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# A Collagen and Silk Scaffold for Improved Healing of the Tendon and Bone Interface in a Rabbit Model

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**Background:** The study aimed to develop a novel orthopedic surgical scaffold made of collagen and silk to repair the tendon and bone interface, and to investigate its influence on tendon and bone healing in a rabbit model.

**Material/Methods:** Four types of surgical scaffold were prepared, including a random collagen scaffold (RCS), an aligned collagen scaffold (ACS), a random collagen scaffold combined with knitted silk (RCSS), and an aligned collagen scaffold combined with knitted silk (ACSS). Rabbit bone marrow stem cells (BMSCs) were cultured and seeded onto the RCS and ACS scaffold. The animal model included four-month-old female New Zealand White rabbits (N=20) that underwent drilling into the rotator cuff of the left supraspinatus muscle tendon, randomized into the ACSS and RCSS groups.

**Results:** Rabbit BMSCs adhered to and proliferated on the RCS and ACS *in vitro*. Transcription levels of the COL I, COL III, and tenascin (TCN) genes were significantly increased in the ACS group compared with the RCS group. Transcription levels of COL I, runt-related transcription factor-2 (RUNX-2) and bone morphogenetic protein-2 (BMP-2) were significantly increased in the RCS group compared with the ACS group. RCSS and ACSS implanted in the rabbit models for eight weeks resulted in more regenerative tissue in the RCSS group compared with the ACSS group, with new cartilage at the tendon and bone interface at 12 weeks.

**Conclusions:** A collagen and silk scaffold improved healing of the tendon and bone interface in a rabbit model.

**MeSH Keywords:** **Bone Marrow Cells • Collagen • Silk • Tissue Scaffolds**

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## Background

The structure of the tendon and bone interface has four layers that include tendon, fibrocartilage, mineralized fibrocartilage, and bone [1]. As the tendon and bone interface contains both tendon-like tissues and bone-like tissues, and the spatial structure is multi-layered, when the tendon and bone interface are damaged, healing can be difficult and surgical is a challenging clinical problem [2,3]. Approximately 20% of the general population may suffer from a rotator cuff tear, with roughly 7% requiring surgery. Following surgery, more than 25% of patients will experience further injury to the rotator cuff. For patients with a large rotator cuff tear, the re-tear rate can be up to 57% [4,5]. Many factors influence the process of rotator cuff repair such as advanced patient age, the presence of tendinosis, and fatty infiltration, but the main factor is the difficulty in achieving healing at the tendon and bone interface [6,7].

For many years, autografts and allografts have been used clinically for tendon and bone healing, however, each method has certain disadvantages. For autografts, additional tissue harvesting procedures and donor site morbidity are difficult issues to resolve. For allografts, there is an increased risk of infection and immune rejection can occur, which limits their use [8,9]. Therefore, since the 1970's, synthetic polymeric scaffolds have been developed to improve healing at the tendon and bone interface and are now widely used [10,11]. Compared with other types of biomaterial, collagen fiber is considered to be a promising graft material because of its biocompatibility. However, the mechanical properties of collagen fiber alone can lack sufficient strength to be used for repairs at the tendon and bone interface [12]. Silk has good mechanical properties but has poor cell recruitment properties [13,14]. Given the advantages and disadvantages of collagen fiber and silk, a promising approach would be to use collagen and silk scaffold to promote healing following repair of the tendon and bone interface.

As the structure of the tendon and bone interface is complex, the spatial structure of the scaffold for tendon and bone healing is very important. Previously published studies have shown that a scaffold with an aligned structure can promote the process of cell migration and tendon differentiation [15,16]. These previous studies have focused mainly on tendon and ligament repair, and unlike tendon and ligaments, the tendon and bone interface is always near cancellous bone, which is hypervascular and multicellular [17]. Also, the structure of the tendon and bone interface is not aligned with the tendon. Therefore, a scaffold with an aligned structure may not be suitable for healing at the tendon and bone interface. It is assumed that the healing at the tendon and bone interface using a scaffold with a random structure would be superior to that of a scaffold with an aligned structure.

The aim of this study was to develop a novel orthopedic surgical scaffold made of collagen and silk to repair the tendon and bone interface and to investigate its influence on tendon and bone healing in a rabbit model. Following manufacture of a collagen and silk scaffold (CSS) four types of surgical scaffold were prepared, including a random collagen scaffold (RCS), an aligned collagen scaffold (ACS), a random collagen scaffold combined with knitted silk (RCSS), and an aligned collagen scaffold combined with knitted silk (ACSS). Rabbit bone marrow stem cells (BMSCs) were cultured and seeded onto the RCS and ACS scaffold.

## Material and Methods

### Ethics statement

This study was approved by the Ethical Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University. Experimental procedures using animals were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

### Manufacture of the random collagen scaffold (RCS), the aligned collagen scaffold (ACS), the random collagen scaffold combined with knitted silk (RCSS), and the aligned collagen scaffold combined with knitted silk (ACSS)

Insoluble collagen I (col I) was isolated from pig Achilles tendon and silk fibers were supplied by Zhejiang Cathaya International Co. Ltd. (x, China) [18]. The random collagen scaffold (RCS) was produced by a routine freezing method, and the aligned collagen scaffold (ACS) was produced by unidirectional freezing method [19,20]. Following mixing with knitted silk, a random collagen scaffold combined with knitted silk (RCSS), and an aligned collagen scaffold combined with knitted silk (ACSS) were produced using an ordinary freezing method and a unidirectional freezing method, respectively [20]. The spatial structures of the RCS, ACS, RCSS, and ACSS were observed by scanning electron microscopy (SEM).

