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Self-assembling peptide and nHA/CTS composite scaffolds promote bone regeneration through increasing seed cell adhesion

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ABSTRACT

Porous scaffolds fabricated with nano-hydroxyapatite (nHA) and chitosan (CTS), are widely used in bone tissue engineering (BTE). However, cell adhesion is relatively poor in nHA/CTS scaffolds, which also do not provide an ideal three-dimensional environment for seed cells. These deficiencies limit the applicability of these BTE scaffolds to repair bone defects. To address these challenges, we designed a composite scaffold that combines nHA/CTS with self-assembling peptide (SAP), a material which is similar to the extracellular matrix. We found that SAP/nHA/CTS scaffolds both increased the adhesion of bone mesenchymal stem cells (BMSCs) and enhanced the mechanical properties of the scaffold. This composite scaffold was then used to repair a femoral condylar bone defect in a mouse model. Healing and mineralization was demonstrated after 12 weeks using H&E staining, microcomputerized tomography, and bone mineral density tests. To our knowledge, this is the first report that SAP/nHA/CTS scaffolds can increase cell adhesion and promote the reconstruction of femoral condylar bone defects. Moreover, this study indicates that BTE using a SAP/nHA/CTS scaffold may be a novel prospective strategy for healing extensive bone defects.

1. Introduction

Repair and restoration of function in large bone defects caused by trauma and tumor are major clinical challenges in orthopedic management. The present gold standard treatment is transplantation of autogenous bone grafts [1]; however, its clinical application is limited due to potential risks including long-term chronic pain, nerve injury, and risk of new fractures [2–4]. Transplantation of allogenic bone grafts is often constrained due to the risk of immunologic rejection and disease transmission [5,6]. Bone tissue engineering (BTE) holds the potential to both avoid associated donor site morbidity and reproduce the precise facial contours necessary for aesthetic repair of the damaged region [7]. BTE may also overcome the problems inherent in autografts and allografts, providing new potential treatments for the repair of bone defects [8]. BTE has undergone substantial development over the last few decades [9]; however, it is still not widely used in clinical practice. One reason is that the seed cells in the scaffolds are limited and unevenly distributed, and only a portion of the cells in the scaffold participate in osteogenesis repair [10].

Previous approaches to improve cell adhesion and uniform cell distribution have focused on scaffold structure, mechanical properties, *etc.* [11–15]. These efforts increased cell adhesion by promoting fibrous protein accumulation to increase the surface area for seed cell adhesion. However, these strategies also inhibited vascularization and nutrient delivery [16]. Thus, it remains challenging to prepare an appropriate scaffold with large porosity, high mechanical strength, and an adequate cell adhesion rate for BTE.

Our previous study found that seed cells can survive in self assembling peptide (SAP), and directly involved in the repair of bone defects [10]. SAP, one type of Semi-solid hydrogel, is biocompatible and biodegradable, and spontaneously forms a three-dimensional (3-D) hydrogel scaffold when in contact with physiological media or salt solutions [17], and has been widely used in tissue engineering [18–21]. Although SAP can be easily made into various shapes and has been used

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to fill the critical size of bone defects in non-load-bearing areas, it does not hold the mechanical strength necessary to support load-bearing functions [7]. Conversely, nano-hydroxyapatite (nHA)/chitosan (CTS) scaffolds, which have strong mechanical properties and an interconnected porous structure, have been widely used in BTE [22–24]. However, nHA/CTS scaffolds share the common drawbacks of other scaffolds; that is, pores that are much larger than those present in the *in vivo* microenvironment, which restrains the cell adhesion. We reasoned that when combined, SAP and nHA/CTS might complement one another perfectly by increasing cell adhesion through the formation of a novel 3-D composite scaffold, thus promoting the regeneration of bone defects.

The essential requirements for 3D scaffold for bone tissue engineering should include interconnected pores network (allowing for vascularisation and nutrient transport), appropriate mechanical properties (to withstand *in vitro* and *in vivo* forces), biocompatibility and biodegradability, adequate surface chemistry and topography (enhancing cell adhesion and migration), and customised external shap (enabling an optimal biomechanical coupling at the implantation site) [25]. Therefore, this study aimed to combine SAP with nHA/CTS to form a new composite scaffold, and then evaluate the effects on BTE both *in vitro* and *in vivo* using bone marrow mesenchymal stem cells (BMSCs) as seed cells. Our *in vitro* experiments evaluated cell adhesion, mechanical properties, degradation rate, and osteogenic differentiation of the novel SAP/nHA/CTS composite scaffold in osteogenic conditions. The *in vivo* study then tested if SAP/nHA/CTS scaffolds improved induction of bone growth in and across a femoral condyle defect relative to traditional nHA/CTS scaffolds. We hypothesized that this novel composite scaffold would enhance the mechanical properties, increase the cell adhesion, and promote the regeneration of the femoral condyle defect.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma–Aldrich, unless otherwise stated. All the reagents were used without further treatment.

