Original Paper

Mechanical Loading Improves Tendon-Bone Healing in a Rabbit Anterior Cruciate Ligament Reconstruction Model by Promoting Proliferation and Matrix Formation of Mesenchymal Stem Cells and Tendon Cells

Fanglong Song Dawei Jiang Tianchen Wang Yi Wang Fengmei Chen
Guoxing Xu Yinquan Zhang Yifan Kang

Department of Orthopedics, Third Affiliated Hospital of PLA Second Military Medical University, Shanghai, China

Key Words

Mechanical loading • Tendon-bone healing • Mesenchymal stem cells • Tendon cells • Co-culture

Abstract

Background/Aims: This study investigated the effect of mechanical stress on tendon-bone healing in a rabbit anterior cruciate ligament (ACL) reconstruction model as well as cell proliferation and matrix formation in co-culture of bone-marrow mesenchymal stem cells (BMSCs) and tendon cells (TCs). **Methods:** The effect of continuous passive motion (CPM) therapy on tendon-bone healing in a rabbit ACL reconstruction model was evaluated by histological analysis, biomechanical testing and gene expressions at the tendon-bone interface. Furthermore, the effect of mechanical stretch on cell proliferation and matrix synthesis in BMSC/TC co-culture was also examined. **Results:** Postoperative CPM therapy significantly enhanced tendon-bone healing, as evidenced by increased amount of fibrocartilage, elevated ultimate load to failure levels, and up-regulated gene expressions of Collagen I, alkaline phosphatase, osteopontin, Tenascin C and tenomodulin at the tendon-bone junction. In addition, BMSC/TC co-culture treated with mechanical stretch showed a higher rate of cell proliferation and enhanced expressions of Collagen I, Collagen III, alkaline phosphatase, osteopontin, Tenascin C and tenomodulin than that of controls. **Conclusion:** These results demonstrated that proliferation and differentiation of local precursor cells could be enhanced by mechanical stimulation, which results in enhanced regenerative potential of BMSCs and TCs in tendon-bone healing.

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F. Song, D. Jiang and T. Wang contributed equally to this work.

Yifan Kang

Department of Orthopedics, Third Affiliated Hospital of PLA Second Military Medical University, #225 Changhai Road, Shanghai 200433 (China)
Tel. +8615821692785, E-Mail yifankangch@sina.com

Introduction

The anterior cruciate ligament (ACL) plays an important role in stabilization of the knee joints and is one of the most commonly injured ligaments, particularly in young athletes [1]. In response to injury, the ACL has limited regenerative potential due to its limited blood supply and unique intra-articular environment [2-4]. Furthermore, ACL injury is often associated with an increased risk of early osteoarthritis of the knee [5, 6]. Several methods of ACL reconstruction using autografts or allografts of the hamstrings or patellar tendon have been under investigation in the last decades for the management of ACL tears [1, 7]. However, the long-term outcomes of current reconstruction methods remain unsatisfactory with high failure rates, thus revisions are necessary [8].

In ACL reconstruction, passing the tendon graft through a bone tunnel is often required, which renders tendon-bone healing challenging, as this process requires the healing of two inhomogeneous tissues [7]. Previous studies have demonstrated that the structure of tendon-bone healing after ACL reconstruction differs dramatically from the native enthesis tissue connecting tendon to bone [9, 10]. The poor quality of tendon-bone healing always leads to formation of scar tissue and inferior mechanical properties [11].

The distinct mechanical stress environment at the interface of the tendon-bone results from the unique tissue structure in this area, which involves two materials of widely different mechanical properties: tendons/ligaments and bone [12-14]. At the same time, mechanical stress is particularly critical for the formation of the tendon-bone junction structure [9, 12, 15]. As often occurs in bone fractures, micromovement around the fragments is beneficial to fracture healing, thus postoperative functional exercise after joint surgeries is often recommended to restore joint function [16, 17]. Continuous passive motion (CPM) therapy, as an effective adjunctive treatment for osteochondral pathologies afflicting the knee, is widely used after joint replacement, fixation of intra-articular fractures, release of arthrofibrosis/adhesive capsulitis, and regenerative surgeries for microfractures [17]. Intensive preclinical animal studies and clinical studies have focused on the role of CPM therapy in ACL reconstruction, but the clinical effects of CPM therapy remain controversial [17-22]. In addition, although CPM therapy of the knee after ACL reconstruction offers a cyclic mechanical environment at the interface of the tendon-bone, there have been few reports on its effect on tendon-bone healing. It seems that the most accepted evaluation methods of the tendon-bone healing quality are histological and mechanical analysis, which can be readily performed in animal studies, but not in human studies. Another reason is that the parameters of CPM therapy, such as initiation time, intensity, frequency, and duration, vary between studies and, at present, there are no widely accepted criteria [17].