### Rabbit bone marrow stem cell (BMSC) isolation and culture

Bone marrow was isolated from the cancellous bone of New Zealand White rabbits. Nucleated cells were aspirated and incubated in a 100-mm culture dish ( $5 \times 10^6$  cells/dish) and cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After two days of culture, adherent cells were continuously cultured, and cells were detached with trypsin and serially subcultured when they reached confluence [21–23].

### Rabbit BMSC differentiation and cell proliferation assay

The differentiation potential of rabbit BMSCs toward osteogenesis, chondrogenesis, and adipogenesis was assessed *in vitro* [24].

**Table 1.** List of primer sequences used in the quantitative polymerase chain reaction (qPCR).

Genes	5'-3'	Primers	Production size (bp)
Collagen I	Forward	GCAAGAACGGAGATGACGGA	296
	Reverse	TTGGCACCATCCAACCACT	
Collagen III	Forward	CCGAACCGTGCCAATATGC	235
	Reverse	AACAGTGCGGGGAGTAGTTG	
TCN	Forward	GGCTGGTGAAGACTCATCCCT	158
	Reverse	TGATGAGTGACCCGGAGAGA	
RUNX-2	Forward	CTGACTTCTGCCTCTGGCCTT	126
	Reverse	GTGAGGGATGAAATGCTTGGG	
BMP-2	Forward	AGCTTTGGGAGACGACAGC	244
	Reverse	GGCTCGTGTCTGATTACC	
GAPDH	Forward	TCACCATCTCCAGGAGCGA	293
	Reverse	CACAATGCCGAAGTGGTCGT	

TCN – tenascin; RUNX-2 – runt-related transcription factor 2; BMP-2 – bone morphogenetic protein-2.

Cell proliferation was assessed by the cell counting kit-8 (CCK-8) assay. The collagen scaffold (0.5 cm diameter) was immersed in a 96-well plate for 24 hours and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Rabbit BMSCs were seeded on the scaffold at  $10^3$  cells/well. The cell proliferation assay of the scaffold was examined at set time points (1, 3, 5, and 7 days) using the CCK-8 method at 450 nm. Histochemical staining with alizarin red was used for bone marrow differentiation, chondrogenic differentiation was identified by Alcian blue staining, and adipocyte differentiation was identified by oil red O (ORO) staining. The structural morphology of the scaffold containing the rabbit BMSCs was observed by scanning electron microscopy (SEM).

#### Preparation of the cell pellets

Cell suspensions were adjusted to  $1 \times 10^5$  cells/mL in culture medium, and 1 mL aliquots were transferred to sterile 15 mL tubes. The tubes were centrifuged in a microcentrifuge (1000 rpm for 5 min) to form cell pellets. After incubation at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for between one and two days, the pellets were detached and carefully seeded on pre-immersed scaffolds. Twenty-four hours after implantation, the scaffolds were fixed and viewed using SEM.

#### RNA isolation and quantitative polymerase chain reaction (PCR)

Rabbit BMSCs were seeded on the RCS and ACS scaffold at  $10^3$  cells/well, as previously described. After seven days and 14 days, RNA ( $n=3$ /group) was isolated followed by a single step phenol-chloroform-isoamyl alcohol extraction. The content

of tendon-related and bone-related genes were assessed by quantitative PCR and included Collagen I, Collagen III, tenascin (TCN), runt-related transcription factor 2 (RUNX-2), and bone morphogenetic protein-2 (BMP-2) [20]. The primer sequences of the genes are listed in Table 1. Each quantitative PCR run was performed in triplicate.

