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Type I collagen and polyvinyl alcohol blend fiber scaffold for anterior cruciate ligament reconstruction

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Abstract
The aim of this study was to perform an evaluation of a braided fiber scaffold for anterior cruciate ligament (ACL) reconstruction. The scaffold was composed of 50% type I collagen (Col-I) and 50% polyvinyl alcohol (PVA). First, the biocompatibility and in vitro weight loss of the scaffold were tested. Then, the scaffolds were used to reconstruct the ACL in China Bama mimi pigs. At 24 weeks post-operation, the mechanical properties and histology of the regenerated ACL were analyzed. The maximum load and tensile strength were 472.43 ± 15.2 N and 29.71 ± 0.96 MPa, respectively; both were ~75% of those of native ACL and ~90% of those of fiber scaffold. This indicated that the scaffold maintained a large portion of native ACL’s mechanical properties, and tissue formation on the scaffold compensated most of the tensile strength loss caused by scaffold degradation. Histology and immunohistology analysis showed the morphology and major extracellular matrix components of the regenerated ligament resembled the native ACL. Thus, the Col-I/PVA blend fiber ACL scaffold showed good potential for clinical applications.

(Some figures may appear in colour only in the online journal)

1. Introduction
The anterior cruciate ligament (ACL) is the most important intra-articular ligament of the knee; it controls the normal motion and acts as a knee joint stabilizer to prevent the tibia from moving forward [1]. But the ACL is extremely susceptible to injury, at least 200 000 ACL reconstructions are performed each year in the United States, with an estimated direct cost of $3 billion dollars annually [2]. The common treatments for ACL injury include autografts and allografts. However, both methods have obvious drawbacks [3, 4]. The source for autografts is limited, and the complications of the donor site are serious. Allografts have problems with the potential for infections and immunoreactions.

The shortcomings of current ACL injury treatments have encouraged tissue-engineered solutions for ACL reconstruction [5]. Many degradable biomaterials have been used to fabricate tissue-engineered ACL scaffolds [6], including type I collagen (Col-I) [7, 8], silk [9–11], poly lactic acid (PLA) [12], poly-l-lactic acid (PLLA) [13–15], polyglycolic acid (PGA) [16] and their copolymers [17, 18]. However, the overall performance of the tissue engineering ACL scaffolds is less than optimal [19]. Recently, two studies using silk-collagen fiber scaffold [20] and poly(lactide)-based fibrous scaffold [21], respectively, have shown good mechanical properties and biocompatibilities in vitro, but their in vivo performances have not been verified yet.
The aim of this study was to investigate the feasibility of Col-I and polyvinyl alcohol (PVA) blend fiber as an ACL scaffold. Col-I promotes cell adhesion and differentiation [22], but its degradation speed [23] and mechanical properties [24] cannot meet the needs for ACL reconstruction scaffold.

We blended PVA with Col-I, for the purpose of integrating collagen’s biocompatibility and PVA’s mechanical strength. Then, we tested the biocompatibility and in vitro weight loss of the ACL scaffold. Finally, we implanted the ACL scaffold in a pig model. After 6 months, we analyzed the histology and immunohistology of the regenerated ACL with surrounding tissues, and evaluated its biomechanical properties.

2. Materials and methods

2.1. Scaffold fabrication

The Col-I (Cosen Biochemical, China) and PVA (Model AH-26, Medicine Group Chemical, China) hybrid was made at the concentration ratio of 50:50. Then, wet spinning technology was used to make the hybrid into Col-I/PVA blend fiber. The scaffold braiding method was based on a previous protocol [25] with some minor changes. Briefly, 180 blend fibers were gathered into a bundle, and three bundles were twisted into a braid with the diameter of 4.5 mm (figure 1). The braided scaffold was cut to a length of 20 cm; both ends of the scaffold were tied by dental steel wire, and fixed by sutures.

