Comparison of Hybrid Scaffolds for Tendon and Ligament Tissue Engineering

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ABSTRACT

Knitted scaffolds and gel-composite scaffolds were unsuitable for anterior cruciate ligament (ACL) reconstruction as cells seeded often get dislodged from the scaffolds in in vivo dynamic conditions. As such, two systems of hybrid silk scaffolds; namely the nanofibrous silk scaffold and the silk sponge scaffold, were developed to counter this problem. The nanofibrous silk scaffold was made of knitted silk integrated within its pores a layer of interlocking silk nanofibers while the silk sponge scaffold comprised of weblike microporous silk sponges formed in the openings of a knitted silk scaffold. The aim of this study was to compare the cellular responses of bone marrow-derived mesenchymal stem cells (BMSCs) on the two systems of hybrid silk scaffolds that were designed for tendon/ligament tissue engineering applications. Rabbit BMSCs were seeded and cultured in vitro for two weeks on the silk scaffolds. The samples were evaluated and compared for their cell seeding efficiency, proliferation, protein expression of soluble collagen and cellular morphology. In addition, mechanical properties of the scaffolds after two weeks of incubation were evaluated. Preliminary results showed that cellular responses to both nanofibrous silk scaffold and silk sponge scaffold were similar and both scaffold systems were able to retain their basic functions of promoting cell proliferation and differentiation, and maintaining the structural integrity of the combined cell-scaffold systems.

INTRODUCTION

The anterior cruciate ligament (ACL) plays a crucial role in the knee joint by guiding normal motion and providing stability. The ACL is frequently injured in athletic accidents and surgical reconstruction is often required to restore knee function. ACL reconstruction surgeries are usually performed using biological grafts but often lead to complications such as donor site morbidity, immunological reaction and undesirable host tissue responses. This has become one of the main concerns of the orthopedic surgeons and doctors today and therefore calls for an urgent need to find an ideal solution.
Advancements in tissue engineering over the last decade has offered researchers today the possibility of creating functionally engineered tissues to treat ACL injuries without many undesirable side effects associated with current surgical procedures and techniques. The tissue reconstruction scaffold has become the center of focus in ligament tissue engineering because of its many requirements. An ideal scaffold should be biodegradable, biocompatible, and porous, exhibit sufficient mechanical strength, and promote the formation of ligamentous tissue (Liu, 2008). Potential ACL scaffolds developed for ligament tissue engineering in recent studies involved biodegradable materials such as collagen and polymeric scaffolds which includes poly-glycolic acid (PGA) and poly-lactic acid (PLA). Although much attention was given to these materials initially, they were unsuitable for use in ligament tissue engineering because collagen lost mechanical strength quickly while PGA and PLA reduced cell adhesion, proliferation and function (Petrigliano, 2006) (Hokugo, 2006). It was not until recent years that silk has become a promising material as scaffold for ligament reconstruction due to its biocompatibility, slow degradability and excellent mechanical properties (Wang, 2006) (Altman, 2003). Our group has developed two silk scaffold systems; one which involves integrating silk nanofibers into the knitted silk scaffold and one that incorporates microporous silk sponges to knitted silk scaffold for tendon/ligament tissue engineering (Liu, 2008). In this paper, a comparative study is performed between the two systems of hybrid scaffolds to assess their respective cellular function and biomechanical properties in the regeneration of tendon/ligament tissues.

MATERIALS AND METHODS

Scaffold Fabrication

Raw Bombyx mori silk fibers (obtained from Mahasarakam University, Thailand) were used to knit the scaffolds in dimensions of 40mm x 25mm with a knitting machine (Silver-reed SK270, Suzhou, China). The fiber used in the fabrication of the scaffold consisted of three yarns of silk and each yarn had 80 fibroins with a diameter each of approximately 8μm. The knitted silk was then mounted onto metal-frames before being immersed into a degumming solution of 0.25% (w/v) Na$_2$CO$_3$ and 0.25% sodium dodecyl sulfate (SDS) with temperature of between 98 and 100°C. The aqueous solution was refreshed every half hour and the process was repeated thrice until majority of the sericin was removed.

Nanofibrous silk scaffold  The nanofibrous silk scaffold was prepared by electrosprinning silk nanofibers into the pores of a knitted silk scaffold. 25% (w/v) of silk solution was prepared by first removing sericin off the raw silk fibers with the aforementioned degumming solution and dissolved in a 250% (w/v) lithium thiocyanate solution. The silk solution obtained was then dialyzed against distilled water using a SnakeSkin Pleated Dialysis Tubing (PIERC, MWCO 3500) for 24 h and frozen at -80°C for 4h before it was freeze-dried for 24 h into silk sponges. Subsequently, 10% (w/v) of electrospinning silk solution was prepared by dissolving the silk sponges in hexafluoroisopropanol (HFIP) on a mechanical shaker for 12 h. The final silk solution obtained was poured into a 5ml syringe placed on a syringe pump and extruded through a flow rate of 0.5ml/h and a potential difference of between 11kV and 15kV into silk nanofibers. The nanofibers were allowed to integrate into the pores of the knitted scaffolds placed approximately 20cm below the tip of the syringe needle. The nanofibrous silk scaffolds obtained were contacted with a 90/10 (v/v) methanol/water solution for 10min and dried overnight in
fume hood to induce an amorphous to silk II conformational change in the nanofibers to prevent resolubilization in the cell culture medium.