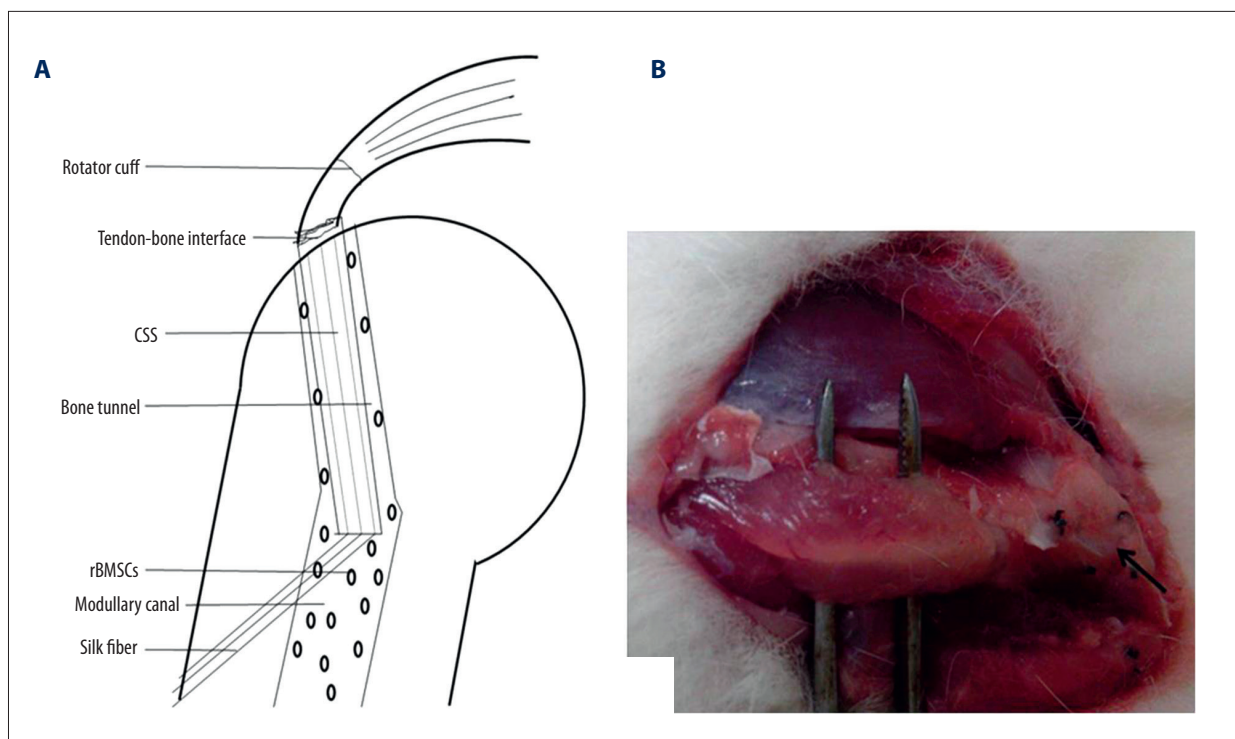
#### Animal model

Four-month-old female New Zealand White rabbits (N=20), weighing 2.0–2.5 kg were randomized into the ACSS and RCSS groups. Following anesthesia, the rotator cuff tendons (the supraspinatus muscle tendon) of the left shoulder of the rabbits were exposed, a 1-cm defect was made from the insertion of the rotator cuff, and a bone tunnel was drilled directly on the insertion site along the normal loading direction. The ACSS or RCSS was then implanted with one end was anchored through the bone tunnel and the other end was sutured with the residual rotator cuff (Figure 1A, 1B) [20]. The incision was then closed, and five samples from the ACSS and RCSS group were harvested at eight weeks and 12 weeks after surgery.

#### Histopathology and immunohistochemistry

Animal tissue specimens were harvested at eight weeks and 12 weeks after surgery. The tissues were fixed in formalin and embedded in paraffin wax, followed by tissue sections cut at 10 µm onto glass slides. Tissue sections underwent histochemical staining for light microscopy with hematoxylin and eosin (H&E) and safranin O (SO) [20].

Histological sections (10 µm) were incubated at 37°C for 30 min in 0.5% pepsin and 5 mM hydrochloric acid, and endogenous



**Figure 1.** Schematic diagram and macroscopic appearance of the rabbit shoulder rotator cuff repair model. **(A)** A diagram of the rabbit shoulder rotator cuff repair model. **(B)** The macroscopic appearance of the rabbit shoulder rotator cuff repair model. The thin black arrow shows the tendon and bone interface. RCS – random collagen scaffold; ACS – aligned collagen scaffold; RCSS – random collagen scaffold combined with knitted silk; ACSS – aligned collagen scaffold combined with knitted silk; BMSCs – bone marrow stem cells.

peroxidase was blocked for 10 min using 3% hydrogen peroxide in methanol. The sections were incubated overnight with a primary antibody to col I at 4°C, then incubated with secondary antibodies for two hours at room temperature, followed by rinsing and incubation with the brown chromogen 3,3'-diaminobenzidine (DAB) for 10 min at room temperature [23].

### Statistical analysis

Statistical analysis was performed using SPSS version 22.0 software. The t-test was used for statistical analysis and a p-value of <0.05 was considered as statistically significant.

## Results

### Manufacture and characterization of the collagen and silk scaffold

The aligned collagen scaffold (ACS) had a brighter white surface, and the random collagen scaffold (RCS) had a sponge-like surface (Figure 2A, 2B). Scanning electron microscopy (SEM) showed that the spatial structure of the ACS was mainly arranged in parallel, while the spatial structure of the RCS was

disorganized (Figure 2C, 2D). Following combination with knitted silk, SEM showed that collagen fibers and silk were alternately linked in both types of scaffold (Figure 2E, 2F).

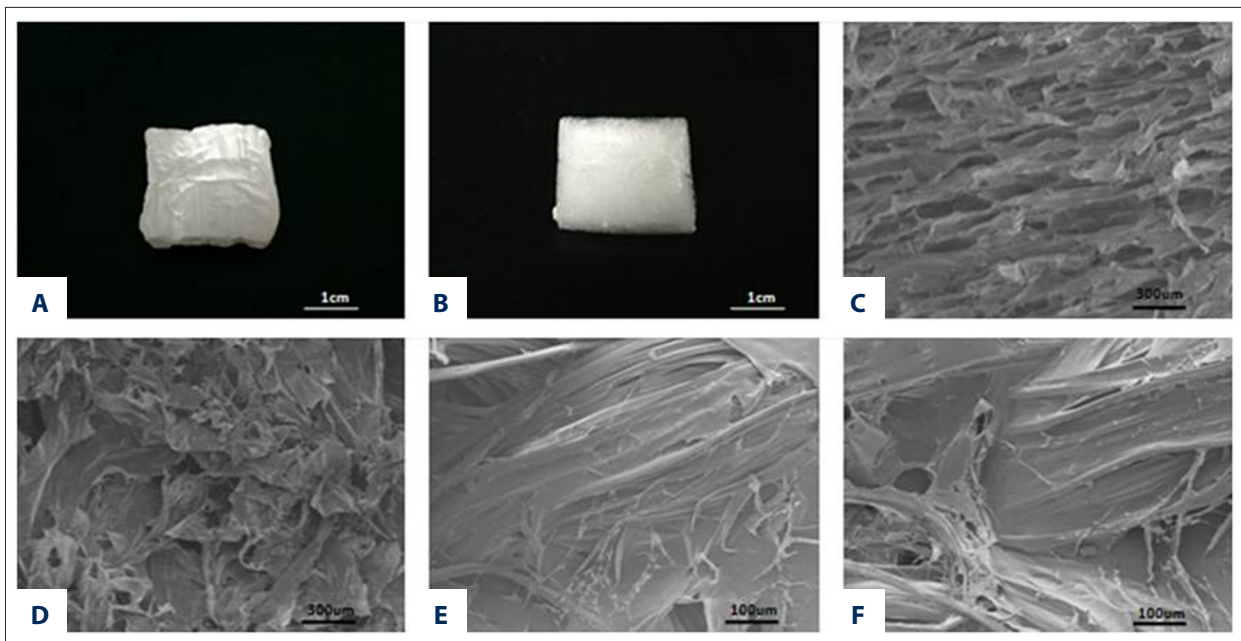
### Characterization and differentiation of rabbit bone marrow stem cells (BMSCs)

