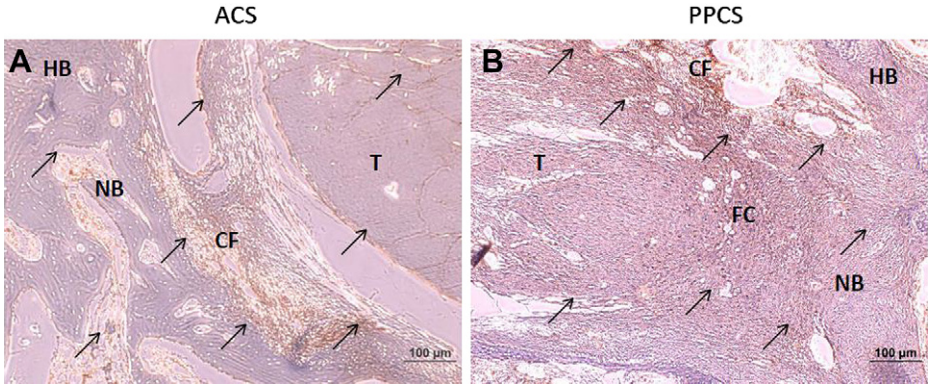

Fig. 6. Immunohistochemical staining showing the expression of type I collagen (black arrows) at the tendon–bone junction at 8 weeks in the acellular collagen sheet (ACS) group (A) and the periosteum progenitor cell sheet (PPCS) group (B). CF = collagen fiber; FC = fibrocartilage; HB = host bone; NB = new bone; T = tendon.

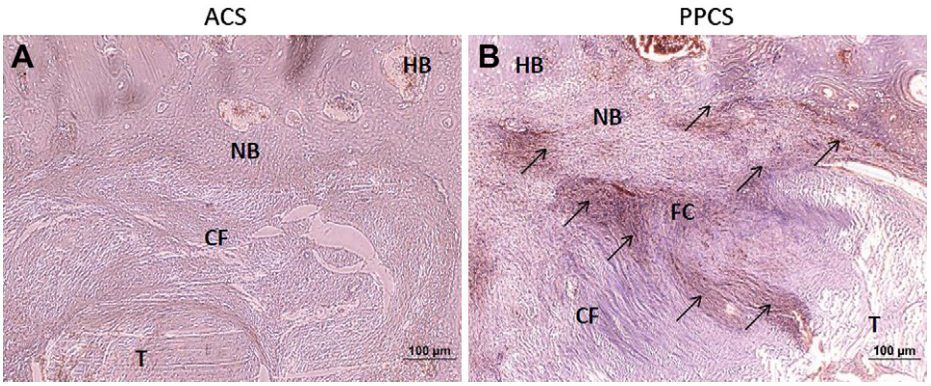

Fig. 7. Immunohistochemical staining showing the expression of type II collagen (black arrows) at the tendon–bone junction at 8 weeks in the acellular collagen sheet (ACS) group (A) and the periosteum progenitor cell sheet (PPCS) group (B). CF = collagen fiber; FC = fibrocartilage; HB = host bone; NB = new bone; T = tendon.

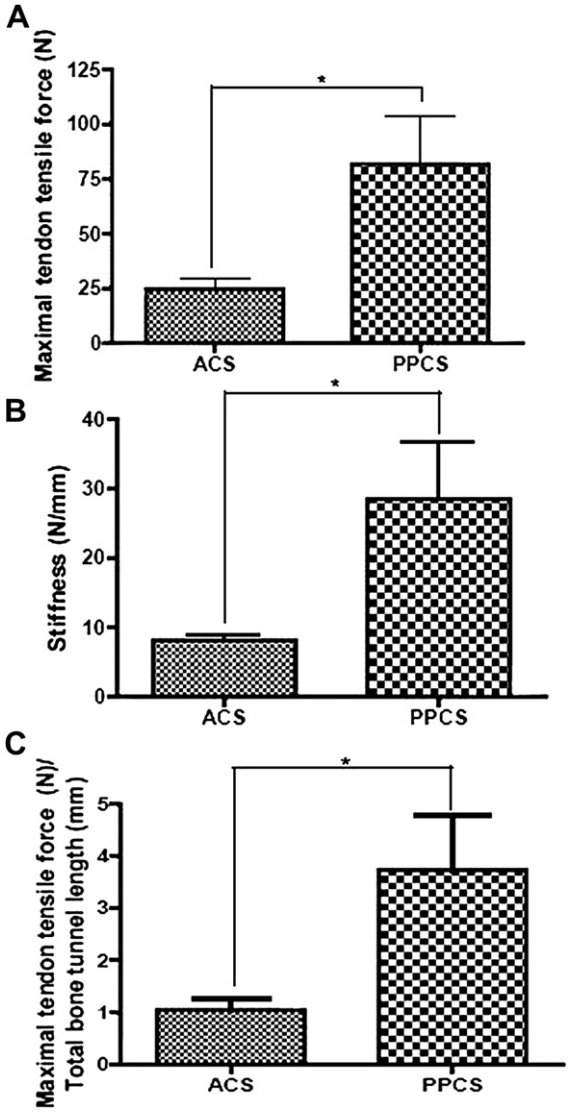

Fig. 8. Maximal tendon tensile force (A), stiffness (B), and maximal tendon tensile force per total bone tunnel length (C) in the periosteum progenitor cell sheet (PPCS) group and the acellular collagen sheet (ACS) groups 8 weeks after reconstruction of the anterior cruciate ligament ($n = 10$, $^{*}p < 0.05$, PPCS greater than ACS).