2.2. Cytocompatibility and in vitro weight loss of scaffold

The Col-I/PVA fibers were sterilized with 70% alcohol for 5 min, followed by 15 min UV irradiation. Then, the fibers (1 ml cm⁻²) were soaked in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, USA) + 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C with 5% CO₂ and 95% humidity for 48 h. After that, a fiber bundle was co-cultured with mouse fibroblast (L-929) cells (5 x 10⁴ cells ml⁻¹, CI.020, Abgent, China) in a 96-well plate (Nunc, Denmark), and medium (100 μl/well) were changed every 2 days. L-929 cell strain is commonly used in toxicity testing; here we used L-929 fibroblasts to test the cytocompatibility and cytotoxicity of the blend fiber scaffold. After 4 days incubation, the images of cells were taken using scanning electron microscope (SEM) (S-3400N, HITACHI, Japan). Within the first week, MTT assay (M2128, Sigma, USA) was done on days 2, 4 and 7 to quantify cell growth in groups with different Col-I/PVA extract concentration (50%, 100%), blank control group (DMEM + 10%FBS) and positive control group (0.5% phenol in DMEM + 10% FBS) (n = 6 for each group).

To measure the in vitro weight loss, Col-I/PVA blend fibers were divided into six groups, and the initial weight (w₀) of each group (n = 6) was measured using electronic balance (BA61, Sartorius, Germany). Then, they were evenly placed in a 24-well plate (Nunc, Denmark), and 1 x 10⁵U lysozyme (ST206, Beyotime, China) in 15 ml PBS was added into each well. For each individual group, fiber weight was examined at 1, 2, 3, 4, 6 and 8 weeks. The dry weight (w₁) of the fibers was weighed again at each time point. The percentage of weight loss of Col-I/PVA fibers was determined as weight loss(%) = (w₀ - w₁)/w₀ x 100%.

2.3. In vivo ACL reconstruction

China Guangxi Bama mini pigs were used as animal models (35–42 kg, 1 year old); they were divided into an experiment group (scaffold implantation) (n = 8) and a control group (ACL resection) (n = 4). The animal experiments and surgeries were conducted in compliance with the protocol approved by Animal Ethics Committee of the Third Military Medical University.

In the control group, the ACL was cut off, and the residual ACL at tibial and femoral attachment point was reserved. In the experiment group, the tibial tunnel was drilled at 5 mm medial from tibial tubercle with a 6.0 mm cannulated reamer (Smith and Nephew, Incorporated, USA) at 90° knee flexion; the femoral tunnel was drilled at the ACL attachment point in intercondylar fossa. A custom-built suture passer was used to guide the scaffold through a tibial and femoral tunnel. Two 4.0 mm screws were applied to fix the scaffold. After that, the redundant of the scaffold was cut off, articular cavity was irrigated by 0.5% iodophor, and then the articular cavity, muscle, subcutaneous tissue and skin were sutured.

While the pigs were under anesthesia after surgery, the knees were examined by digital radiography (DR, SIEMENS, Germany) to check the position of the screws, making sure the scaffolds were fixed correctly (figure 2). All animals were allowed to move freely without immobilization after surgery, and in order to prevent infection, benzathine intramuscular injection was adopted twice a day for 6 days. The pigs were killed at 24 weeks postoperatively; computed tomography (CT, SIEMENS, Germany) scanning of the bone tunnel was done. Then, we removed the metal screws in the bone tunnel, and took magnetic resonance imaging (MRI, SIEMENS MAGNETOM Trio 3.0T, Germany) and SEM images of the regenerated ACL.

2.4. Mechanical test

The maximum load, tensile strength and the elastic modulus of the regenerated ACL were measured. The tensile strength was defined as the maximum load divided by the cross-sectional area of the sample; the Young’s modulus was defined as stress divided by strain, in which the stress was defined...

Figure 2. Digital radiography of a pig knee in the experiment group showing the right position of the metal screws. (A) Anteroposterior view. (B) Lateral view.