The rabbit bone marrow stem cells (BMSCs) were successfully extracted from rabbits and showed an elongated spindle-like morphology (Figure 3A). Osteogenic differentiation was confirmed by alizarin red staining after induction for four weeks, indicating mineralized calcium deposits (Figure 3B). After induction for three weeks, chondrogenic differentiation was shown histochemically by Alcian blue staining, which showed blue-green new cartilage cells in the pellet (Figure 3C), and adipocyte differentiation was shown by oil red O (ORO) staining with the accumulation of red lipid droplets in the cells (Figure 3D).

### Rabbit bone marrow stem cell (BMSC) *in vitro* studies

The rabbit BMSCs were seeded in the collagen fiber scaffolds. The cell counting kit-8 (CCK-8) assay on days 1, 3, 5, and 7 after seeding showed that the cells proliferated well, indicating that the scaffolds were non-toxic. No significant difference





**Figure 2.** Preparation and characterization of the scaffolds. (A) The macroscopic structures of the aligned collagen fiber scaffold (ACS). (B) The macroscopic structure of the random collagen scaffold (RCS). (C) Scanning electron microscopy (SEM) of the aligned collagen scaffold (ACS). (D) SEM of the RCS. (E) SEM of the ACS. (F) SEM of the random collagen scaffold combined with knitted silk (RCSS). ACS – aligned collagen fiber scaffold; RCS – random collagen fiber scaffold; ACSS – aligned collagen fiber in knitted silk scaffold; RCSS – random collagen fiber in knitted silk scaffold. Scale bars: 1 cm (A, B), 300 μm (C, D), 100 μm (E, F).

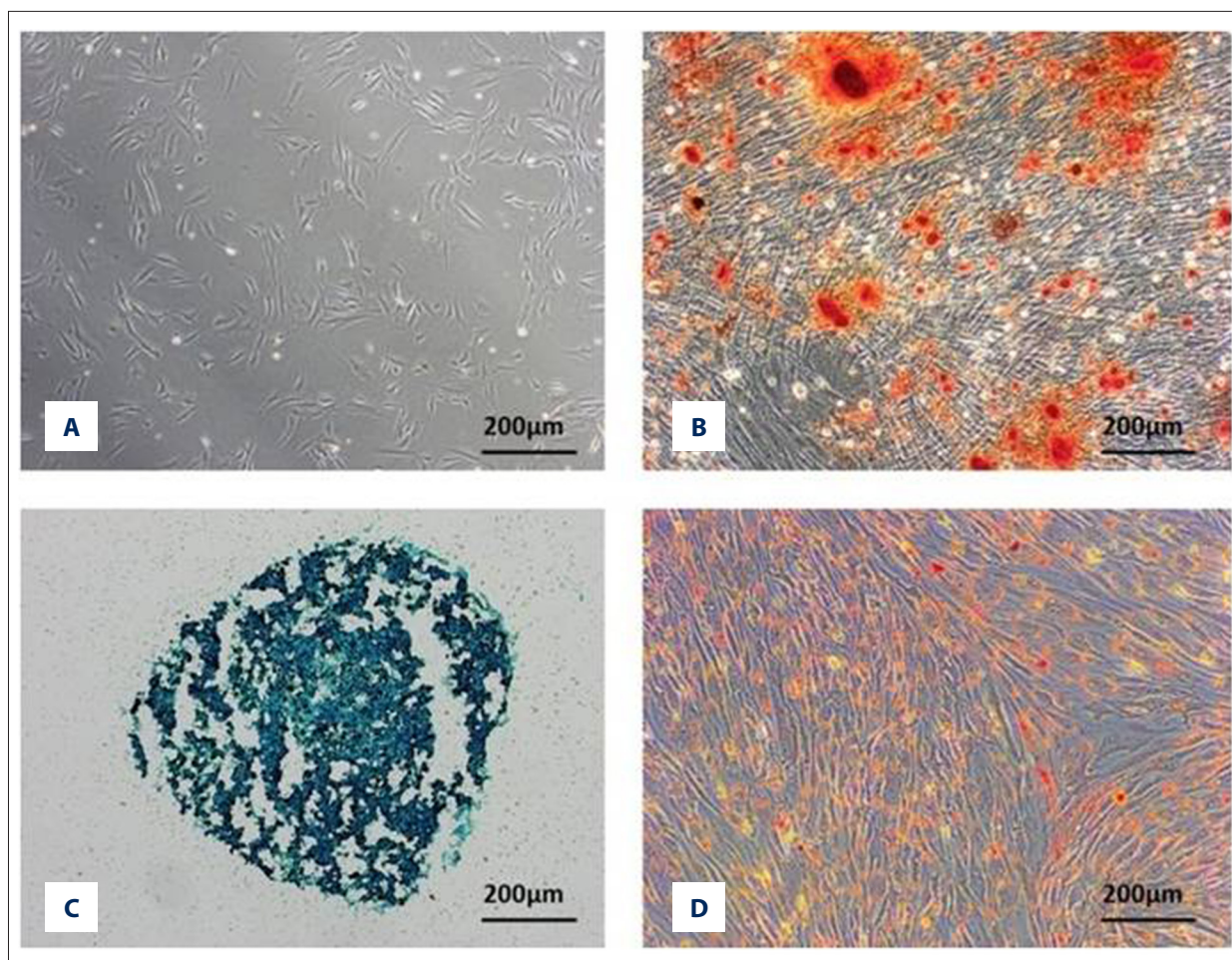
in the CCK-8 assay was observed between the RCS and ACS groups (Figure 4A). SEM showed that rabbit BMSCs attached and spread well on both kinds of scaffold. In the ACS group, rabbit BMSCs were elongated and almost parallel to the direction of collagen fibers (Figure 4B). In contrast, in the RCS group, rabbit BMSCs had a polygonal morphology with random orientation (Figure 4E). The cell migration assessment showed that the different spatial configuration of the scaffold influenced the speed of migration of the cells. Following the direction of the ACS, more rabbit BMSCs migrated from the cell pellet 24 hours after implantation, and the mean migration distance was increased. In the direction perpendicular to the ACS, fewer rabbit BMSCs migrated from the cell pellet with most cells located at the edge, indicating that the mean migration distance was short (Figure 4C, 4D). However, the cell number and migration distance showed no significant difference between the different directions in the RCS (Figures 3F, 4G).