Mesenchymal stem cells (MSCs) are regarded as a key factor in response to tendon-bone injury, as they pass through holes drilled at the tendon footprint through the bone marrow, infiltrate around the tendon graft, and participate in the healing [23]. Tendon grafts reportedly develop necrosis followed by revascularization and tendon cells (TCs) have limited potential for proliferation and differentiation, thus precursor cells, such as tendon-derived MSCs (TDMSCs), are important for formation of the fibrocartilage junction in tendon-bone healing [7]. Since the two sources of MSCs in the tendon-bone interface possess efficient features for the reconstruction and remodeling during tendon-bone healing, recent intensive studies have focused on the use of MSCs to accelerate early tendon-bone healing and have shown promising results [24-27]. MSCs exposed to mechanical stimulation have been demonstrated to exert enhanced proliferation and differentiation, as well as inhibition of apoptosis [28-32]. Thus, mechanical loading may improve tendon-bone healing by promoting the regenerative potential of MSCs.

The hypothesis of the present study was that cyclic mechanical loading might enhance tendon-bone healing by promoting cell proliferation and matrix formation of precursor cells at the tendon-bone interface. To test this hypothesis, the contribution of CPM therapy to tendon-bone healing after ACL reconstruction surgery was evaluated in a rabbit model by histological and biomechanical evaluations and reverse transcription polymerase

chain reaction (RT-PCR) analysis of gene expressions of Collagen I (COL1A1), Collagen II (COL2A1), Collagen III (COL3A1), alkaline phosphatase (ALP), osteopontin (OPN), Tenascin C and tenomodulin (TNMD) at the tendon-bone interface. Furthermore, the effects of mechanical stretch on a co-culture of bone marrow-derived MSCs (BMSCs) and TCs were also determined by detecting the proliferation and matrix synthesis of the two cell types.

Materials and Methods

Study designing and parameter setting

All the animal experiments were approved by the Animal Research Ethics Committee of PLA Second Military Medical University. A total of 72 male New Zealand white rabbits (6-month-old) weighing between 3-3.5kg were purchased from the PLA Second Military Medical University Animal Administration Center.

For animal experiment, all the experimental animals undergoing unilateral (right) knee ACL reconstruction with autologous semitendinosus tendon were randomly divided into two groups: control group (n=36) and CPM-treated group (n=36). ACL reconstructions in the control group were performed following by free activities in cages; animals in CPM-treated group underwent CPM of the right knees two weeks after the operation. Parameters of the CPM: 40°–110° of flexion; 1cycle/30s×60min; every other day; 3 weeks. 18 specimens from each group were harvested at 6 and 12 weeks postoperatively and evaluated using histological observation (n=6), biomechanical test (n=6), and RT-PCR assay (n=6), respectively.

For *in vitro* experiment, bone marrow and semitendinosus tendon were harvested from rabbits for isolation and culture of BMSCs and TCs. Differentiation assay was performed to identify the multiple differentiation potential of BMSCs. Co-culture system of the two cells (1:1) seeded on collagen I-coated six-well Bioflex plates was used to explore the effect of mechanical stretch on the proliferation and synthesis of extracellular matrix in the two sources of cells. Parameters of the mechanical stretch: magnitude: 10% elongation; duration: 48h; frequency: 10 cycles/min with each cycle consisting of 2s of stretch and 2s of relaxation. After mechanical stimulation, cell proliferation was determined and total RNA was isolated from the cultured cells and RT-PCR was performed.

ACL reconstruction

All animals were operated under general anesthesia with 3% intraperitoneal sodium phenobarbital (1.0mL/kg). A middle anterior incision was made and the semitendinosus tendon (2.5cm) was isolated and harvested from posteromedial part of the knee joint through a small incision (Fig. 1A). As weaving suture was prepared on each end of the tendon graft with a 2-0/T silk braided non-absorbable suture (Ethicon; Johnson & Johnson, USA) (Fig. 1B), the cavity of knee joint was exposed through a medial parapatellar approach and the native ACL was fully resected with a sharp surgical blade. Then the bone tunnels were drilled through femur and tibia at the footprint of the native ACL using a 1.5mm Kirschner wire. The tendon

Fig. 1. Surgical procedures for ACL reconstruction using autologous semitendinosus tendon. (A, B) The semitendinosus tendon was harvested and weaving sutures were prepared on each end of the tendon autograft. (C) Tendon graft insertion into tibial and femoral tunnels. (D) Straddle nails made with a needle of a 10ml syringe. (E, F) The tendon graft was fixed adjacent to the tunnel exit by knotting at straddle nails.