Table 1. The relative growth rate (RGR) of L-929 cells on Col-I/PVA scaffold by MTT assay (blank control: DMEM + 10% FBS, positive control: 0.5% phenol, n = 12 per group, mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>OD (490 nm)</th>
<th>RGR</th>
<th>OD (490 nm)</th>
<th>RGR</th>
<th>OD (490 nm)</th>
<th>RGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td>Day 4</td>
<td></td>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td>50%Col-I/PVA</td>
<td>0.54 ± 0.02</td>
<td>90.73%</td>
<td>1.23 ± 0.10</td>
<td>97.61%</td>
<td>1.65 ± 0.14</td>
<td>123.61%</td>
</tr>
<tr>
<td>100%Col-I/PVA</td>
<td>0.53 ± 0.02</td>
<td>90%</td>
<td>1.2 ± 0.12</td>
<td>94.64%</td>
<td>1.34 ± 0.27</td>
<td>100.45%</td>
</tr>
<tr>
<td>DMEM + 10%FBS</td>
<td>0.59 ± 0.04</td>
<td>100%</td>
<td>1.26 ± 0.29</td>
<td>100%</td>
<td>1.33 ± 0.28</td>
<td>100%</td>
</tr>
<tr>
<td>0.5% phenol</td>
<td>0.09*</td>
<td>16.01%</td>
<td>0.06 ± 0.003*</td>
<td>5.15%</td>
<td>0.08 ± 0.004*</td>
<td>6.3%</td>
</tr>
</tbody>
</table>

*Statistically different from all other groups (p < 0.05).

as the loading force divided by the cross-sectional area. The pig knees in the experiment group were dissected of all the soft tissue and ligaments except for the regenerated ACL to form a femur-scaffold-tibia complex. The length and diameter of the ACL was measured. Then, each knee was positioned at 180° extension, clamped and mounted in an RGT-5KN microcomputer-controlled electron biomechanical testing machine (Greg’s instrument, China). The load to failure test was performed at a strain rate of 2% per second. The maximum load was recorded by the microcomputer, and then the tensile strength and Young’s modulus were calculated according to their definitions. The same test was done on the blend fiber scaffolds and the native pig knees, both of which served as comparison groups.

2.5. Histological and immunohistological analysis

The regenerated ligaments in the experiment group were fixed with 10% formalin, embedded in paraffin, cut into sections of 5 μm, and then stained with hematoxylin and eosin (H.E.). The extracellular matrix (ECM) components of the regenerated ligament were analyzed using immunohistochemistry staining, in which monoclonal antibodies for Col-I (Sigma, USA), type III collagen (Chemicon, USA) and tenascin-C (Chemicon, USA) were used. The specimens of regenerated ligament–bone junctions were fixed with 10% formalin, embedded in paraffin, cut into sections of 5 μm, and then treated by fast-green staining.

2.6. Statistical analysis

All data were analyzed using PASW Statistics 18.0 and P-values of <0.05 were considered to be significant. The data were presented as mean ± standard deviation (SD). For mechanical tests and MTT assay, one-way analysis of variance (ANOVA) followed by Tukey’s test was done to determine significant differences between groups.

3. Results

3.1. Cytocompatibility and in vitro weight loss of scaffold

The results of MTT assay showed the relative growth rate (RGR) was defined as the growth rate in experiment groups normalized to growth rate in blank control) of L-929 cells ranged from 90% to 123.61% within 1 week, and there was no significant difference between the 50% Col-I/PVA extract group and 100% Col-I/PVA extract group (table 1). SEM imaging showed the cells attached to the scaffold and spread as a spindle shape after 4 days culture (figures 3(A), (B)). The cytotoxicity of scaffolds in DMEM + 10%FBS (RGR100%) and 50%Col-I/PVA (average RGR103.98%) groups were graded Level 0 (RGR≥100%, no cytotoxicity), and scaffolds in 100%Col-I/PVA group (average RGR95.03%) was graded Level 1 (RGR75%~99%, slight cytotoxicity) according to China national standard of biological evaluation of medical devices—Test for in vitro cytotoxicity (ISO 10993-5:1999, IDT).