Tendon-associated genes including Col I, Col III, and tenascin (TCN) and bone-associated genes including Col I, runt-related transcription factor 2 (RUNX-2), and bone morphogenetic protein 2 (BMP-2) were analyzed by quantitative polymerase chain reaction (PCR). Following seeding of the rabbit BMSCs in the collagen fiber scaffold and tendon induction for one week, the transcript levels of Col I, Col III, and TCN of the ACS group were significantly increased when compared with those

of the RCS group by 2.48-fold ( $p < 0.05$ ), 1.31-fold, and 1.21-fold, respectively. After two weeks, the transcript levels were significantly increased by 1.04-fold, 1.15-fold, and 1.81-fold ( $p < 0.05$ ), respectively (Figure 5A). In contrast, after osteogenic induction for one week, the transcript levels of Col I, RUNX-2, and BMP-2 of the RCS group were significantly increased compared with the ACS group by 1.13-fold, 2.78-fold ( $p < 0.05$ ), and 2.96-fold ( $p < 0.05$ ), respectively. After two weeks, the transcript levels were increased by 1.86-fold ( $p < 0.05$ ), 1.26-fold ( $p < 0.05$ ), and 1.17-fold, respectively (Figure 5B).

### Rabbit model *in vivo* study findings

The random collagen scaffold combined with knitted silk (RCSS) and the aligned collagen scaffold combined with knitted silk (ACSS) were implanted within the rotator cuff of the rabbit model and harvested eight weeks and 12 weeks after surgery. The effect of the scaffolds on rotator cuff repair was assessed by light microscopy with hematoxylin and eosin (H&E) and safranin O (SO) staining. At eight weeks post-surgery, a mass of polygonal cells and regenerative tissue appeared in the RCSS group. However, there was only a small amount of sporadic tissue in the ACSS group. Also, increased chondrogenesis was seen at some sites of the tendon and bone interface in the RCSS group (Figures 6A, 6B, 6E, 6F). At 12 weeks post-surgery, dense regenerative tissue was seen in the RCSS group, and



**Figure 3.** Characterization and multipotent differentiation of rabbit bone marrow stem cells (BMSCs). (A) Morphology of rabbit bone marrow stem cells (BMSCs) at P2. (B) Alizarin red staining shows osteogenic differentiation of rabbit BMSCs. (C) Alcian blue staining showed chondrogenic differentiation of rabbit BMSCs. (D) Oil red O (ORO) staining showing adipogenic differentiation of rabbit BMSCs. Scale bars, 200 µm.

the new cartilage increased and matured. In contrast, the regenerative tissue in the ACSS group was still sparse (Figures 6C, 6D, 6G, 6H). The tendinous and osteogenic abilities of both types of scaffold were further assessed by immunohistochemistry. Compared with the ACSS group, increased expression of Col I was found in the RCSS group at eight weeks post-surgery, and this difference increased further by 12 weeks post-surgery (Figures 6I–6L).