Table 1. Modified Histologic Scoring System for Tendon-Bone Healing

Cellularity	1 Marked	2 Moderate	3 Mild	4 Minimal
Mature cartilage cells	<25%	25-50%	50-75%	>75%
Extent of fibrocartilage tissue surrounding tendon	Slightly surrounded (<25%)	Partially surrounded (25-50%)	Moderately surrounded (50-75%)	Mostly surrounded (>75%)
Interface tissue transition from bone to tendon	Slightly indistinct (<25%)	Partially indistinct (25-50%)	Moderately indistinct (50-75%)	Mostly indistinct (>75%)

graft was then passed through both the bone tunnels using a bearded needle (Fig. 1C). The femoral side was fixed by knotting at a straddle nail made with a needle of a 10ml syringe fixed adjacent to the tunnel exit (Fig. 1D, E). The tibial side was fixed similar to the femoral side at a 30° of flexion of the knee after pre-tensioning (Fig. 1F). The joint was irrigated with 0.9% saline solution followed by the capsule and skin closed with interrupted sutures. Postoperatively, all the animals received an intramuscular injection of 50000U of Gentamicin every 8 hours for the first 3 days following surgery to prevent infection.

Continuous passive motion (CPM)

Animals in CPM-treated group underwent CPM of the right knees two weeks after the operation. The apparatus and parameters of CPM used in our study are modified based on descriptions by Zarnett et al. [33]. Briefly, during the following three weeks, animals' knees were moved passively by the CPM apparatus for 60min every other day in an awakened state. A range of motion from 40°–110° at a rate of 1cycle every 30s was adopted. At other times, animals in CPM-treated group together with the control animals were allowed for free activities in cages after surgery until they were sacrificed.

Histological analysis

Rabbits were sacrificed using air injection intravenously into ear margin at 6 and 12 weeks post-surgery. Rabbit knee joints were removed and fixed in 10% formalin solution followed by decalcification for 2 months. The tibial parts of the tissues were then embedded in paraffin, and serial 4mm thick coronal sections across the middle of the tendon were made. For histologic examination, the tissue sections were stained with H&E (hematoxylin and eosin) and toluidine blue. The stained sections were analyzed to determine the fibrocartilage formation at the tendon-bone interface which was comparable in locations in each group. The histological evaluations were performed independently using light microscopy by two observers in a blinded fashion. According to the criteria of the previous studies [34, 35], we developed a modified scoring system based on the subcategories of cellularity at the interface tissue, mature cartilage cells, extent of fibrocartilage tissue surrounding tendon and interface tissue transition from bone to tendon (Table 1). There were 6 specimens in each group at each time point for histological analysis. Representative histological images matching the conclusions were presented.

Biomechanical analysis

Rabbit knee joints were isolated and all periarticular soft tissues containing synovial membrane, collateral ligaments and capsule of joint that connecting the femoral and tibial part were removed except the grafted tendon. All scar tissues, sutures and internal fixations at the tibial tunnel exits were carefully removed. The femur-tendon graft-tibia complexes were mounted onto the mechanical testing machine allowing tensile loading along the axis of the graft (STA1224; Orientec, Tokyo, Japan). After preconditioning of the constructs, a load-to-failure test was performed at an elongation speed of 40mm/min. If the site of graft failure was at femoral tunnel or mid-substance, the test would continue until the maximal pullout load of the graft in tibial tunnel was recorded (ultimate load to failure).

Isolation and culture of cells

BMSCs and TCs were isolated from 5-month-old male New Zealand white rabbits under general anesthesia. For BMSCs isolation and culture, an aliquot of bone marrow (10 ml) was aspirated from the proximal tibia end of donor rabbits with a 20ml heparinized sterile syringe. Then the aspirate was centrifuged at 1500rpm for 20min. Precipitated cells were suspended in general medium DMEM/F12 (Ham's F12: high glucose DMEM 1:1; Hyclone, Pittsburgh, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Pittsburgh, USA) and 1% penicillin/streptomycin (P/S) (Hyclone, Pittsburgh, USA) (complete

culture medium), plated in a 25cm² plastic culture flask and cultured in a humidified atmosphere incubator containing 95% air and 5% CO₂ at 37°C. After 7 days, red blood cells were washed with phosphate buffered saline (PBS) and fresh medium added. After 3 days, cells reached 90–95% confluence and were passaged by trypsinization and recultured as passage one at a density of approximately 10⁴cells/cm². For TCs isolation and culture, a 10mm long semitendinosus tendon from the same rabbit was harvested. The tendon sheath and surrounding paratenon were carefully removed, the tendon tissue was washed in sterile PBS and then aseptically minced into pieces about 1mm³ and digested in 0.2% collagenase Type I (Sigma, St. Louis, MO, USA) for 60min at 37°C. Next, the resulting cell solution was filtered through a 70-mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1500rpm for 5 min. The isolated cells were washed in PBS and resuspended in complete culture medium and seeded at a density of 10⁴cells/cm² in a 25cm² plastic culture flask and cultured in a humidified atmosphere incubator at 37°C with 5% CO₂. After 5 days, the cells were washed with PBS to remove the non-adherent cells and fresh medium was added with medium changed every other day. Cells were passaged by trypsinization when they were 90–95% confluent and seeded at a density of 10⁴cells/cm² as passage one. All cells used in the present experiment were passage one.

