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# Long-term effects of knitted silk–collagen sponge scaffold on anterior cruciate ligament reconstruction and osteoarthritis prevention

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ABSTRACT

Anterior cruciate ligament (ACL) is difficult to heal after injury due to the dynamic fluid environment of joint. Previously, we have achieved satisfactory regeneration of subcutaneous tendon/ligament with knitted silk–collagen sponge scaffold due to its specific "internal-space-preservation" property. This study aims to investigate the long-term effects of knitted silk–collagen sponge scaffold on ACL regeneration and osteoarthritis prevention. The knitted silk–collagen sponge scaffold was fabricated and implanted into a rabbit ACL injury model. The knitted silk–collagen sponge scaffold was found to enhance migration and adhesion of spindle-shaped cells into the scaffold at 2 months post-surgery. After 6 months, ACL treated with the knitted silk–collagen sponge scaffold exhibited increased expression of ligament genes and better microstructural morphology. After 18 months, the knitted silk–collagen sponge scaffold-treated group had more mature ligament structure and direct ligament-to-bone healing. Implanted knitted silk–collagen sponge scaffolds degraded much more slowly compared to subcutaneous implantation. Furthermore, the knitted silk–collagen sponge scaffold effectively protected joint surface cartilage and preserved joint space for up to 18 months post-surgery. These findings thus demonstrated that the knitted silk–collagen sponge scaffold can regenerate functional ACL and prevent osteoarthritis in the long-term, suggesting its clinical use as a functional bioscaffold for ACL reconstruction.



## 1. Introduction

Anterior cruciate ligament (ACL) plays an important role in providing stability to the knee and enabling functional movements [1]. Due to its biomechanical function, ACL is one of the most frequently damaged tissue during vigorous sports activities. In the USA alone, there are 100,000–200,000 ACL injuries annually [2,3]. The repair of ACL remains a formidable clinical challenge because of its very limited self-regenerative capacity. Current surgical procedures for ACL injuries such as autografting, allografting, and xenografting exhibit obvious drawbacks, such as autograft donor site morbidity, ligament laxity, lengthy rehabilitation, potential disease transmission and immune-rejection with allografts and xenografts [4,5]. Most notably, it was reported that 40%–90% of patients with ACL injuries go on to develop knee osteoarthritis (OA), and that most treatments cannot alleviate the pathological condition but instead exacerbate it [6–8]. Hence, more effective reconstructive techniques for ACL repair need to be developed.

Artificial ligaments are emerging as a promising alternative treatment approach. There are a number of studies on the use of artificial ligaments in ACL injuries, as well as several commercial products available, such as Leeds-Keio®, Proplast®, Gore-Tex® and

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Stryker-Dacron® [4]. Those products were developed in the 1970s–1990s, and were fabricated from non-degradable synthetic materials. Although artificial ligaments overcome the deficiencies of autografts and allografts and exert satisfactory effects in the short term, they induce certain adverse effects in the long term, including graft rupture, OA occurrence, chronic synovitis, and foreign body reaction [4,9–13]. Hence, research and development of an ideal biological scaffold for ACL reconstruction is still ongoing.

Recently, silk-based biomaterials are being widely utilized in soft tissue engineering due to their good biocompatibility and remarkable mechanical strength [14]. We have previously fabricated a knitted silk–collagen sponge scaffold and achieved promising results in treating Achilles tendon [15], medial collateral ligament (MCL) [16] and shoulder rotator cuff tendon defects [17]. The knitted silk scaffold combined with collagen matrix enhanced regeneration with more collagen matrix deposition, better mechanical properties, more native microstructure with larger diameter collagen fibrils compared to silk scaffold alone. But its effect on ACL, the most frequently encountered site of injury in clinical practice, remains unclear. Although the essence of tendon and ligament tissue is similar, ACL injuries are more difficult to deal with due to the dynamic fluid environment and lack of blood supply and seed cells within the joint cavity. Therefore, the efficacy of utilizing the knitted silk–collagen sponge scaffold for ACL reconstruction needs to be further investigated.

In 2009, Fan and colleagues utilized silk scaffold seeded with mesenchymal stem cells (MSCs) for *in vivo* ACL regeneration and the results at 24 weeks showed enhanced repair of ACL, compared to silk scaffold alone [18]. This suggested that the silk scaffold needs to be further improved for ACL reconstruction. Besides, the long-term effects of ACL repair through implantation of biomaterials within articular cartilage have never been evaluated. Hence, this study aims to investigate the long-term effects of the knitted silk–collagen sponge scaffold on ACL regeneration and osteoarthritis prevention.

## 2. Experimental section

### 2.1. Preparation and characterization of scaffolds

#### 2.1.1. Scaffold fabrication

Raw silk fibers (*Bombyx mori*) were purchased from Zhejiang Cathaya International Inc. (Hangzhou, China). The knitted silk scaffold and silk–collagen sponge scaffold was fabricated as described previously [16]. Briefly, the knitted silk was fabricated using 12 yarns (1 filament/yarn) of silk fibers on a knitting machine. Plain knitted silk scaffolds were manufactured with 21 stitches per centimeter. The knitted silks were treated with an aqueous solution containing 0.02 M $Na_2CO_3$ at 90 and 100 °C for 60 min to extract sericin. Insoluble type I collagen was isolated and purified from pig Achilles' tendon using neutral salt and dilute acid extractions. The sericin-extracted knitted silk mesh was immersed in an acidic collagen solution (type I, pH 3.2, 1 wt%, 3 mm depth), and frozen at 80 °C for 12 h. It was then freeze-dried under vacuum (Heto PowerDry LL1500) for 48 h to allow the formation of collagen sponges. Then the scaffolds were crosslinked by dehydrothermal treatment and cut into 20 × 80-mm pieces. The structural morphology of scaffolds after processing was characterized by scanning electron microscopy (SEM) and light microscopy.