The in vitro weight loss of the Col-I/PVA blend fibers in the presence of lysozyme was relatively fast for the first two weeks (p < 0.05). From the third week, weight loss speed began to slow down. The percentage of weight loss at weeks 1, 2, 3, 4, 6 and 8 was 8.28%, 15.14%, 16.26%, 16.88%, 17.17% and 17.44%, respectively (figure 4).
Figure 3. Scanning electron microscope imaging of cells and tissues. 4 days after seeding, L-929 cells attached and spread on the fiber bundle (A, ×500), (B, ×200) (n = 6). 24 weeks after scaffold implantation, fibrous tissues formed on (C, ×2000) and between (D, ×4000) the fibers within scaffold (n = 6).

Figure 4. The percentage of weight loss of the Col-I/PVA blend fiber scaffold in vitro at weeks 1, 2, 3, 4, 6 and 8 (mean ± SD, n = 6, *p < 0.05).

3.2. Imaging and macroscopical observation

At 24 weeks postoperatively, CT scanning showed that in the experiment group the bone tunnel did not expand and instead there was moderate mineralization on the tibial and femoral tunnel wall (Figures 5(A), (B)). In MRI, the regenerated ACL (Figure 5(D)) could be seen, and it resembled the native ACL (Figure 5(C)) in morphology. Besides, the intensity of articular cartilage was homogeneous, indicating no cartilage injury in the experiment group. SEM imaging showed a considerable amount of fibrous tissue formed on (Figure 3(C)) and between the fibers within the scaffold (Figure 3(D)).

From macroscopic observation, the resected ACL in the control group (Figure 5(E)) was not repaired, and the ACL nub was wrapped up with synovial tissue. While in the experiment group, the ligament (Figure 5(F)) was regenerated in the original ACL location, and both the morphology and color of the regenerated tissue were similar to that of native ACL (Figure not shown).

3.3. Biomechanical analysis

The mechanical properties of the regenerated ligament were compared with those of native ACL and blend fiber scaffold. The load-deformation curves (Figure 6) of native ACL reached peak value via concave slopes, while regenerated ACL and fiber scaffold reached maximum load via convex slopes. At 24 weeks postoperatively, the maximum load of the regenerated ACL was 472.43 ± 15.2 N, ~88% maximum load of ACL scaffold and ~75% maximum load of native ACL (p < 0.05) (Table 2). In terms of the tensile strength, the trend was almost the same, with regenerated ACL having 29.71 ± 0.96 MPa, which was ~88% of scaffold strength and ~78% of native ACL strength (p < 0.05) (Table 2). These results showed that native ACL had better load-bearing ability over fiber scaffold and regenerated ACL. The behaviors of the
Table 2. Mechanical properties of Col-I/PVA scaffold, regenerated ligament and native ACL (n = 4, mean ± SD).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximum load (N)</th>
<th>Tensile strength (MPa)</th>
<th>Young’s modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-I/PVA scaffold</td>
<td>534.93 ± 49.26</td>
<td>33.63 ± 3.10</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Regenerated ligament</td>
<td>472.43 ± 15.20</td>
<td>29.71 ± 0.96</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Native ACL</td>
<td>630.07 ± 76.11*</td>
<td>37.43 ± 2.13*</td>
<td>0.01 ± 0’</td>
</tr>
</tbody>
</table>

*Statistically different from other groups (p < 0.05).

Reconstructed ligament and the scaffold were not significantly different (table 2), indicating that the reconstructed ligament maintained the mechanical properties of the scaffold in spite of in vivo degradation.