Differentiation assay

BMSCs were subjected to osteogenic, adipogenic, and chondrogenic differentiation media to identify the multiple differentiation potential of BMSCs. Briefly, for adipogenic and osteogenic differentiation, BMSCs were incubated in six-well plates and expanding the cells to 100% confluency. Adipogenic differentiation was induced by medium containing DMEM/F12, 10% FBS, 1μM dexamethasone, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 10μM insulin, 200μM indomethacin (each purchased from Sigma, St. Louis, MO, USA) and 1% P/S. Medium was changed every 3 days. After 2 weeks, cells were fixed in 4% paraformaldehyde and stained with Oil-red O solution. Osteogenesis was induced by medium containing DMEM/F12, 5% FBS, 10nM dexamethasone, 10mM β-glycerophosphate and 200μM L-ascorbic acid (each purchased from sigma, St. Louis, MO, USA) and 1% P/S. Medium was changed every 3 days. After 2 weeks, cells were fixed in 4% paraformaldehyde and stained with Alkalinephosphatase (ALP). Chondrogenesis was assessed by the pellet culture. A cell pellet was made by centrifugation of 1×10⁶ cells at 1500rpm for 5min and the pellet seeded onto PLGA (8mm×8mm×2mm; polylactide (PLA): polyglycolic acid (PGA) 9:1; porosity 90%; pore size 100μm) and cultured with medium containing DMEM/F12, 5% FBS, 10ng/mL TGF-β (PeproTech, NJ, USA), 100nM dexamethasone, 10μM insulin (all purchased from sigma, St. Louis, MO, USA) and 1% P/S. The medium was changed every 2–3 days. After 3 weeks, the pellet was fixed in 4% paraformaldehyde and embedded in paraffin and sectioned. Representative sections were stained with toluidine blue to assess the glycosaminoglycan formation in the extracellular matrix of cells.

Co-culture assay

For direct-contact co-culture of BMSCs and TCs, the cells were seeded on collagen I-coated six-well Bioflex plates (Flexcell International, Hillsborough, NC, USA) with a density of 10⁴ cells/cm² at the ratio of 1:1 (BMSCs/TCs, n=3). The complete culture medium was used for all co-culture experiments and was changed every 3 days.

Mechanical stretch application

For mechanical stretch, cells were seeded onto collagen I-coated six-well Bioflex plates at a density of 10⁴cells/cm². When cells were at 95% confluence after 48h, the medium was changed with DMEM/F12 for serum-starving for 24h and then subjected to cyclic mechanical stretch using the Flexcell FX-5000T strain system (Flexcell International, NC, USA). Cells were stretched with a 10% elongation magnitude for 48h at a frequency of 10cycles/min, each cycle consisting of 2s of stretch and 2s of relaxation. Cells cultured on the same kind of plates without stretch loading were used as control cells.

Cell proliferation assay

Alamar Blue (AB, Alamar Biosciences, Sacramento, CA, USA) assay was performed to obtain the index of cell growth after stretch was applied to the mixed cells. In brief, after mechanical stimulation, fresh culture medium containing 10% AB solution was added to cells and incubated at 37°C for 2h. When the AB mixture turned to a purplish or reddish shade, triplicates of 100μL AB mixture from each well were transferred to

Table 2. Primer sequences for RT-PCR analysis

Gene	Primer 5' to 3'	Fragment (bp)
COL1A1	F:ACCTGGTCCCCAAGGTTTCCAA	247
XM_017348831.1	R:CTTGGCACCATCCAAACCACTG	
COL2A1	F:TGCAGGAGGGGAAGAGGTATAATG	223
NM_001195671.1	R:GGTTCTCCTTTCTGCCCTTTGGT	
COL3A1	F:TGGTCTTCTGGTAAAACGGA	196
XM_002712333.3	R:TTCACCCTTAGCACCAGGGGAT	
ALP	F:TGTGCTGTGCAGCCTTGGGTGAGT	240
XM_017346489.1	R:TGTGCAGAGAGAGGGGCAGTCAGT	
OPN	F:GCAGCAACCACAGTTTTCACGT	222
D16544.1	R:TACATTACAGGTGCTGAGCCACT	
Tenascin C	F:AGGGCTTTGAGGAAAGTGAACC	216
FJ480400.1	R:TCAGAGCATACTCCACCGTGTT	
TNMD	F:GAACAAATGAGCAGTGGGTGGTC	272
NM_001109818.1	R:TTGCAAGGCATGATGACACGACAG	
β -actin	F:CAACTGGGACGACATGGAGAAG	152
NM_001101683.1	R:TGAACGTCTCGAACATGATCTG	

a 96-well plate. Then, the absorbance was detected using an enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, USA) at 570 and 600nm. The number of viable cells was expressed as percentage of AB reduction (% AB reduction) which was calculated according to the manufacturer's protocol.