Silk sponge scaffold. The silk sponge scaffold was prepared by forming microporous silk sponges in the openings of a knitted silk scaffold. 1% (w/v) of silk solution was obtained by first extracting raw silk fibers from the degumming solution and dissolving in a CaCl$_2$-CH$_3$CH$_2$OH-H$_2$O (mole ratio = 1:2:8) solution at 78 ± 2°C. The silk solution was then dialyzed against distilled water for 24h before knitted sericin-free silk scaffolds were immersed into the silk solution. The immersed scaffolds were frozen at -80°C for 12h and subsequently freeze-dried for 24h to allow the formation of microporous silk sponges within the openings of the knitted scaffold. Finally, the silk sponge scaffolds were contacted in 90/10 (v/v) methanol/water solution for 10min and dried overnight in fume hood.

Cells and Cell Culture
Primary bone marrow-derived mesenchymal stem cell (BMSC) from New Zealand White rabbits were cultured in DMEM (Gibco, Invitrogen) containing 10% FBS (HyClone, Logan, UT). The cultures were grown to confluence at 37°C and 5% CO$_2$. Cell culture medium was replaced every three days and cells obtained at passage 3 were used in this study.

Cell Seeding and Culture on Scaffolds in vitro
Both systems of scaffolds were kept in an air-tight glass urn and sterilized by exposure to 37% formaldehyde for 24 h. The sterilized scaffolds were then allowed to degas by leaving them in the bio-safety cabinet for about 4 h. The scaffolds were transferred into the wells of a custom-made 6-well culture chamber and seeded each with 1ml of 5.0 x 10$^5$ cells. The chambers were incubated for 4 h to allow cell attachment before each well was topped-up with another 2ml of cell-free medium. The scaffolds were grown in vitro in a 5% CO$_2$ incubator at 37°C for 2 weeks, with the medium being replaced every 3 days. Manual cell count was performed for the medium in each well after the first 36 h using a disposable haemocytometer to evaluate the cell seeding efficiency. The cell seeding efficiency was expressed as the number of cells attached to the scaffold as a percentage of the total number of cells seeded (5 x 10$^5$ cells).

Cell Viability and Morphology
Cell viability was observed with fluorescent microscopy after live cell staining with 5-chloromethyl fluorescein diacetate (CMFDA) dye. The scaffolds were first rinsed thoroughly with PBS and then added with 6μl of CMFDA (5mg/ml) in 2ml of PBS before being incubated for 20 min. The scaffolds were subsequently washed twice in PBS and observed by fluorescent microscopy. CMFDA dye stained viable cells green.

Cell Proliferation
Quantitative cell proliferation assay was performed using Alamar Blue assay (BioSource International, CA, USA) on day 3, 7, 10 and 14 after cell seeding. Briefly, at each time point, the cell medium was removed and 10% (v/v) Alamar Blue in DMEM solution was added into each well and incubated for 3 h. The absorbance of the reduced Alamar Blue dye was measured in 96-well plates at absorbance wavelengths of 540nm and 600nm using a microplate reader (TECAN Microplate Reader, Magellan Instrument Control and data Analysis Software). The percent reduction was computed using the Alamar Blue standard formula to give an indirect
estimate of the number of cells in the scaffold. 4 samples were used to conduct the test at each time point.

**Soluble Collagen Production**

The soluble collagen produced by the BMSCs was quantified at day 3, 7, 10 and 14 using Sircol collagen dye binding assay Kit (Biocolor Ltd., Newtownabbey, Ireland). Briefly, 200μl of culture medium was removed from each well and the removed solution was added with 1ml of dye reagent and mixed for 30 min at room temperature. The mixture was then centrifuged at 13500g for 10 min to precipitate the pellet of dyed collagen from the culture medium. After the supernatant was removed, the collagen pellet was dissolved in 1ml of releasing reagent. The absorbance of re-dissolved dye was measured in 96-well plates at absorbance wavelength of 540nm using a microplate reader (TECAN Microplate Reader, Magellan Instrument Control and data Analysis Software). Subsequently, the collagen amount was extrapolated from a standard curve and the collagen in each sample was presented as the amounts produced in μg/ml. 4 samples were used to conduct the test at each time point.