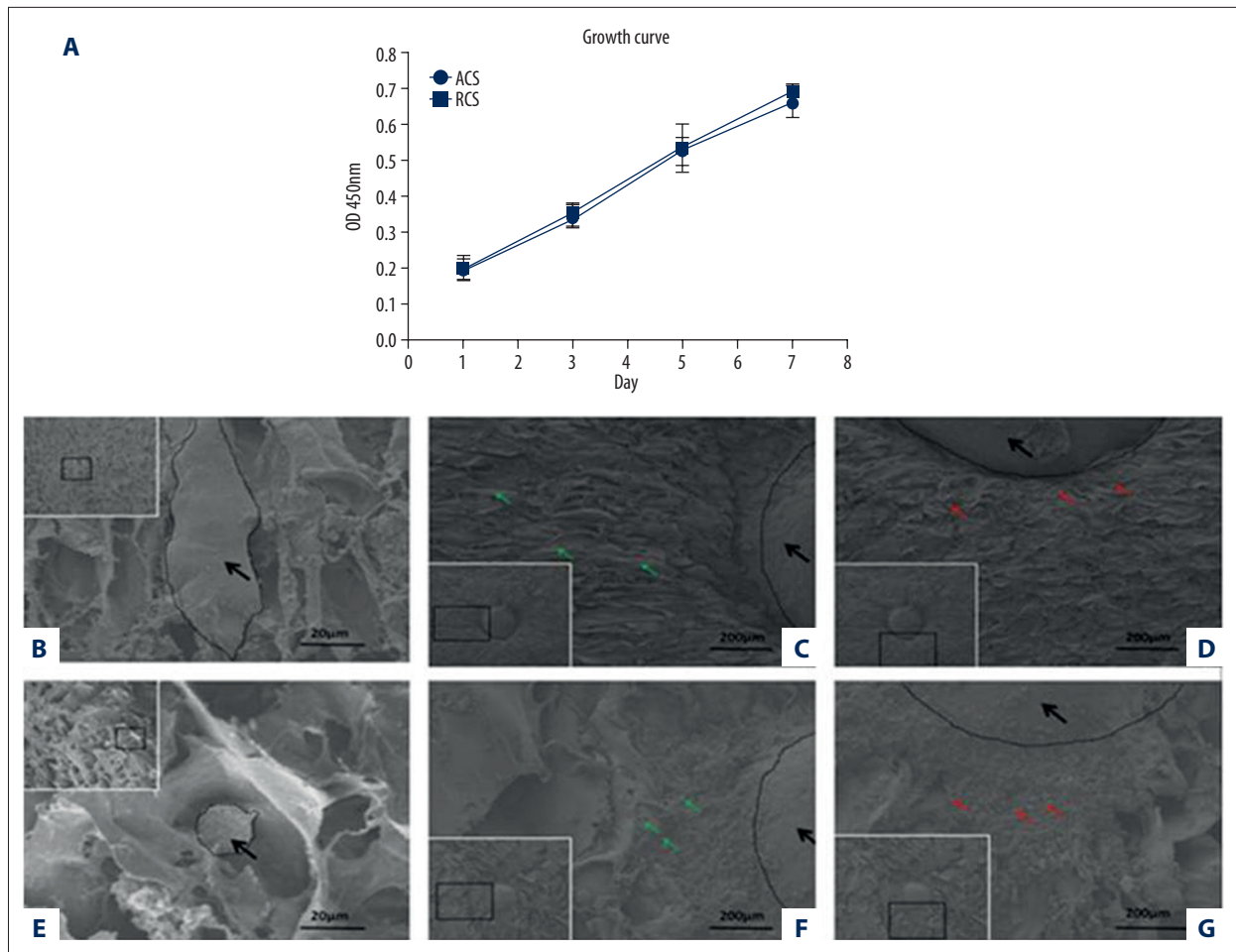
## Discussion

Healing at the tendon and bone interface is a challenging clinical problem, and previous studies have focused on tissue engineering strategies [25,26]. Collagen fiber is considered a promising biomaterial because of its ideal biocompatibility. Also, the use of silk can provide satisfactory mechanical properties in terms of tendon strength [27,28]. Previous studies have

investigated the use of a collagen and silk scaffold and have demonstrated its efficacy in tissue reconstruction [14,20,29]. Compared with the random scaffold, the spatial structure of the aligned scaffold has been shown to be similar to that of normal tendon and ligament, and adhered cells were prone to tendon differentiation [24,30]. However, in previous studies, the tendon and bone interface was not aligned to tendon or close to cancellous bone, and osteogenic differentiation is important for healing at the tendon and bone interface [1,31]. Therefore, in this study scaffolds were manufactured with different spatial structures and their effects were compared in promoting healing at the tendon and bone interface.

In the present study, four types of surgical scaffold were prepared, including a random collagen scaffold (RCS), an aligned collagen scaffold (ACS), a random collagen scaffold combined with knitted silk (RCSS), and an aligned collagen scaffold combined with knitted silk (ACSS). Rabbit bone marrow stem cells (BMSCs) were