RT-PCR

Total RNA was isolated from tendon-bone interfacial samples of the proximal tibia at the site in accordance with histologic examination or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A spectrophotometer was used to determine RNA concentrations, and samples with values between 1.7 and 2.0 were used. Complementary DNA (cDNA) synthesis was carried out from RNA using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) with specific primers to determine the gene expression levels of different samples. The specificities of the PCR products were confirmed by conducting melting curve analysis, and the levels of mRNA were calculated as target gene expression normalized to β -actin. The primers used are shown in Table 2.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD). A Student's t-test was used to evaluate results between two groups except for the evaluations of differences of gene expressions between groups, which a nonparametric Wilcoxon signed-rank test was performed. All the data analysis was performed with SPSS analysis software (SPSS Inc, 16.0). $p < 0.05$ was regarded as statistically significant.

Results

Clinical observations

All animals recovered after surgery in 1-2h. There was no evidence of infection, immobilization or unloading of the joint in any of the animals during the experiments.

Histological analysis of the tendon-bone healing after ACL reconstruction

As shown in Fig. 2, 6 weeks after surgery, there was an obvious zone with highly cellular, disordered fibrovascular tissue at the tendon-bone interface in the control specimens and no evidence of cartilage tissue formation existed (Fig. 2Ba, e). In the CPM-treated specimens, healing occurred with the formation of new cartilage tissue between the tendon and bone. The tendon was moderately surrounded with chondrocytes with irregular cell shape (hypertrophic chondrocytes), however, the arrays of chondrocyte were along the direction of the force on tendon and the chondrocytes showed a vigorous extracellular matrix production. Fiber continuity occurred and the borderline between the tendon and bone was vague (Fig. 2Bb, f).

12 weeks after surgery, occasional cartilage formed and the amount of cellular and vascular tissue decreased at the interface zone in the control specimens compared with that of 6 weeks. However, arrays of cells were irregular and less extracellular matrix of the chondrocytes synthesized compared with the CPM-treated specimens (Fig. 2Bc, g). 12 weeks after surgery, specimens in CPM-treated group demonstrated a typical direct insertion of ACL with progressive formation of new fibrocartilage and the cartilage tissue was more mature at the tendon-bone interface. Chondrocytes arranged in neat rows along the direction of the force on tendon, a vague tidal line formed between the fibrocartilage zone and calcified cartilage zone (Fig. 2Bd, h).

Of the two groups, histologic scores of tendon-bone healing increased significantly with time respectively ($p < 0.05$). At 6 and 12 weeks postoperatively, the CPM-treated group displayed a statistically significant higher histologic score, compared with that of control group respectively ($p < 0.05$), which indicated that CPM was a beneficial factor for the regeneration of tendon-bone junction (Fig. 3).

Biomechanical testing of the joint after ACL reconstruction

Biomechanical testing was performed 6 and 12 weeks after surgery (Fig. 4). The results showed that the average ultimate load to failure increased significantly in the two groups with time, respectively ($p < 0.05$). The pullout load of specimens in the CPM-treated group

Fig. 2. Histological observations of tendon-bone healing. (A) Representative photographs of the direct insertion of normal ACL. Typical four layers of direct insertion of ACL were shown: tendon, fibrocartilage, calcified cartilage and bone and a clear tidal line between the fibrocartilage zone and calcified cartilage zone was observed. (B) Representative histological photographs of specimens of control and CPM-treated groups 6 and 12 weeks postoperatively. (a, e) There was an obvious zone with highly cellular, disordered fibrovascular tissue at the tendon-bone interface and no evidence of cartilage tissue formation existed. (b, f) New cartilage tissue formed between the tendon and bone. The tendon was moderately surrounded with chondrocytes and the arrays of which were along the direction of the force on tendon. (c, g) Occasional cartilage formed and the amount of cellular and vascular tissue decreased at the interface zone and arrays of cells were irregular and less extracellular matrix of the chondrocytes synthesized. (d, h) Progressive formation of new fibrocartilage and the cartilage tissue was more mature at the tendon-bone interface. Chondrocytes arranged in neat rows along the direction of the force on tendon and a vague tidal line formed between the fibrocartilage zone and calcified cartilage zone. N=6 from each group. Bars=100µm. T, tendon; B, bone; IF, interface.

Fig. 3. Histologic scores of tendon-bone healing. Boxplots showing the histologic scores of control and CPM-treated groups at weeks 6 and 12 postoperatively. * $p < 0.05$ versus control group at week 6; ** $p < 0.05$ versus control group at week 12; # $p < 0.05$ versus CPM-treated group at week 6. $N = 6$ from each group.

Fig. 4. Biomechanical testing of the joints after ACL reconstruction. Boxplots showing the ultimate load to failure of control and CPM-treated groups at weeks 6 and 12 postoperatively. * $p < 0.05$ versus control group at week 6; ** $p < 0.05$ versus control group at week 12; # $p < 0.05$ versus CPM-treated group at week 6. $N = 6$ from each group.

was significantly greater than that of control at 6 and 12 weeks after surgery, respectively ($p < 0.05$).

