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Silk fibroin porous scaffolds for nucleus pulposus tissue engineering

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ABSTRACT

Intervertebral discs (IVDs) are structurally complex tissue that hold the vertebrae together and provide mobility to spine. The nucleus pulposus (NP) degeneration often results in degenerative IVD disease that is one of the most common causes of back and neck pain. Tissue engineered nucleus pulposus offers an alternative approach to regain the function of the degenerative IVD. The aim of this study is to determine the feasibility of porous silk fibroin (SF) scaffolds fabricated by paraffin-sphere-leaching methods with freeze-drying in the application of nucleus pulposus regeneration. The prepared scaffold possessed high porosity of 92.38 ± 5.12% and pore size of 165.00 ± 8.25 μm as well as high pore interconnectivity and appropriate mechanical properties. Rabbit NP cells were seeded and cultured on the SF scaffolds. Scanning electron microscopy, histology, biochemical assays and mechanical tests revealed that the porous scaffolds could provide an appropriate microstructure and environment to support adhesion, proliferation and infiltration of NP cells *in vitro* as well as the generation of extracellular matrix. The NP cell–scaffold construction could be preliminarily formed after subcutaneously implanted in a nude mice model. In conclusion, The SF porous scaffold offers a potential candidate for tissue engineered NP tissue.

1. Introduction

Intervertebral disc (IVD) as a complex connective tissue lies between adjacent vertebrae in the spine and is responsible for both load transmission and flexibility during motion of the spine [1,2]. IVD consists of two major and distinct regions: nucleus pulposus (NP) and annulus fibrosus (AF). There are significant differences between NP and AF in biochemical ingredients, mechanical behaviors and cellular type. NP and AF work together to implement mechanical function of IVD, The NP primarily provides the IVD with compressive properties, whereas the AF surrounding the NP provides the shear and tensile properties [3].

The NP is a water-rich gelatinous central region of IVD containing a large amount of negatively charged proteoglycans (PGs) and collagen II [4,5]. As a common disease with age, the degeneration of IVD mainly involves the dehydration and fibrosis of NP-matrix [6], which results in NP collapse and the reduction of IVD height. Consequently the IVD cannot fully pressurize and resist applied load. The clinical symptoms are low back pain and eventually disability. It is reported that of adults aged 50 years and older, 85% suffers from disc degeneration [7,8].

The aims of NP therapies are to restore the structure and function of NP tissue so that the IVD can pressurize properly. Current clinical treatments mainly involve conservative management (medication and physical therapy) or surgical intervention (spine fusion, total disc replacement, or nucleus pulposus (NP) replacement), However, these methods only gain symptomatic relief to some extent rather than completely restore the function of IVD [9]. Injection of growth factors (e.g. TGF-βand BMP-2) or cells (MSCs and chondrocyte) may be an effective therapy to a certain extent at the early stage of IVD degeneration, but at more advanced stage, the healing efficacy is limited and even helpless [10–14].

The development of cell-based tissue engineering technique provides a promising alternative for repair of degenerated IVD [11,14]. The degeneration of IVD is believed to originate from the chronic pathological, biological and anatomical changes of NP. Thus, many tissue engineering studies are directed toward treatment of the NP [15]. The objective of NP tissue engineering is to generate *in vitro* a complex structure containing materials and cells for the replacement of degenerated NP. The scaffold serving as extracellular matrix (ECM) provides an optimal microenvironment for cell attachment, proliferation and migration [16]. Therefore the selection of scaffold materials and optimization of scaffold architecture are critical for NP tissue engineering. Currently, injectable hydrogels such as collagen, alginate, chitosan, silk fibroin and hyaluronic acid (HA) have been proposed as scaffolds for NP tissue engineering because of their similarity to the native NP tissue. Most of them are used alone or in combination with cells or growth factors to *in situ* gelate in the site of NP tissue [10–13]. However, the application of such hydrogels to construct tissue engineered NP structure is limited to some extent due to: ① relative weak mechanical strength, which cannot provide a sufficient physical support to withstand physical loading

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of spine when the tissue engineered NP structure is implanted in body [17]; ② relative small pore size, which makes it very difficult to *in vitro* directly seed cell inside hydrogels [18]; ③ relatively fast degradation rate, which cannot well match the slow secretion rate of NP ECM [13,19]. Based on these reasons, the versatile porous scaffold which is more suitable for NP tissue engineering needs to be further developed, however, there are few reports concerning the problem [20–22].

Silk fibroin (SF) as a natural biomaterial possesses many outstanding features such as good biocompatibility, proper mechanical properties, low immunogenicity and controllable degradation rate. SF scaffolds have been applied to various tissue engineering [23,24], including cartilage, nerve, vascular, ligament and so on. Recently, more and more attentions were paid on the application of SF materials in IVD tissue engineering [25–30]. For example, Park et al. used the silk fibroin/hyaluronic acid composite gels to regenerate the NP [27]. Chang et al. used porous SF scaffolds for tissue engineering AF [30]. Due to the high similarity of NP to cartilage tissue in terms of biochemical composition, biomechanical function and cellular type [31–34], we attempted to use porous SF scaffolds with interconnected pores for NP tissue engineering.

