A Preclinical Evaluation of the Functionalized Biphasic Silk Fibroin Scaffold for Complete Bone-Ligament-Bone Regeneration

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Introduction: Anterior Cruciate Ligaments (ACL) restoration has been a prevalent orthopaedic procedure, with the use of tendon autografts remaining as the gold standard. The limitations to this solution are primarily the inherent donor site morbidity and the lack of graft integration with the bone tunnel.

In view of the need for robust tissue engineered graft integration, we propose the use of a functionalized biphasic silk scaffold for complete bone-ligament-bone tissue regeneration. The ends of this hybrid silk scaffold system are incorporated with nanoparticles of low crystallinity hydroxyapatite (nHA) and loaded with bone morphogenetic protein 2 (BMP-2) to stimulate bone tunnel and enthesis regeneration, while the central one-third supports ligament regeneration. It is hypothesized that the complementing synergy between the osteoinductive BMP-2 and the osteoconductive nHA will encourage bone regeneration.

Methods: Knitted scaffolds (240 fibroins, 60 × 20 mm for in vitro characterization and rabbit implantation; 480 fibroins, 100 × 20 mm for pig implantation) were first fabricated from raw Bombyx mori silk and subsequently degummed. Aqueous silk fibroin (SF) solution was first obtained by dissolution. Three different types of aqueous SF-based solutions were made by blending: pure aqueous SF (2.6 % w/v), aqueous SF (2.6 % w/v) with nHA (0.78 mg/end), and aqueous SF (2.6 % w/v) with nHA (0.78 mg/end) and BMP-2 (29 µg/end). Sequential cast and freeze process of these aqueous solutions were made over the knitted SF, with the central one-third of the scaffolds casted in pure aqueous SF solution and the ends casted in either SF/nHA solution (control group, Ctrl) or the SF/nHA/BMP-2 solution (experimental group, Exp). After lyophilization and methanol treatment, the completed scaffolds were characterized for scaffold morphology, mechanical properties at the two phases and elution kinetics (n = 5).

For in vitro characterizations, the scaffolds were cut at the phase boundaries to form 3 hybrid scaffold groups: pure SF, SF with nHA (SF/nHA), and SF with nHA/BMP-2 (SF/nHA/BMP-2). Rabbit bone marrow derived MSCs (P3, 1 × 10^6/scaffold) were seeded and statically cultured over 28 days. The cellular viability, proliferation, gene expression and collagen deposition levels were performed (n = 3).

For in vivo characterizations, the two groups of complete scaffolds (experimental: with SF/nHA/BMP-2 ends, control: with SF/nHA ends) were each seeded with rabbit MSCs (P3, 3 × 10^5/scaffold for rabbit implantation) or porcine MSCs (P2, 10 × 10^5/scaffold for pig implantation) and statically cultured to allow cell adhesion for a day prior to implantation. The animal experiments were approved by Institutional Animal Care and Use Committee of National University of Singapore. Forty-eight New Zealand White rabbits (12 weeks old, 2.5-3.0 kg) and fourteen Yorkshire pigs (~60 kg) were each randomly divided into experimental and control groups respectively.

Standard ACL reconstruction was performed after excision of the native tissue and the animals sacrificed at the respective postoperative timepoints (rabbits: 2, 4, 6 months; pigs: 6 months). The knee joints were collected immediately and kept at -80°C. The samples were then scanned using micro-CT prior to histological preparations (n = 3) and mechanical tests (n = 5).

Results: The fabricated biphasic silk scaffolds were shown to be porous with interconnected pores. nHA and BMP-2 were observed to be securely incorporated in the lyophilized SF sponges. BMP-2 bioactivity was ascertained after the fabrication process and was shown to be eluting with an initial burst release, followed by a lowered sustained release. MSCs were observed to be viable and proliferative in all three groups of the in vitro study. Uppregulation of osteogenic genes (such as COL 1, ON, OPN) persisted in the SF/nHA and SF/nHA/BMP-2 groups compared with the pure SF group. This was phenotypically supported by the increased deposition of collagen in the two groups compared to the pure SF group. Gross observation of the excised rabbit and porcine knee joints showed no signs of osteoarthritis and that the ligament portion was regenerated (Fig. 1). Bone tunnel narrowing was observed in Exp compared to Ctrl as indicated by micro-CT images (Fig. 2A, B). Individual slice measurements (n=30) of each specimen indicated significantly better bone tunnel healing in the Exp group (~68%) as compared to Ctrl (~6%). Histological characterizations further indicated presence of new bone formation in Exp with development of Sharpey’s fibers in the earlier post-implantation stages (Fig. 2C, D). Better graft to bone integration was also observed from the superior pull-out strength of Exp compared to Ctrl in the rabbit models, while for the Exp in the porcine model mechanical failure initiated at the mid-substance of the ligament portion during tensile testing and not due to pull out.

Discussion: With the aim of addressing the issue of osteointegration in tissue engineered ACL grafts, we have developed a BMP-2 eluting biphasic silk scaffold that releases BMP-2 in a manner that resembled the physiological conditions at fracture. Based on
our knowledge, this study is the first to investigate the biphasic silk scaffold enhanced with blended osteoinductive BMP-2 and osteoconductive HA nanoparticles, and carried through in vitro analysis, small animal study and large animal preclinical study. It was found that BMP-2 and nHA synergistically complemented each other in stimulating osteogenic differentiation of MSCs, resulting in bone tunnel narrowing with new mineralized tissues observed in both the small and large animal model. Consequently, there was enhanced graft-host integration by 6 months resulting in mechanical properties closer to that of the native bone-ACL-bone construct. The BMP-2 eluting biphasic silk scaffold was thus shown in this series of bench work, small animal study and large animal preclinical trial to be promising as a tissue engineering solution for complete ACL-bone regeneration.

Significance: We have developed a biphasic silk scaffold with clear tissue type zone demarcation for complete bone-interface-ligament tissue regeneration. Clinically, this scaffold system aims to overcome the limitation of non-fusion or non-anchorage of the regenerated soft tissue (ligament) with the hard tissue (native bone), which will affect clinical outcome and recovery duration.

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