**Biomechanical Testing**

After 14 days of culture, mechanical testing was conducted on the cell-scaffold constructs. The cell-scaffold constructs were carefully rolled up along their short axis and secured at both ends with silk to produce a tightly wound shaft with a dimension of about 40mm long and 5mm in diameter. The constructs were then tested under tension using an Instron testing machine (Model 3345, Instron Inc., MA) with a maximum load of 1000N. The gauge length was set at 20mm and the samples were tested at a speed of 10mm/min. The maximum tensile load, stiffness, young’s modulus and ultimate tensile strength were determined. Results were obtained from an average of 4 measurements.

**Statistical Analysis**

Data were analyzed using MS Excel, expressed in mean ± standard deviation, and tested for significance using Student’s T tests (p<0.05 being considered significant).

**RESULTS AND DISCUSSION**

Manual cell count with haemocytometer 36 h after seeding revealed that both nanofibrous silk scaffold and sponge silk scaffold exhibited high cell seeding efficiency; the nanofibrous scaffold with 89.33% attachment and the sponge scaffold with 86.33% attachment (Fig.1). The slightly lower percentage of cell attachment observed with sponge scaffold could be due to the freeze-dried silk being more porous than the intertwining silk nanofibers.

![Figure 1. Percent cell attachment on scaffolds.](image-url)
Cell proliferation was assessed with Alamar Blue assay and the results showed that BMSCs grown on both nanofibrous silk scaffolds and silk sponge scaffolds demonstrated similar growth pattern; percent reduction in Alamar Blue decreased from about 31% at day 3 to 26% at day 7 then increased to about 37% at day 14 (Fig. 2). The initial decrease at day 3 and increase at day 7 in percent reduction of Alamar Blue corresponded to the lag phase and logarithmic growth stage of the growth pattern of the BMSC respectively (Dai, 2007). The increase in percent reduction of Alamar Blue by day 14 also showed that the cells remained viable and that both systems of scaffolds were equally capable in supporting the growth and proliferation of the BMSCs.

The amount of soluble collagen produced by cells grown on each scaffold was evaluated with Sircol collagen dye at each time point and it was found that the amount of soluble collagen produced by the BMSCs grown on sponge scaffold was significantly higher (41.27μg/ml) than that produced by BMSCs grown on nanofibrous scaffold (23.30μg/ml) at day 3 (Fig. 3). This could be attributed to the less compact and higher porosity nature of the sponge scaffold (Fig. 5) which resulted in the soluble collagen produced by the cells to diffuse into the surrounding culture medium much easier than those in the dense layers of nanofiber coated scaffolds. Another possible cause to this observation could be that the nanofibers actually mimicked the extra-cellular matrix (ECM) much better than the microporous sponge layer in the sponge scaffold and therefore circumvented the need for the BMSCs to secrete more collagen while they were in the initial stages of growth in the scaffold. In addition, the high surface area to volume ratio and good hydrophilicity of the electrospun polymeric nanofibers (Li, 2002) could retain the soluble collagen within its matrix better; leading to less collagen being released into the culture medium. By day 14, the ECM would have become very developed and functional as the amount of soluble collagen produced by the BMSCs grown on both the nanofibrous scaffolds and sponge scaffolds decreased sharply to 10.62μg/ml and 14.79μg/ml respectively (Fig. 3).

Results obtained from the mechanical testing of the hybrid scaffolds revealed that although the silk sponge scaffold was significantly stiffer (5.53N/mm) than the nanofibrous scaffold (3.61N/mm) as shown in Figure 4, other mechanical properties of the two systems of scaffold were similar. While the sponge scaffold had higher ultimate tensile strength (12.65MPa), max load (210.52N) and Young’s Modulus (17.17MPa) as compared to the nanofibrous scaffold (Fig. 4), the differences were found to be statistically insignificant. However, these two hybrid
scaffolds showed better mechanical properties compared to PLGA and PLLA based knitted scaffolds developed in our previous studies, which is desirable for tendon/ligament tissue engineering applications because the whole process requires a scaffold to provide and maintain strong mechanical support throughout the healing period (Sahoo, 2007). Therefore, silk-based hybrid scaffold systems on knitted silk scaffolds are better suited for tendon and ligament tissue engineering.

![Figure 4. Mechanical properties of nanofibrous silk scaffold and sponge silk scaffold at day 14.](image)

![Figure 5. Fluorescence images after live cell staining of (A) and (B) nanofibrous silk scaffolds and (C) and (D) sponge silk scaffolds.](image)
CONCLUDING REMARKS

Cellular responses in terms of cell seeding efficiency, cell proliferation and collagen production were similar in both nanofibrous- and sponge-coated silk scaffolds. By the end of the experiment, both scaffolds retained their basic functions of promoting cell proliferation and differentiation while maintaining structural integrity of the cell-scaffold composite. In addition, the hybrid scaffolds possessed good mechanical properties, essential for tendon and ligament tissue engineering.

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