2.2. Harvest and culture of rat BMSCs (BMSCs)

All of our procedures were approved by the Southern Medical University Animal Care and Use Committee. BMSCs were harvested from the marrow of the femora of 4-week-old male Sprague Dawley rats as previously described [26], and the harvested BMSCs were then cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum. The medium was replaced every 3 days, and non-adherent cells were discarded. When the primary cultured cells reached approximately 90% confluence, the cells were passaged. After the second passage, cells were allowed to reach 90% confluence, and then were harvested and used for experiments.

2.3. Osteogenic differentiation of BMSCs in vitro

Osteogenic differentiation of P_3 BMSCs was induced at a cell density of 8×10^4 cells/coverslip in osteogenic medium composed of DMEM/F-12 (Corning, Guangzhou, China) supplemented with 10% (v/v) fetal bovine serum (Corning, Guangzhou, China), 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate. The differentiation medium was changed every 3 days. Fourteen days later, Alizarin Red S and alkaline phosphatase (ALP) staining was performed to evaluate osteogenic products as previously described [27]. Briefly, the induced cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After fixation, 2% (v/v) of Alizarin Red S and ALP staining solution were respectively added to fixed cells and incubated in the dark at 37 °C for 45 min. The staining solution was removed and washed three times with phosphate-buffered saline (PBS, Corning), and imaged using a microscope.

2.4. Fabrication of nHA/CTS scaffolds

The scaffolds were prepared *via* a freeze-drying method similar to published protocols [28]. Briefly, a 2 ± 0.05% CTS solution was prepared in 1% acetic acid with 1 ± 0.05% nHAP nanoparticles (nHAP-NH2 or nHAP). To crosslink the CTS matrix, 2% glutaraldehyde was added to the CTS solutions and stirred for 1 h. The mixtures were then placed into a disposable syringe, and frozen at −20 °C overnight. Frozen mixtures were then removed from the mold and lyophilized at −80 °C for 24 h to obtain porous scaffolds. The lyophilized scaffolds were then neutralized with 2% NaOH and 5% NaBr for 2 h, further washed with distilled water, and pre-frozen in the mold shelf at −20 °C overnight. Finally, the scaffolds were removed from the mold and stored in centrifuge tubes at −20 °C for further use.

2.5. Characterization of combined scaffolds

2.5.1. Preparation of SAP/nHA/CTS combined scaffolds

SAP, which is a commercialized hydrogel, consists of standard amino acids (1% w/v) and 99% water. Under physiological conditions, especially triggered by Ca^{2+}, the peptide component self-assembles into a 3D hydrogel with a nanometer-scale fibrous structure rapidly, which is very similar to extracellular matrix (ECM). However, the SAP can't be applied to bone tissue engineering alone because of its mechanical property is not enough to meet the need of bone tissue. We try to combine SAP with nHA/CTS to form a novel composite scaffold, which could provide a suitable micro-environment which is similar to ECM for seed cells, as well as has better mechanical properties than each of them at the same time. Specific methods are as follows, the nHA/CTS with large pore size prepared in the earlier period was placed in culture dish, nHA/CTS was washed fully with aseptic PBS, and dried under UV irradiation in Clean Bench. Then SAP was injected slowly to nHA/CTS at low temperature, and the culture dishes were immediately placed in the 37 °C incubator for half an hour so that SAP could fully self-assemble in nHA/CTS. Then the SAP/nHA/CTS was freeze dried and stored in special airtight containers.

2.5.2. Scanning electron microscopy (SEM)

Scaffold surfaces and inner morphologies were evaluated by scanning electron microscopy (SEM; S-3000N), using 10 nm gold-palladium coating as previously reported [29]. For SAP/nHA/CTS scaffolds, SAP was injected into the nHA/CTS scaffold on the ice surface. The composite scaffolds, SAP/nHA/CTS and nHA/CTS, were freeze-dried prior to scanning electron microscopy after the SAP assembled.

2.5.3. Porosity measurement

A liquid displacement method was used to determine the porosity percentage in each sample. Porosity (ε%) was evaluated through subtraction of scaffold weight in the wet (Ww) and dry (Wd) state. For Ww measurements, scaffolds were immersed in absolute ethanol until reaching saturation, then weighed. The overall porosity of the scaffold was determined using the following equation, where ρ refers to the alcohol density (g/cm^3), and V to volume before immersion.

$$\varepsilon\% = (Ww - Wd)/\rho V \times 100$$

2.5.4. Mechanical testing

The compressive strength and the modulus were tested using a Zwick/Roell machine according to the ASTM D5024-95a protocol. The specimens were cylinders with a 4 mm diameter and 5 mm thickness. Samples were immersed in PBS for 12 h prior to mechanical testing, which was carried out at a crosshead speed of 0.5 mm/min. The modulus value was determined from the linear region of the acquired