3.4. Histological and immunohistological analysis

24 weeks after the operation, H.E. staining showed that there was cellular infiltration around the scaffold fibers; the cells (figure 7(B)) in articular cavity were similar to those of the native ACL (figure 7(A)). H.E. and fast-green staining of the ligament–bone junction (figure 7(C)) showed that the interface was filled with abundant fibrous tissues which contained calcium salts. And the newly formed ligament tissue bonded well with bone tissue (figure 7(D)). Moreover, the Col-I/PVA scaffold could still be discerned in the regenerated ACL and ligament–bone junction, implying partial degradation of the scaffold. The immunohistochemistry staining (figure 8) of the ACL scaffold and adjacent tissues in the experiment group showed that the stainings for Col-I and tenascin-C were strong positive, while the staining for type III collagen was weak positive. This result indicated that the ECM components of the regenerated ligament contained much Col-I and tenascin-C, but a little type III collagen.

4. Discussion

The selection of biodegradable scaffold materials is crucial to ligament tissue engineering. Though many biomaterials have been reported for their potential in ACL reconstruction, none of them yet can be successfully applied on humans [26, 27]. The ideal scaffold for ACL reconstruction should be biocompatible and biodegradable; in addition, it should have an appropriate degradation rate that matches the growth rate of regenerated tissue and similar mechanical properties to native ACL [28]. The engineered ACL scaffold qualified in all these aspects is still under investigation [29].

In this study, ACL was reconstructed by implanting Col-I/PVA blend fiber scaffold in a pig model. The scaffold was composed of 540 braided blend fibers at a ratio of 50% Col-I to 50% PVA. PVA is nontoxic and fully degradable, and it has high tensile strength and flexibility [30]. All these characteristics make PVA fiber a logical candidate for fabricating an ACL reconstruction scaffold. Here, we blended Col-I with PVA for the purpose of retaining and integrating their advantages. Indeed, the blend fiber scaffold well supported cell/tissue attachment and ingrowth, and the regenerated ACL at 24 weeks post-operation reached ~75% of the mechanical properties of the native pig ACL.

Before in vivo evaluation of the scaffold, we tested its cytocompatibility and in vitro weight loss. We co-cultured L-929 fibroblasts with Col-I/PVA fiber bundles for 1 week, and found that the toxicity of the material to fibroblasts was trivial. SEM imaging showed L-929 cells adhered to the scaffold, and cell morphology was typical of fibroblast. The in vitro weight loss experiment indicated that the scaffold lost weight slowly and smoothly after 2 weeks, allowing enough time for tissue formation rather than losing mechanical integrity too
Figure 6. Load-deformation curves measured at 2% strain per second. (A) Native pig ACL ($n = 4$). (B) Col-I/PVA scaffolds ($n = 4$). (C) Regenerated ACL at 24 weeks post-implantation ($n = 4$).

Figure 7. Histology of regenerated ligament (H.E.) and ligament–bone junction (fast-green). (A) Native ACL (H.E. × 200). (B) Regenerated ligament in articular cavity (H.E. × 100, purple indicates cells, arrow points to one of the scaffold fiber). (C) The ligament–bone junction was filled with calcified fibrous tissues (fast-green × 40) (arrow points to one of the scaffold fiber). (D) The regenerated ligament tissue bonded well to bone tissue (fast-green × 40, red indicates regenerated tissue, blue indicates bone tissue).

Figure 8. The immunohistochemistry of regenerated ligament 24 weeks after ACL reconstruction (arrow indicates one of the scaffold fiber). (A) Staining for tenascin-C. (B) Staining for type I collagen. (C) Staining for type III collagen.
quickly. This was then confirmed by regenerated ACL having comparable mechanical properties with the fiber scaffold. Therefore, the scaffold we fabricated was safe and suitable for ligament reconstruction. Next, we chose a pig model for ACL reconstruction, because pig knees were more similar to human knees than other large laboratorial animals [31]. In the first week after ACL reconstruction surgery, all pigs were not restricted in movement, but they did not bear weight on their surgical legs. The surgical sites were swelling, and the food intake was slightly less than usual; 2 weeks after surgery, the pigs regained appetites and the post-surgical swelling went down; 4 weeks post-operation, the animals’ gait returned to normal.

