Biomedicine & Pharmacotherapy 97 (2018) 600–606

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Composite scaffolds loaded with bone mesenchymal stem cells promote the repair of radial bone defects in rabbit model

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ARTICLE INFO

Keywords:
Silk fibroin
Chitosan
Nano-hydroxyapatite
Bone marrow derived mesenchymal stem cells
Osteogenesis
Bone defect

ABSTRACT

This study aimed to investigate the efficacy of three-dimensional scaffolds of silk fibroin/chitosan/nano-hydroxyapatite (SF/CS/nHA) and bone marrow derived mesenchymal stem cells (BMSCs) on the repair of long segmental bone defects in rabbits. BMSCs were cultured with SF/CS/nHA in vitro, and cell proliferation, alkaline phosphatase activity and $Ca2+$ content were examined. A 15 mm segmental defect in the radius was generated in 12 New Zealand White rabbits, which were divided randomly into three groups ($n = 4$): experimental group with SF/CS/nHA scaffold of induced BMSCs; control group with SF/CS/nHA scaffold; and blank group without any materials. Postoperatively at 12 weeks, osteogenesis effect and the degradation and absorption of SF/CS/nHA were evaluated by X-ray, hematoxylin eosin staining, and scanning electron microscopy. In vitro, SF/CS/nHA scaffolds exhibited good biocompatibility and no toxicity. SF/CS/nHA promoted adhesion, growth, and calcium nodule formation of BMSCs compared to control ($P < 0.05$). In vivo, we observed gradual new bone formation and bone defect gradually recovered at 12 weeks in experimental and control group, but more new bone was formed in experimental group ($P < 0.05$). In blank group, limited bone formation was observed and bone defect was obvious. In conclusion, SF/CS/nHA scaffolds loaded with BMSCs achieve high efficacy to repair segmental defect in the radius.

1. Introduction

Large segmental bone defect remains a big problem for orthopedic surgeons, and the best choice of treatment is autologous bone graft at present [1]. However, this procedure has many disadvantages such as trauma surgery and pain. In addition, limited sources for the graft and complications associated with obtaining the bone prevent the wide application [2]. Although allograft bone grafts and xenograft bone grafts overcome the disadvantages of autogenous bone grafts, they pose the strong possibility of a rejection reaction and the potential spreading of disease [1,3].

Recently, considerable efforts have been focused on materials with physicochemical properties similar to natural bone that have good biological compatibility and activity and induce osteogenesis. These bone graft materials must contain two basic components: (1) bone mineral, namely calcium phosphate hydroxyapatite; and (2) organic matter such as the main collagen fiber and osteogenesis growth factor [1], and include hydroxyapatite (HA), nano-hydroxyapatite (*n*-HA), calcium phosphate, chitosan and artificial polymers. HA is a good alternative material for bone tissue repair due to good biocompatibility and osteogenic activity [4,5]. However, there are both advantages and disadvantages associated with all kinds of single scaffolds used for bone tissue repair. Therefore, it is important to adjust the proportion of material and the corresponding combination to create ideal scaffold materials [6]. BudiRaharjo et al. used chitosan scaffold material coated with nano-hydroxyapatite cultured with stem cells and demonstrated good biocompatibility and osteogenesis induction ability [7]. Da Silva et al. described composite scaffolds composed of chitosan and silk fibroin cultured with bone marrow derived stem cells (BMSCs) in vitro, and they found that these scaffolds could induce cartilage cell differentiation [8].

Wang et al. reported that biomimetic bone substitutes of collagen-silk fibroin/hydroxyapatite exhibited good biocompatibility and strong ability to stimulate new bone formation [9]. Yang et al. prepared nanocomposites using silk sericin and hydroxyapatite crystals, and showed that the nucleation of hydroxyapatite crystals mediated by silk sericin promoted osteogenic differentiation [10,11]. Therefore, nanocomposites composed of silk fibroin and hydroxyapatite show promise in tissue engineering.

In this study, three-dimensional scaffolds of silk fibroin/chitosan/nano-hydroxyapatite (SF/CS/nHA) alone or in combination with BMSCs were used to repair radial bone defects in rabbit model. SF/CS/

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http://dx.doi.org/10.1016/j.biopha.2017.10.110
Received 8 September 2017; Received in revised form 17 October 2017; Accepted 21 October 2017
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nHA was evaluated in vitro and in vivo for the ability to repair bone defect.

2. Materials and methods

2.1. Fabrication of three-dimensional composite scaffold materials

Silk fibroin, chitosan, and nano-hydroxyapatite (Sigma, St. Louis, MO, USA) were dissolved in 2% solution and then mixed at a volume ratio of 1:1:1 with constant stirring at 55 °C. The solution was then quickly injected into an orifice plate of 9 holes using a 20-ml syringe and frozen at −80 °C for 24 h. The frozen scaffolds were sealed quickly with sealing membrane, which was suctioned using a vacuum dryer for 36 h after creating scaffolds with pores in the corresponding area. After the dried scaffolds were stored in a 75% volume fraction of methanol and 1 mol/L sodium hydroxide, they were again dried in a vacuum after washing with ultrapure water. The scaffolds were soaked for 10 h in EDC and NHS crosslinking agent (Sigma, St. Louis, MO, USA) after the wet composite scaffolds were frozen at −80 °C, dried for 36 h under a vacuum. The prepared three-dimensional composite scaffolds were sealed for storage and then used in the applications [12]. The wafer-shaped SF/CS/nHA materials were prepared and physical and chemical properties were evaluated at Sichuan University. All materials were sterilized during the preparation.