#### 2.1.2. SEM imaging

The specimens were dehydrated in increasing concentrations of alcohol (30–100% v/v) and were critically point-dried. They were mounted on aluminum stubs and coated with gold, then viewed under a Hitachi S-3000N SEM at an accelerating voltage of between 15 kV.

#### 2.1.3. Assessment of biomechanical properties

Mechanical testing was performed using an Instron tension/compression system with Fast-Track software (Model 5543, Instron, Canton, MA, USA) as described previously [16,19]. Each specimen was tested in tension at a rate of 10 mm/min until the mesh ruptured. The biomechanical properties of each mesh were represented by failure force ($N$) ($n = 4$).

#### 2.1.4. Cell proliferation

Cell proliferation was assessed using Cell Counting KIT-8 (CCK-8, Dojindo, Kumamoto, Japan). Primary MSCs from rabbits weighing 2.5–3.0 kg were explanted and cultured as described previously [16]. The first to third passage cultures were utilized for all subsequent experiments, and cell culture media was replaced once every three days. The MSC-seeded scaffold was incubated in CCK-8 solution in a 5% $CO_2$ incubator at 37 °C for 3 h, at various culture timepoints (1d, 3d, 7d, 10d and 14d; $n = 3$). The intense orange-colored formazan derivative formed by cell metabolism is soluble in the culture medium. The absorbance was measured at 450 nm and cell numbers were correlated to optical density (OD).

### 2.2. Animal model

Fifteen New Zealand White rabbits weighing 2.5–3.0 kg were utilized in this study. The animals were subjected to general anesthesia. Lateral parapatellar arthrotomy was used to expose the right knee joint of the rabbit. After the native ACL was excised, the tibial and femoral bone tunnels were created with a 2.0 mm diameter drill-bit. The full length of tunnel from anterolateral femur, across the joint, to anteromedial tibia was about 30 mm. The silk scaffolds with or without collagen sponge were carefully rolled up along their short axis to produce a tightly wound shaft of 80 mm in length and 2.0–4.0 mm in diameter. Both ends of the shaft were sutured with 2-0 polyester suture (MERSILK, Johnson & Johnson, US) in a whipstitch style. The shaft was passed through the tunnel and both ends were fixed by sutures tied over screws in the femur and tibia. The left limbs were replaced with the knitted silk scaffold, and the right limbs were replaced with silk–collagen sponge scaffold. After surgery, animals were allowed free cage activity. Upon animal sacrifice, five limbs from each experimental group were utilized for histological evaluation and gene expression analysis at the 2, 6 and 18 months timepoints. Specimens were imaged by transmission electron microscopy (TEM) to assess the collagen fibril diameter after 2 and 18 months post-surgery, and were scanned with microCT at 18 months. Treatment of animals was in accordance with standard guidelines approved by the Zhejiang University Ethics Committee.

### 2.3. Macroscopic and histological assessment

The cartilage surface was stained with Indian ink for macroscopic observation. Specimens were fixed, dehydrated, and embedded within paraffin blocks. Histological sections (8 μm) were prepared using a microtome, and subsequently deparaffinized with xylene, hydrated using decreasing concentrations of ethanol, and then subjected to hematoxylin and eosin (HE) staining, Masson trichrome and safranin O staining. The quantity of fibroblast-like cells, immunological cells and blood vessels within the newly-formed ACL tissue was assessed by a modification of techniques utilized in a previous study [20]. Histological evaluation was performed using the modified Mankin's score.

### 2.4. Collagen content assay

The amount of deposited collagen on the scaffold was quantified by using a collagen quantitative assay kit (Jiancheng Inc., Nanjing, China) following the manufacturer's protocol.

### 2.5. RNA isolation and RT-PCR

Total tissue RNA ($n = 3$) was isolated by lysis in TRIZOL (Invitrogen Inc., Carlsbad, CA, USA) followed by a one-step phenol chloroform–isoamyl alcohol extraction as described by the manufacturer's protocol. Real-time PCR analysis of five genes including COLLAGEN I (COL I), BIGLYCAN (BGN), DECORIN (DCN), TENASCIN (TNC) and GAPDH was performed using Brilliant SYBR Green QPCR Master Mix (TakaRa) with a Light Cycler apparatus (ABI 7900HT), as described previously [21]. The primer sequences used in this study are listed in Table 1. Each real-time PCR run was performed with at least three experimental replicates, and the results are presented as target gene expression normalized to GAPDH.

**Table 1**
List of primer sequences utilized for real-time polymerase chain reaction.

| Genes | 5′–3′ | Primers | Production size (bp) |
|---|---|---|---|
| Collagen I | Forward | CTCCAAGGCCAAGAAGCATG | 400 |
| | Reverse | AGCGCCACCGATGTCCAAA | |
| BIGLYCAN | Forward | GATGGCCTGAAGCTCAA | 407 |
| | Reverse | GGTTTGTTGAAGAGGCTG | |
| DECORIN | Forward | ACTGGGCACCAACCCTCTGA | 497 |
| | Reverse | ATCTGAAGGTGGATGGCTGGA | |
| TENASCIN | Forward | CGTGAAAAACAATACCCGAGGC | 160 |
| | Reverse | GCCGTAGGAGAGTTCAATGCC | |
| GAPDH | Forward | TCACCATCTTCCAGGAGCGA | 293 |
| | Reverse | CACAATGCCGAAGTGGTCGT | |