Gene expressions at the interface between tendon and bone

Relative mRNA expressions for bone, cartilage and ligament matrix formation at the interface between tendon and bone were evaluated using RT-PCR (Fig. 5). Expressions of COL1A1, ALP, OPN, Tenascin C and TNMD increased significantly with time respectively ($p < 0.05$) and their expressions in the CPM-treated group were significantly greater than that of control at different time after surgery, respectively ($p < 0.05$) (Fig. 5A, D-G). In the control group, gene expression of COL2A1 at week 12 was greater than that of week 6 ($p < 0.05$). However, COL2A1 expression decreased with time from week 6 to week 12 in the CPM-treated group ($p < 0.05$). Furthermore, CPM led to a significant up-regulation of COL2A1 at week 6 and down-regulation of it at week 12, compared with that of control ($p < 0.05$) (Fig. 5B).

Expression of COL3A1 in the two groups decreased significantly with time respectively ($p < 0.05$) and CPM induced a significant down-regulation of it ($p < 0.05$) at each time point respectively which showed an opposite tendency with COL1A1 (Fig. 5C).

Cell observation and identification

As shown in Fig. 6, BMSCs and TCs in the first passage were almost fibroblast-like cells with adherent growth ability (Fig. 6A, B). Oil-red staining of the BMSCs showed that moderate intracellular lipid droplets formed after adipogenic induction for 2 weeks (Fig. 6C). ALP staining after induction of osteogenesis for 2 weeks demonstrated the osteogenic potential of BMSCs (Fig. 6D). BMSCs pellet cultured in chondrogenic differentiation medium for 3 weeks were stained with toluidine blue, which indicated the secretion of proteoglycan (Fig. 6E). Results above suggested the multipotency of the BMSCs.

Effect of mechanical stimulation on cell proliferation and gene expressions

Co-culture of BMSCs with TCs treated with 10% mechanical strain for 48h showed a higher rate of cell proliferation than control cells ($p < 0.05$) (Fig. 7). Additionally, mechanical strain influenced the expressions of the matrix-forming genes significantly (Fig. 8). Gene expressions of COL1A1, COL3A1, ALP, OPN, Tenascin C and TNMD in the Co-culture system were significantly up-regulated after stretching respectively ($p < 0.05$), however, expression of COL2A1 was not influenced by mechanical stretch (Fig. 8).

Fig. 5. Gene expressions at the interface between tendon and bone. Relative mRNA expressions for (A) COL1A1, (B) COL2A1, (C) COL3A1, (D) ALP, (E) OPN, (F) Tenascin C and (G) TNMD at the interface of tendon-bone in each group were analyzed at weeks 6 and 12 postoperatively. Levels of mRNA were normalized to β -actin and then related to the control (labeled as "1.00"). * $p < 0.05$ versus control group at week 6; ** $p < 0.05$ versus control group at week 12; # $p < 0.05$ versus CPM-treated group at week 6. N=6 from each group.

Discussion

In the present study, the efficacy of CPM therapy of the knee after ACL reconstruction surgery was investigated. Ideal ACL reconstruction should provide firm attachment of the tendon graft to the bone and minimize tendon-bone displacement under cyclic mechanical loading. The high initial stability of the tendon-bone junction allows earlier rehabilitation and quicker return to full activity [36]. Although the attachments of the two ends of the tendon graft are firm, the long distance between the biomechanical point of action of the ACL and the insertion site may result in relative movement of the tendon-bone junction during a large range of motion of the knee joint, which leads to expansion of the bone tunnel and loosening of fixations [37]. Additionally, it has been suggested that a delayed mechanical stimulation would provide an improved tendon-bone healing response compared with immediate initiation of mechanical loading or prolonged immobilization after ACL reconstruction [38]. Patients who had undergone early, aggressive rehabilitation after ACL reconstruction had been reported significantly greater tunnel widening compared with those who had undergone a conservative rehabilitation program [39]. Considering that early aggressive postoperative rehabilitation may have detrimental effects on graft-to-bone healing and any benefit of CPM therapy must be weighed against the risk of graft-tunnel motion, the intervention of therapy in this study was started 2 weeks after surgery to avoid blocking the infiltration of inflammatory cells and in-growth of vascular tissues, as well as to maintain the initial stability of the tendon graft.

