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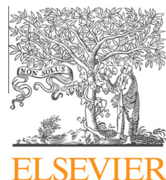

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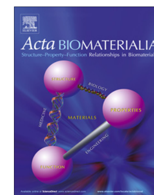

Time-dependent bladder tissue regeneration using bilayer bladder acellular matrix graft-silk fibroin scaffolds in a rat bladder augmentation model

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ABSTRACT

With advances in tissue engineering, various synthetic and natural biomaterials have been widely used in tissue regeneration of the urinary bladder in rat models. However, reconstructive procedures remain insufficient due to the lack of appropriate scaffolding, which should provide a waterproof barrier function and support the needs of various cell types. To address these problems, we have developed a bilayer scaffold comprising a porous network (silk fibroin [SF]) and an underlying natural acellular matrix (bladder acellular matrix graft [BAMG]) and evaluated its feasibility and potential for bladder regeneration in a rat bladder augmentation model. Histological (hematoxylin and eosin and Masson's trichrome staining) and immunohistochemical analyses demonstrated that the bilayer BAMG-SF scaffold promoted smooth muscle, blood vessel, and nerve regeneration in a time-dependent manner. At 12 weeks after implantation, bladders reconstructed with the BAMG-SF matrix displayed superior structural and functional properties without significant local tissue responses or systemic toxicity. These results demonstrated that the bilayer BAMG-SF scaffold may be a promising scaffold with good biocompatibility for bladder regeneration in the rat bladder augmentation model.

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1. Introduction

Various congenital and acquired urinary tract diseases, such as bladder exstrophy, bladder cancer, and neurogenic bladder dysfunction, require bladder reconstruction [1]. Currently, bladder reconstruction is still one of the greatest surgical challenges in the field of urology. While enterocystoplasty is still considered the gold standard and has played a major role in bladder reconstruction for decades [2], it is associated with a series of complications, such as recurrent urinary tract infections, electrolyte imbalance, urinary incontinence, perforation and urolithiasis, severely impacting the quality of life of patients [3].

Tissue engineering (TE) approaches provide a potential strategy to minimize these consequences and have great promise for bladder repair and reconstruction [4,5]. In recent studies, different types of scaffolds, constructed mainly from naturally derived materials and synthetic polymers, are used widely for bladder reconstruction [6,7]. Bladder acellular matrix grafts (BAMGs) are intact collagen-based xenogenetic biomaterials derived from pigs that are prepared using rigorous techniques to remove all cells [8]. Moreover, BAMGs exhibit good biocompatibility and support tissue regeneration [9], and several studies have indicated that BAMG is a suitable scaffold for bladder reconstruction [6,10,11]. In addition, BAMGs have also been shown to prevent permeation of luminal contents into the abdominal cavity [12]. Although multilayered urothelial tissues have been shown to be generated by implantation of a simple BAMG scaffold in the bladder, these dense and smooth BAMGs cannot promote satisfactory smooth muscle regeneration, vessel formation, and nerve tissue regeneration, particularly for seeding cells or host tissue penetration into the scaffold [10,13]. Silk fibroin (SF) is a unique natural fibrous protein

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that has a unique array of properties, including high structural strength and elasticity, diverse processing plasticity, and tunable biodegradability [14]. Unfortunately, the simple and compact SF structure is not easily degraded and increases the risk of urinary stones [15,16].

The ideal scaffold for the regeneration of the urinary bladder must fulfill specific requirements pertaining to three-dimensional (3-D) architecture, microenvironmental needs of the various types of cells in the bladder wall, barrier function, and structural integrity of the bladder [17]. To reach this goal, we have developed a bilayer scaffold by adding SF directly on the BAMG and lyophilizing the sample, resulting in the SF-dependent generation of an internal interconnected porous architecture; this structure was then shown to increase the extent of degradation and infiltration of host bladder tissue [16]. We believe that this bilayer BAMG-SF scaffold may represent a novel promising biomaterial for bladder reconstruction.

Therefore, in the present study, we examined the feasibility of the bilayer BAMG-SF scaffold for bladder reconstruction in rats. The morphological features of the bilayer scaffold were characterized by scanning electron microscopy (SEM), and biological performance of the bilayer scaffold was analyzed at 2, 4, and 12 weeks after implantation into the rat bladder. Furthermore, we also evaluated the extent of SF scaffold degradation in vivo, inflammatory responses, and systemic safety.