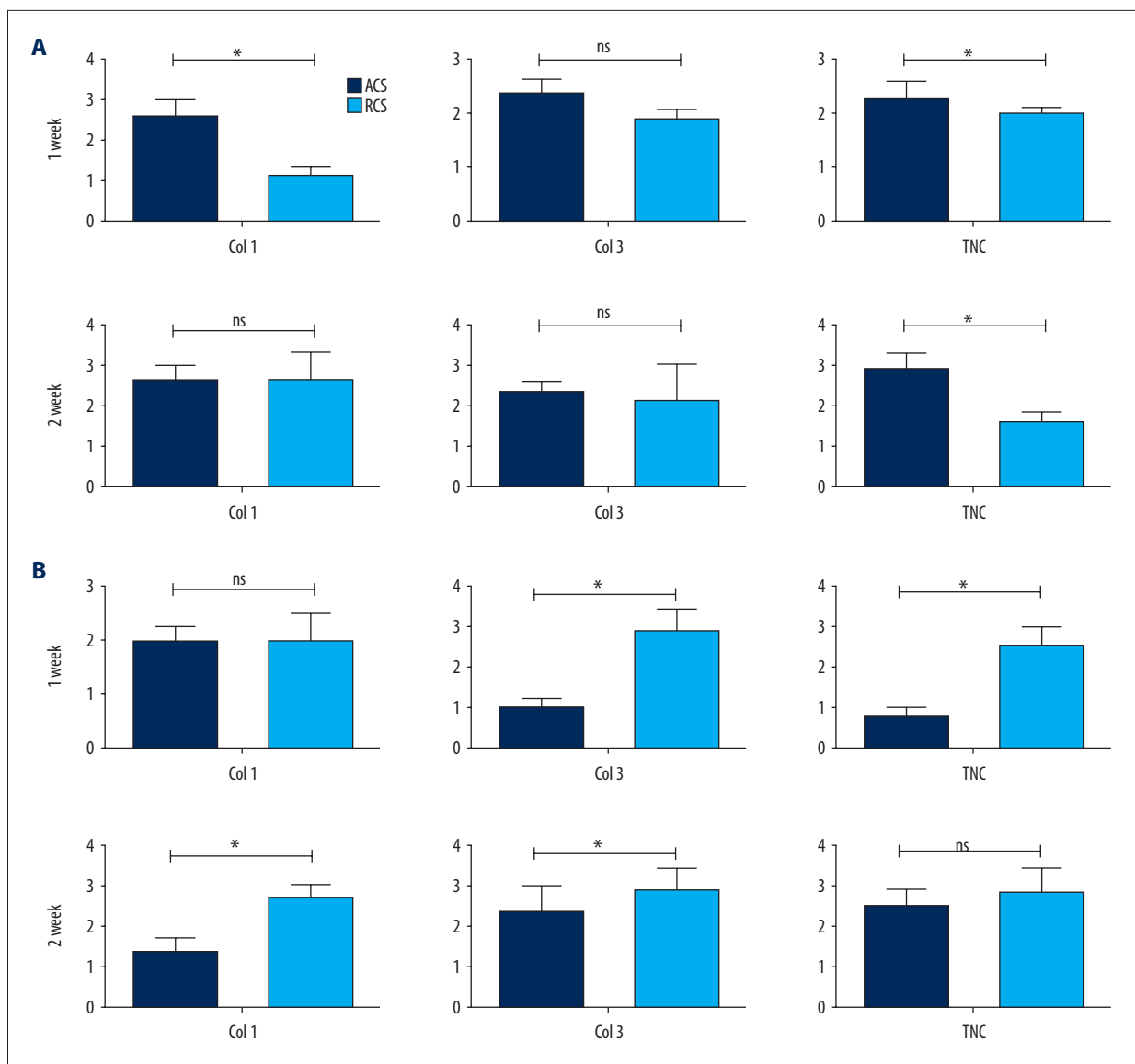
**Figure 4.** Cell proliferation, morphology, and migration *in vitro* assays. **(A)** Proliferation of rabbit bone marrow stem cells (BMSCs) on the aligned collagen fiber scaffold (ACS) and the random collagen scaffold (RCS) scaffolds. **(B)** Scanning electron microscopy (SEM) of rabbit BMSCs in ACS, the bold black arrow showed the morphology of rabbit BMSCs. **(C, D)** SEM of cell migration after cell pellet implantation 24 hours in ACS, the bold black arrow showed the cell pellet, the green thin arrow showed the cell migration following the direction of ACS, the red thin arrow showed the cell migration perpendicular to the direction of ACS. **(E)** SEM of rabbit BMSCs in RCS; the bold black arrow shows the morphology of rabbit BMSCs. **(F, G)** SEM of cell migration after pellet implantation for 24 hours in RCS. ACS – aligned collagen fiber scaffold; RCS – random collagen fiber scaffold. Scale bars: 20  $\mu\text{m}$ , 200  $\mu\text{m}$ .

cultured and seeded on the RCS and ACS scaffold. Both types of scaffold improved healing of the tendon and bone interface in a rabbit model and showed positive effects on cell proliferation, as measured by the cell counting kit-8 (CCK-8) assay. Using quantitative polymerase chain reaction (PCR), different spatial structures of the scaffolds showed different results on tendon and osteogenic induction. Tendon-related genes, including Col I, Col III, and tenascin (TCN) were highly expressed in the ACS group. In contrast, bone-related genes, including Col I, bone morphogenetic protein-2 (BMP-2), and runt-related transcription factor-2 (RUNX-2) genes were highly expressed in the RCS group.

The scanning electron microscopy (SEM) findings in this study showed that the morphology of most cells from the ACS group

appeared as long spindle cells, similar to tenocytes. The morphology of most cells from the RCS group appeared as polygonal cells, similar to chondrocytes. These findings supported that spatial structure played an important role in cell differentiation. The aligned scaffold promoted the induction of tendon cells, and the random scaffold promoted the induction of osteogenic cells [32,33]. However, owing to the complex structure of the tendon and bone interface, it remains unknown which spatial structure is better for healing at the tendon and bone interface.

The findings of this study showed that, *in vivo*, more fibrous and cartilage tissues were induced in the bone tunnel in the RCS group compared with the ACS group after scaffold