4. Discussion

The normal insertion of the ACL at the tendon–bone junction is described as being of the direct type, which has four morphologically distinct phases: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone.[34,35] Ideally, tendon–bone healing will also be a direct-type insertion, the intermediate transitional zone of fibrocartilage serving as the gradation from tendon to bone. But how to regenerate the normal tendon bone insertion of the tendon graft within the bone tunnel is still a big issue. Recent work has demonstrated that both biological and mechanical factors drive the development and morphogenesis of tendon–bone healing.[36] In our previous study, the periosteum-wrapped tendon graft showed better tendon–bone healing, and the periosteum was able to form fibrocartilage at the interface.[10,11]

In the current study, the histological findings showed there was fibrocartilage and collagen fiber formation at the tendon–bone interface in the PPCS group at 8 weeks. In the ACS group, however, only collagen fibers filled in the interface. The PPCS group, unlike the ACS group, displayed periosteal progenitor cells that were capable of differentiating into fibrocartilage. Type I collagen exists mainly in bone. Both the PPCS and the ACS group showed type I collagen at the tendon–bone interface, with some new bone ingrowth. Drilling of the cancellous bone for the pull-through tendon graft can cause osteogenesis in the bone tunnel where type I collagen is deposited.

Type II collagen is the main component of cartilage. Only the PPCS group stained positively for type II collagen and glycoaminoglycans. This correlated with fibrocartilage formation in PPCS group. This fibrillar network of type II collagen allows cartilage to entrap proteoglycan aggregates as well as providing tensile strength to the tissue. The above histological results demonstrate that PPCS cells have the ability to differentiate into chondroblasts and form fibrocartilage.[9,19]

In this study, we used PPCS, whose cells possessed the ability to differentiate, and disposed of the excess tissue, which could use up space in the bone tunnel and theoretically decrease the final strength of the healed tendon–bone junction. On the other hand, an ACS can act as a scaffold and carry multiple growth factors, providing a suitable environment for tissue growth as these growth factors and cytokines might enhance the healing process. However, because of the lack of progenitor cells, it cannot correctly direct tendon–bone healing towards the ideal end-product – fibrocartilage.

The cytokines contained in the ACS may stimulate the fibroblasts and some local bone marrow progenitor cells near the tendon–bone junction to generate collagen fibers, but these cells

do not have the potential to differentiate into fibrocartilage-producing cells. Hence the tendon–bone junction was filled with fibrous tissue in the ACS group, but with fibrocartilage in PPCS group. In addition, the SIS was composed mainly of collagen. After grafting, this connective tissue was destined to degrade and be replaced by fibrous tissue, which would take up space at the tendon–bone junction, thus reducing the strength of healing. This therefore resulted in the ACS group showing suboptimal tendon–bone healing.

On biomechanical testing, the PPCS group (maximum load 81.8 ± 54.3 N) was able to sustain three times the maximum load of the ACS group (24.6 ± 9.5 N) at 8 weeks. The maximum load per total bone tunnel length in the PPCS group (3.7 ± 2.6 N/mm) was higher than that of the ACS group (1.0 ± 0.5 N/mm; $p = 0.040$). The presence of fibrocartilage at the tendon–bone junction demonstrated better strength after healing.

In previous studies, it has been demonstrated that autoperiosteum can promote tendon–bone healing.[10,12] Chen et al demonstrated a maximum load for a periosteum-wrapped tendon graft at the tendon–bone junction 8 weeks after surgery to be 61.38 ± 5.10 N.[10] According to the biomechanical tests, the single-layer PPCS in our study produced better tendon–bone healing than treatment with intact periosteum, PPCS containing only progenitor cells still showing the ability to enhance tendon–bone healing. The following may thus be deduced. First, periosteal tissue not containing progenitor cells is unnecessary for enhanced tendon–bone healing. Second, the periosteal progenitor cells are crucial in promoting fibrocartilage formation compared with the presence of only growth factors. Single-layer PPCS significantly improved tendon–bone healing in comparison with ACS, with superior histological and biomechanical results.

5. Conclusion

According to the above results, single-layer PPCS significantly promoted tendon–bone healing over ACS in ACL reconstruction. PPCS maintains the capacity for cell differentiation to promote fibrocartilage production. Therefore, PPCS can offer a novel possible therapeutic method to enhance tendon–bone junction healing in ACL reconstruction. This technology could be clinically utilized in future.

Acknowledgments

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