Functional exercise is often recommended in clinical practice to accelerate rehabilitation after ACL reconstruction [16]. CPM therapy, although a classical choice for rehabilitation of knee injury, shows controversial effectiveness in returning knee function after ACL reconstruction [17-22, 40]. However, the effect of CPM therapy on the quality of tendon-bone

Fig. 6. Observation of BMSCs and TCs and identification of BMSCs by multi-lineage differentiation. (A, B) BMSCs and TCs at the first passage were almost fibroblast-like cells with adherent growth ability. Bars=50µm. (C) Oil red stain shows lipid droplets formed in BMSCs after adipogenic induction for 2 weeks. Bar=20µm. (D) ALP staining positively in BMSCs after induction of osteogenesis for 2 weeks. Bar=20µm. (E) BMSCs pellet cultured in chondrogenic differentiation medium for 3 weeks were stained positively with toluidine blue. Bar=200µm.

Fig. 7. Effect of mechanical stretch on cell proliferation of co-culture system of BMSCs and TCs. Data are presented as the mean ± standard deviation of three independent experiments in triplicate. MS, mechanical stretch. * $p < 0.05$ versus control group.

Fig. 8. Effect of mechanical stretch on gene expressions in co-culture system of BMSCs and TCs. Levels of mRNA were normalized to β -actin and then related to the control (labeled as "1.00"). Boxplots showing 6 independent experiments performed with different donors. * $p < 0.05$ versus control group.

healing has been rarely reported. As it is impossible to conduct quantitative and standard active functional exercise studies using rabbits, CPM therapy, therefore, seems to be an optimal method to investigate the effect of mechanical stress on tendon-bone healing. The standard criteria of CPM therapy for animals after ACL reconstruction at present is limited and there is high variability in the composition and time ranges of CPM protocols, which may lead to confusing results. Therefore, the parameters of CPM therapy were set as mentioned in the materials and methods section based on previous reports, although most of those studies focused on outcomes of injuries to the articular cartilage rather than the ACL [17, 33]. The results of this study showed that appropriate CPM therapy enhanced early tendon-bone healing and promoted the formation of fibrocartilage tissue of the tendon-bone junction.

Significant differences in the tendon-bone healing process are evident in different regions of the bone tunnel, resulting from variable biological and mechanical environments

[36]. At the interface of the tendon-bone, compressive stress is thought to occur on the lower side and tensile stress on the upper side (inner hole of the bone tunnel), while shear force is dominant inside the tunnel [15]. Results of histological analysis have revealed that compressive stress enhances cartilage formation and stretch promotes the process of tendon-bone healing, while shear force has little effect on tendon-bone junction regeneration [10, 15]. Stasiak et al. once designed a novel knee joint fixation/distraction system to generate a cyclic axial loading on the tendon-bone interface in a rat model of ACL reconstruction [41]. In the present study, to obtain consistent variables, biomechanical evaluations of the tendon-bone healing were all performed at the tendon graft and tibia, and tissues for histological examination and PCR analysis were obtained at the interface of the tendon graft and proximal lateral tibia, where mechanical stretch is dominant. It has been suggested that mechanical stretch enhances the process of tendon-bone healing and promotes proliferation and differentiation of MSCs [15, 42]. As the two most important cell sources at the tendon-bone interface [43], BMSCs and TCs participate in the process of tendon-bone healing in unique biomechanical environments. Therefore, in accordance with the stress environment of the tendon-bone junction, the effect of mechanical stretch on MSCs and TCs was explored.

In the present study, the effects of mechanical loading on each of the two sources of cells were not investigated. A 1:1 co-culture of BMSCs and ACL cells was reported to have the highest ratio of COL1A1 and Tenascin C expression, as compared with other ratios [43]. Therefore, a 1:1 co-culture system of BMSCs and TCs was used to investigate mechanical stimulation on cells. Although the co-culture system offers as a specific biological environment for the two cell types, in which interactions could also lead to enhanced proliferation and differentiation of either type [43], the results of this study demonstrated that mechanical stimulation promoted proliferation and synthesis of tendon- (COL1A1, Tenascin C and TNMD) and bone- (COL1A1, ALP and OPN) related genes of the co-culture system. As has been verified by many studies, mechanical stretch up-regulates tendon-related genes in TCs and bone-related genes in BMSCs. Zhang et al. reported that mechanical stretch (4%) promoted the expression of tendon-related genes, including COL1A1 and TNMD, in TCs and high mechanical stretching (8%) increased the expression of both tendon-related (COL1A1 and TNMD) and non-tendon-related genes, such as lipoprotein lipase (adipocytes), Sox9 (chondrocytes) and Runx2 (osteocytes) in TDMSCs [44]. In ACL cells, mechanical loading induced up-regulation of the tenocyte-relevant genes Tenascin C, fibronectin, COL1A1 and COL3A1. However, no stretch-induced effects on the expression of tendon-related genes were detected in BMSCs [45]. Intensive studies have demonstrated that physical stimuli can induce osteogenic lineage commitment of stem cells [28, 46]. As the cells used in this study were a co-culture of BMSCs and TCs, the results reflected the effects of mechanical stimulation on these two different cell types. Therefore, both the tendon- and bone-related genes were up-regulated as an effect of mechanical stretch in the co-culture system. However, in both types of MSCs, expression of COL2A1, which represents the chondrogenesis potential in BMSCs, was unchanged by stretch stimulation, which showed a different trend from the research of Zhang et al. which used TDMSCs [44]. This discrepancy may due to the following three reasons: (1) BMSCs have little spontaneous chondrogenic potential when cultured in general medium, especially when cultured in monolayers instead of three-dimensionally [47]; (2) Mechanical stress, especially compression, has a strong effect on chondrogenesis of BMSCs, which stretch stimulation may not [48, 49]; (3) Differentiation of BMSCs may be affected by the biological environment of the co-culture system, which contains a large amount of TCs. As a consequence, tenogenic potential was enhanced instead of chondrogenesis, as confirmed by the results of this study.