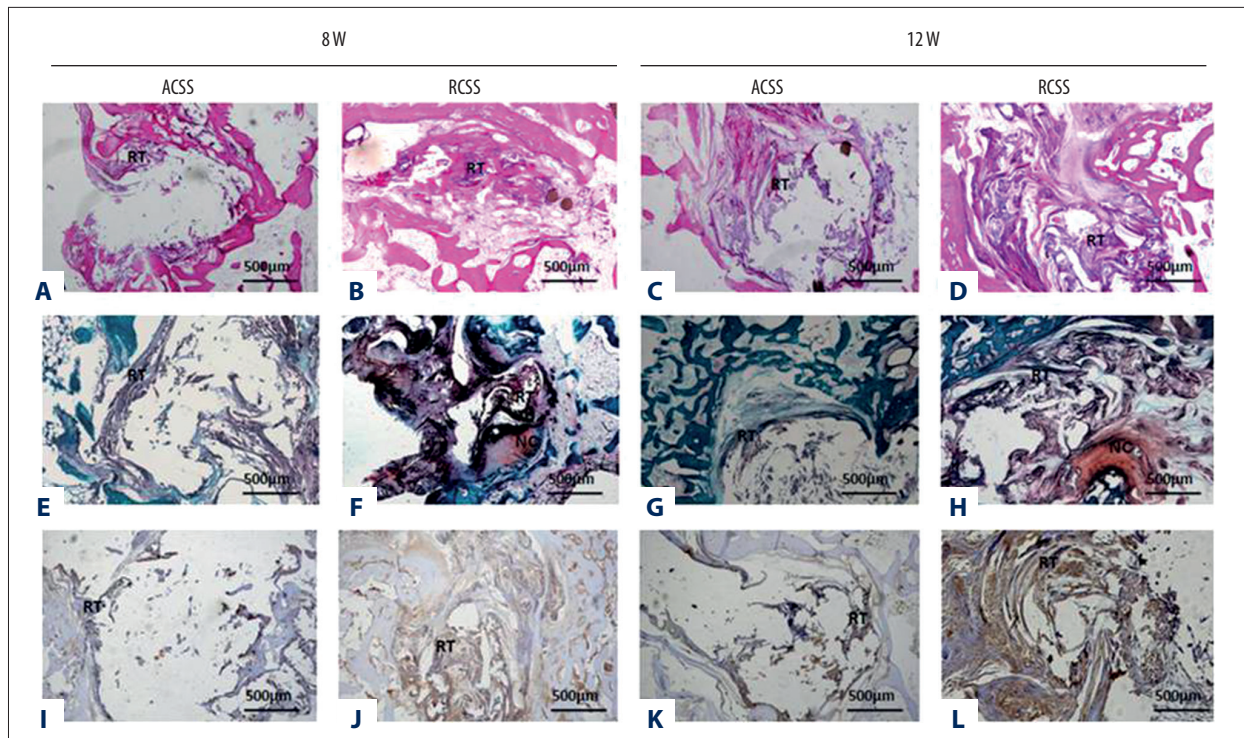


**Figure 5.** Quantitative polymerase chain reaction (PCR) analysis of gene expression of COL I, COLIII, TNC, RUNX-2, and BMP-2 *in vitro*. **(A)** Quantitative polymerase chain reaction (PCR) analysis of gene expression of COL I, COLIII, TNC, RUNX-2, and BMP-2 *in vitro* at one week. **(B)** Quantitative PCR analysis of gene expression of COL I, COLIII, TNC, RUNX-2, and BMP-2 *in vitro* at two weeks. COL I – collagen I; COL III – collagen III; TNC – tenascin; RUNX-2 – runt-related transcription factor 2; BMP-2 – bone morphogenetic protein-2; ACS – aligned collagen fiber scaffold; RCS – random collagen fiber scaffold. Statistically significant at \*  $p < 0.05$ .

implantation for eight weeks and 12 weeks. According to the immunohistochemistry findings, the expression of Col I (a marker of both ossification and tendon formation) was significantly increased in the tissue samples from the animal model treated with RCSS, indicating the increased formation of progenitor tissue. The SEM findings from the cell migration in the RCS group showed that the rabbit bone marrow stem cells (BMSCs) from the cell pellet could migrate in all directions. However, in the ACS group, the rabbit BMSCs mainly migrated in the direction parallel to the collagen fibers. Similarly,

after implantation *in vivo*, the RCSS was able to recruit more cells from the site of injury, including in both the walls and the bottom of the bone tunnel. However, since the ACS was anisotropic, infiltration of the cells from the walls of the bone tunnel was difficult [23,34]. With the lack of cells to produce components to form a sufficient extracellular matrix (ECM), it might be difficult for regenerative tissue such as fiber and cartilage to form, which had an adverse effect on the healing of bone and tendon [35].





**Figure 6.** Rotator cuff tendon repair eight weeks and 12 weeks post-surgery. (A–D) Photomicrographs of the histology of the regenerative tendon outer and inner zone. Hematoxylin and eosin (H&E). (E–H) Photomicrographs of the histology of safranin O (SO) staining of the regenerative tendon outer and inner zone. (I–L) Photomicrographs of the immunohistochemistry staining for the localization of Col I of the regenerative tendon outer and inner zone. (A, E, I) Aligned collagen scaffold combined with knitted silk (ACSS) implantation at eight weeks post-surgery. (B, F, J) Random collagen scaffold combined with knitted silk (RCSS) implantation at eight weeks post-surgery. (C, G, K) ACSS implantation at 12 weeks post-surgery. (D, H, L) RCSS implantation at 12 weeks post-surgery. RT – regenerative tissue; NC – neonatal cartilage; ACSS – aligned collagen fiber in knitted silk scaffold; RCSS – random collagen fiber in knitted silk scaffold. Scale bars: 500 µm.

This study had several limitations. First, the collagen fiber in the manufactured scaffold may degrade too quickly to match adequate deposition of new tissue. Second, as the structure of the tendon and bone interface has four layers, the layered scaffold may be better at producing healing at the tendon and bone interface. Therefore, further studies involving larger animal models should be conducted before these types of surgical scaffold are studied clinically.

## Conclusions

This study demonstrated that a collagen scaffold combined with knitted silk (CSS) had a positive effect on healing at the tendon and bone interface in a rabbit model. The scaffold showed ideal biocompatibility, degradability, and mechanical properties. Furthermore, compared with the aligned scaffold, the random scaffold or random collagen scaffold combined with knitted silk (RCSS), was superior in terms of cell adhesion and osteogenic induction, which implied improved efficacy of the use of for tendon and bone healing.

## Conflict of interest

None.

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