In the present study, we fabricated SF porous scaffolds with interconnected macropores and high porosity by the combination of paraffin-sphere-leaching and freeze-drying methods. The NP cell attachment, proliferation and migration on this type of silk fibroin scaffold were investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Preparation of porous silk fibroin scaffold

Scaffolds were prepared by the combination of paraffin-sphere-leaching and freeze-drying. Briefly, paraffin spheres with about 170 μm in diameter were added to a Teflon mold (cylindrical vial with diameter of 15 mm and depth of 15 mm). The top surface of the paraffin spheres was leveled by use of a flat head metal block with proper pressure. The mold containing the paraffin spheres was carefully moved to a preheated oven for 50 min at 55 °C, and cooled to room temperature creating a paraffin sphere assembly. The 13% (w/v) of aqueous SF solution which was prepared according to previous procedures [35,36] was added to the paraffin-sphere assembly under vacuum so that the solution could fill all the space between the paraffin spheres and then the mold was frozen at −70 °C and freeze-dried to obtain the dried paraffin–silk-fibroin complexes. The complexes were immersed in 80% (v/v) methanol for 2 h. The paraffin spheres in the complexes were dissolved in boiling hexane and residual paraffin was further extracted by using the Soxhlet extractor (SXT-06). The leached SF scaffolds were dried at room temperature under vacuum for 3 days to completely remove the hexane residue.

2.2. Scaffold characterization

The scaffold specimens were cut into cross-sections with 1 mm thickness by using a scalpel blade. The cross-section microstructure was examined by optical microscopy (Leica, M205A,Germany) and scanning electron microscopy (SEM, Hitachi X-650, Japan) after coating with gold–palladium. The average pore size was determined by measuring 50 random pores from SEM images with Image J software (Wayne Rasband, National Institute of Health, USA). The porosity of scaffold was determined by liquid displacement method [35].

2.3. In vitro studies

2.3.1. Isolation, culture and seeding of NP cells

To isolate NP cells, the spine between T10 and L5 was obtained from 4-week-old New Zealand White rabbits. After removal of the muscle and tendon tissues surrounding the AF, the AF was surgically opened, NP tissue was then taken out by using aseptic curette. The resulting NP tissue was placed in a vial and digested by 0.2% collagenase type II (Sigma–Aldrich) solution at 37 °C for 4 h, followed by centrifugation at 1000 rpm for 7 min to harvest primary NP cells. Both cell number and viability were determined by trypan blue test. To obtain more NP cells, the primary NP cells were cultured and proliferated under the conditions of 5% CO_2 atmosphere and 37 °C in High glucose-Dulbecco's modified Eagle's medium (H-DMEM; Gibco) with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic–antimycotic (Gibco) to obtain passage one of NP cells for next use.

In order to create NP cell–scaffold complex, the above generation one of NP cell (2×10^7/mL) were seeded on both top and bottom of the porous scaffolds with diameter of 5 mm and height of 3 mm. The cell-seeded scaffolds were incubated for 4 h to allow the cells to attach to the surface of the scaffolds, then a certain volume of H-DMEM including 10% FBS was added and continually cultured for 1 and 3 weeks. The obtained cell–scaffold complexes would be used for histology and biochemistry analysis.

2.3.2. Total DNA content

Total DNA content was measured to assess the NP cell proliferation in the scaffolds. Six cell-seeded scaffolds were cultured for 1 day, 1 week and 3 weeks, respectively. The cultured specimens were digested in papain solution (P4762, Sigma–Aldrich) at a concentration of 0.1 mg/mL with Hank's balanced salt solution (H2387, Sigma–Aldrich) at 60 °C for 16 h. Total DNA of the digested sample was quantified using a fluorescence assay with Hoechst 33258 dye according to the proposed manual (Sigma–Aldrich).

2.3.3. Cell viability and observation on the scaffold

Cell viability of adherent NP cells in the scaffold was assessed using a Live/Dead cell viability assay kit (Molecular Probes; Eugene, OR) after 48 h of culture. After incubation with the Live/Dead staining solution for 30 min, the scaffolds with adherent NP cells were gently rinsed in sterilized PBS, the live cells (green) and dead cells (red) were observed under Leica confocal microscope.

For the SEM observation, the cell–scaffold samples after 1 week of culture were fixed for 6 h with 2.5% glutaraldehyde, dehydrated in a graded series of ethanol, dried and coated with gold, finally were examined by SEM.

2.3.4. Histology and qualitative biochemistry analysis

NP cell–scaffold samples were fixed with 10% formalin, dehydrated, paraffin-embedded and was cut into sections with 5 μm thick using a microtome (Leica, RM2016 Germany). The resulting sections were stained using hematoxylin for 4 min and eosin for 30 s, the images of H&E staining were taken by inverted light microscopy (Ix53, Olympus, Japan). For observation of PGs, the sections were stained with toluidine blue (Sigma). Immunohistochemistry was used to examine type II collagen by the following procedure: The sections were deparaffinized, dehydrated and treated by 3% H_2O_2 solution, then incubated with 1% rabbit serum albumin for 30 min at 37 °C, followed by incubating with primary antibody, washing and incubating with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), Finally the treated sections were developed with diaminobenzidine (DAB) (Vector Laboratories, CA) and observed under an inverted microscope.

2.3.5. Quantification of proteoglycan and type II collagen

PGs and type II collagen contents were quantitatively measured by using ELISA kits (Blue Gene). The cell–scaffold samples ($n = 5$) were rinsed in PBS, minced and homogenized in PBS using a glass grinder. The resulting suspensions underwent 2 freeze–thaw cycles to further break the cell membranes, then the homogenates were centrifuged for 15 min at 5000 rpm and the supernatants were removed immediately. According to the protocols of ELISA kits for PGs and type II collagen,