When compared with cellular experiments, gene expression profiles of tissues at the tendon-bone interface showed an interesting phenomenon. First, gene expression of COL2A1 in the control group increased with time. However, in response to mechanical loading, COL2A1 expression was decreased from week 6 to week 12, significantly enhanced at week 6, and then downregulated at week 12, when compared with controls. It has been reported that cell proliferation occurs in the early phase of tendon-bone healing and COL2A1

expression is up-regulated, then synthesis of COL2A1 decreases as the fibrocartilage tissue become mature [50]. The results of the present study suggest that mechanical stimulation may enhance the healing process and promote the mature phase. In addition, the differences of COL2A1 expression between cell and animal experiments may result from the more complex microenvironment, including expression of cytokines in different phases of the healing process, changes of the distributions and magnitudes of mechanical stress, and a greater variety of precursor cell lineages such as synovial-derived stem cells *in vivo*.

Second, COL3A1 expression decreased with time in the control group and was significantly down-regulated *in vivo* by mechanical loading, which differed from the results of the cellular experiment. It is reported that COL3A1 expression increased at week 6 and decreased at week 12 in a dog model of ACL reconstruction [51], which is in accordance with the results of the present study. During tendon healing, TCs produce large amounts of COL3A1 instead of COL1A1, which often results in scar formation [52]. In this study, significant regeneration accompanied by fibrocartilage formation, rather than scar formation, at the interface of tendon-bone was observed in the CPM-treated group, which suggested a more mature healing process. These results imply that although mechanical loading up-regulated expression of COL3A1 in the co-culture system *in vitro*, the unique regenerative microenvironment *in vivo* may play a more important role than mechanical loading alone in the regulation of COL3A1 expression.

Although there were discrepancies in COL2A1 and COL3A1 gene expression levels, consistencies in expression profiles of tendon- and bone-related genes between the cellular experiment and animal study indicated that mechanical stimulation improved tendon-bone healing, at least partially, by promoting the regenerative potential of MSCs and TCs.

It has been suggested that regulation of the biological environment at the tendon-bone interface is crucial to improve regeneration of native tissues and to prevent scar formation [53]. The unique biological environment at the tendon-bone junction mostly promotes the natural course of endogenous growth factors [54, 55]. Since it is difficult to mimic the kinetics of release of these factors at different stages of regeneration, MSCs isolated from bone marrow as well as many other tissues, such as adipose tissue [56], umbilical cord [34] and tendon tissues [7] have continued to attract attention in recent decades. MSCs have been reported to provide tendon-bone healing with an ideal bio-environment and accelerate tendon reintegration into bone to re-establish biomechanical properties. Other studies have shown that the high potential of MSCs to promote tendon-bone healing may result from: (1) the spontaneous migration of MSCs to an injured site due to the local inflammation environment [57]; (2) the biological and mechanical environment of the tendon-bone junction, which enhances proliferation and differentiation of MSCs into multiple lineages to repair damaged tissues [58, 59]; and (3) the “trophic” effects of MSCs by the secretion of different cytokines and molecules that promote angiogenesis, modulation of the inflammatory response, prevention of scar formation and recruitment of tendon intrinsic progenitor cells to the interface [60]. The results of this study demonstrated that proliferation and differentiation of local precursor cells could be enhanced by mechanical stimulation, which results in enhanced regenerative potential of MSCs and TCs in tendon-bone healing.

The several limitations to this study included (1) a relatively short observation time; (2) the need to further define the parameters of CPM therapy; (3) the failure to investigate the effects of mechanical loading on each of the two cell sources; (4) the lack of studies on the mechanisms of mechanical loading underlying the cell proliferation and matrix formation of the co-culture system; (5) since mechanical stimulation did not promote chondrogenic differentiation of the co-culture system, the formation of fibrocartilage at the tendon-bone interface is not well explained.

Altogether, the results of the present study attaches importance to mechanical loading in tendon-bone healing, as well as proliferation, differentiation and matrix formation of cells at the tendon-bone interface. Moreover, this study provides additional evidence to support the viewpoint that early rehabilitation exercise intervention promotes tendon-bone healing after ACL reconstruction.

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Disclosure Statement

The authors declare that they have no competing interests.

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