2. Materials and methods

2.1. Fabrication of bladder acellular matrix grafts (BAMG)

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Affiliated 9th People's Hospital of Shanghai Jiao Tong University School of Medicine. Porcine bladder tissues were harvested from 3-month-old pigs and rinsed with phosphate-buffered saline (PBS; pH 7.2–7.4) to remove blood. The bladder samples were then transported in ice-cold PBS to the laboratory immediately. Fat tissues and fascia around the urinary bladder were removed with scissors. The urothelium, muscle, and serosal layers were grossly removed by surgical delamination and washed in distilled water in a stirring flask (200 rpm) for 48 h at 4 °C, followed by treatment with 0.03% trypsin for 1 h. The bladders were then soaked for 7 days at 37 °C in 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) and 0.1% (v/v) ammonium hydroxide. The solution was refreshed every day. The resulting matrix was washed with distilled water for 2 days at 4 °C and sterilized with 75% ethanol. The complete elimination of cellular nuclei in the BAMG was confirmed by histological evaluation and quantification of residual DNA content.

2.2. Evaluation of the decellularization efficacy

The BAMG were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. For histological examinations, sections (5 mm) of the BAMG were deparaffinized and stained with H&E and Masson's trichrome stainings to evaluate the cellular nucleus and ECM components. The total DNA in freeze-dried native porcine bladders and BAMG was extracted using a DNA isolation kit for tissues (Roche Applied Sciences). The samples were weighed, finely minced using scissors, and digested with RNase and proteinase K. The remaining DNA was collected and quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit according to the manufacturer's protocol. The fluorescence of test solution was measured on a fluorescence microplate reader (excitation, 480 nm; emission, 520 nm; Thermo Scientific Multiskan FC). The amount of DNA in the native porcine bladders and BAMG was quantified against a DNA standard curve and expressed as μg/mg dry weight of the test samples. We randomly and respectively selected 6 native porcine bladders and BAMGs to carry out the DNA content assay. All the evaluations were repeated 3 times.

2.3. Fabrication of the bilayer BAMG-SF scaffold

The bilayer BAMG-SF scaffold was composed of 3-D porous SF and BAMG. First, the BAMG was washed with deionized water 10 times to remove the alcohol component, which could negatively affect the adhesion of SF on the BAMG. Then, the BAMG was trimmed into 15 mm × 15 mm square pieces and placed in a prepared rectangular casting vessel (bottom: 15 mm × 15 mm), where the muscle layer surface of the BAMG is directly exposed to the SF solution. Preparation of silk fibroin solution and fabrication of highly interconnected porous silk fibroin scaffolds following the procedure described previously [18,19]. Next, 300 μL of SF solution (2% w/v) was slowly poured into the vessel. The whole material was transferred to the refrigerator and frozen at −20 °C for 2 days. The frozen composite was then lyophilized to remove the water and the other solvents to form a porous SF scaffold. Finally, the scaffolds were dried at room temperature under vacuum for 2 days, which were then sterilized in 75% ethanol, rinsed in PBS, and subjected to surgical procedures described below.

2.4. SEM

Structural analysis of the matrix prior to implantation was performed by SEM in order to gain insights into the scaffold surface morphology, interfiber space, and thickness. The samples were frozen with liquid nitrogen, cut into small pieces, and vacuum dried overnight. The samples were then sputter-coated (Balzers Union 07120/135, Germany) with 10 nm of platinum/gold, and the images were recorded with a JEOL 6360 LV microscope (Tokyo, Japan) at 20–25 kV with different magnifications. The average scaffold thickness was measured at random sites through the analysis of cross-section, top-view, and bottom-view SEM images.

2.5. Mechanical testing

Uniaxial tensile tests were performed as previously described [16] on an Instron5542 testing frame (Norwood, MA, USA) equipped with a 1000 N capacity load cell and Biopuls pneumatic clamps. We randomly selected 6 pieces of BAMGs to evaluate the mechanical testing. The bilayer BAMG-SF ($N = 6$) were trimmed into rectangular strips (40 mm × 10 mm) and hydrated in PBS for at least 24 h to reach a swelling equilibrium prior to testing. Each material was cut into a dog-bone shape before testing. Test samples were submerged in a temperature-controlled testing container (Biopuls) filled with PBS (37 °C). Tensile testing was performed by clamping each prepared specimen into a custom fabricated tensile tester with a 50-N load cell. The displacement control mode with a crosshead displacement rate of 10 mm/min was used. The initial elastic modulus (EM), ultimate tensile strength (UTS), and % elongation to failure (ETF) were calculated from stress/strain plots. The EM was calculated using a least squares fit within the linear elastic region. The UTS was determined as the highest stress value attained during the test, and the %ETF was the last data point before the load decreased by more than 10%. Data were presented as the mean ± standard deviation.

2.6. Animals

Biomaterials were evaluated in a bladder augmentation model using adult male Sprague Dawley rats (8 weeks old) following IACUC-approved protocols, as previously described [20]. The

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