24 weeks post-implantation, the pigs were sacrificed. Histology analysis of specimens in the experiment group showed infiltration of fibroblast-like cells, and the scaffold was wrapped by the newly formed tissue. At the ligament–bone junction, a large amount of mineralized fibrous tissue filled the gap; the interface integration between bone tissue and ligament was compact. This finding may present new ideas for improving the ligament–bone integration of the commercially available artificial ligaments.

We set the ratio of Col-I to PVA at 1: 1 in this tentative study; the mechanical properties of blend fiber scaffold turned out to be acceptable. However, further experiments need to be done to determine the optimal ratio of the scaffold composition. To date, various kinds of materials with different modifications have been tried to fabricate a ligament scaffold. Lu et al [16] compared the in vitro performance of PGA, PLLA and (polylactic-co-glycolic acid, 82:18) PLAGA scaffolds coated with fibronectin; they found that the PLLA scaffold showed the best cellular response, degradation and mechanical properties. Freeman et al [32] further evaluated the PLLA scaffold combined with different concentrations (10%, 15% and 20%) of (polyethylene glycol diacrylate) PEGDA; they revealed that the PLLA scaffold soaked in 10% PEGDA had the best chemical release and mechanical properties. Another recent study by Walters et al [33] focused on the crosslinking and the gelatin of the Col-I scaffold; results showed that the 1-ethyl-3-(3-dimethylaminopropyl) (EDC) crosslinked scaffold without gelatin was the most similar with human ACL in mechanical properties.

Although the in vitro investigations such as the aforementioned provide useful references for ligament scaffold fabrication, in vivo evaluations of the scaffold using large animal models are indispensable prior to clinical applications. Here we compared our results with some other studies which used large animal models. Robayo et al [34] implanted Col-I scaffolds in goat knees; after 6 months, the scaffolds were vascularized and populated with cells coming from the surrounding tissues; however, the material properties of the scaffolds were not evaluated. Tovar et al [35] used scaffold composed of 75% polarylate fibers and 25% Col-I to reconstruct ACL in sheep knees; after 3 months they also found cellular infiltration and vascularity, but the maximum load of the regenerated ACL was quite small (42 ± 22 N compared with 1219 ± 376 N of native ACL). A previous study by Fan et al [36] reconstructed ACL in a pig model using a silk scaffold and mesenchymal stem cells. After 6 months the maximum load of the silk scaffold was 52.6% of native ACL control, while in our study the maximum load showed better results (75% of native ACL control). The regenerated tissues and cells on the silk scaffold were thought to be contributed by the implanted stem cells; in our study, we speculated that the fibroblast-like cells might migrate from surrounding tissues or even be differentiated from bone marrow mesenchmal stem cells in bone tunnels.

The limitations of this study are as follows. First, the scaffolds have not completely degraded at 24 weeks post-implantation, so we are unable to observe and analyze the final substitute. Thankfully, some other pigs with scaffold implantation are on feed, the regenerated ligaments will be retrieved for analysis at longer time periods. Second, metal screws were used to fix the scaffold in pig ACL reconstruction, which caused inconvenience for MRI scanning. We will adopt absorbable screws [37] in the next set of experiments, so that the live imaging of the ligament tissue formation could be carried out at the arranged time points. Nonetheless, to our knowledge, this is the first study that reports ACL scaffold with PVA composition. In future studies, we will firstly optimize the composition ratio of Col-I to PVA; secondly we will improve the braiding method of the scaffold if a better protocol is available; finally, we will analyze whether there is necessity of implanting seed cells [38] in our animal experiments.

5. Conclusion

In summary, a braided Col-I/PVA blend fiber ACL scaffold was implanted into a pig model, and it showed very promising results. After 24 weeks, there was obvious tissue ingrowth in and around the scaffold, and the ligament–bone junction was integrated with fibrous tissue. In addition, the reconstructed ACL maintained a large portion of native ACL’s mechanical properties. Thus, we demonstrated here the feasibility of using this blend scaffold for ACL reconstruction. Further investigations will be done not only to optimize the scaffold design, but also to provide insights to fabricating ligament scaffolds for clinical applications.

Acknowledgments

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