2.2. Culture and identificationof BMSCs

Rabbit BMSCs were provided by Cyagen Biosciences Inc. (Guangzhou, China). Flow cytometry showed molecular markers as follows : CD90: 90.64%, CD45: 1.24%, CD14: 0.36%. The frozen BMSCs were thawed by rapid thawing method, followed by incubation at a density of 1×10^4 cells/ml at 37 °C in 5% CO_2 and 95% air. Third generation cells in logarithmic phase were selected as the test cells, and osteoblasts were identified by direct morphological observation under an inverted phase contrast microscope and Alizarin red staining of mineralized nodules.

SF/CS/nHA scaffolds were washed with phosphate-buffered saline (PBS), and then placed in 24-well flat bottom tissue culture plates. BMSCs were seeded at 3×10^4 cells/well and cultured for 10 days.

2.3. Scanning electron microscopy

SF/CS/nHA scaffolds were rinsed and placed into culture plates, BMSCs were then inoculated and cultured. On days 5 and 10 the scaffolds seeded with BMSCs were harvested, fixed by 2.5% glutardialdehyde for 1 h and then dehydrated by graded ethanol (50, 70, 90, 95, 100%). The samples were coated with gold and the morphologies of BMSCs on the surface of variable scaffolds were observed under scanning electron microscope at 20 kV (JEOL JSM-5910, Japan).

2.4. MTT assay

BMSCs were seeded in 96-well plates at 4×10^3 cells/well and allowed to attach for 4 h. The cells were cultured for 14 days. During the period, the cells were labeled with MTT (Sigma) and cell proliferation was detected by MTT assay. Microplate reader (Perkin Elmer HTS7000plus, USA) was used to determine the absorbance value of cultured cells under the wavelength of 570 nm.

2.5. Assay of alkaline phosphatase (ALP) activity and Ca^{2+} content

BMSCs were digested with 0.25% trypsin for 5 min, and then collected by centrifugation followed by treatment with Triton-X. By freezing and thawing the cells three times, the lysates were collected and centrifuged at 15,000 rpm at 4 °C for 15 min. ALP activity and Ca^{2+}content in the supernatants were determined by colorimetric kits (Abcam, Cambridge, MA, USA) following the manufacturer's protocols.

2.6. Construction of animal model of radial bone defect

Animal experiments were approved by the Animal Care Committee of the First People's Hospital of Zunyi City (Zunyi, China). New Zealand White rabbits (4 months old, weighed 2.5–3.0 kg) received intravenous injection of pentobarbital (30 mg/kg). In the middle radius, 3 cm long longitudinal incision was generated to expose the radial diaphyseal, and 15 mm long radial cut was produced with a micro-oscillating saw to cause radial defect. The rabbits were then divided randomly into three groups (n = 4): experimental group (treated with $15 \times 3 \times 3\ mm^3$ scaffold consisting of BMSCs), control group (treated with 15 × 3 × 3 mm [3] scaffold) and blank group (no scaffold). After the closing of the wounds with sutures, the rabbits received intramuscular injection of penicillin at 50 U/kg for 3 days to prevent possible infection.

2.7. Radiographic examination

On day 1 and weeks 4, 8, and 12 postoperatively, all models were given X-ray on the surgical area, with uniform exposure conditions to evaluate osteotylus growth and bone defect healing at 42 kV, 100 mA/s, 1.2 m. The images were evaluated for SF/CS/nHA material absorption and degradation, bone formation and bone defect recovery based on Lane and Sandhu scoring system [13].

2.8. Histological examination

Postoperative 12 weeks, four rabbits were sacrificed in each group. Then samples were collected, including 1 cm of the bone surrounding the scaffolds, to be fixed in 10% formalin, decalcified, embedded in paraffin, and cut into 5 μm sections. The sections were then stained with collagen type I antibody (Abcam, Cambridge, MA, USA) and counterstained with hematoxylin and eosin (H & E). New bone growth and collagen expression in the interface between the scaffold and host bone were analyzed under microscopy.

In addition, the heart, liver, lung, and kidney tissue samples were collected, fixed in 10% formalin, embedded in paraffin, and cut into sections for H & E staining. All sections were evaluated independently by two pathologists in a blind manner.

2.9. Statistical analysis

All data were expressed as the mean ± standard deviation and analyzed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). The differences among the groups were analyzed by two-way ANOVA followed by post-hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Morphology of BMSCs cultured with SF/CS/nHA

BMSCs were cultured with SF/CS/nHA materials for different periods and observed under inverted microscope. BMSCs grew significantly better in experimental group compared with blank group (Fig. 1A and B). In addition, we observed increased number of cells and nuclei, and two to three nuclei in some cells. Thus SF/CS/nHA had good biocompatibility and could induce osteogenesis.

Scanning electron microscopy showed that three-dimensional composite scaffolds were porous. The pore connectivity of the internal scaffolds was good, with an aperture of 100–300 μm and a porosity of 90.18 ± 0.96% (Fig. 1C and D). BMSCs in three-dimensional composite scaffolds were fusiform or polygon shaped. In addition, we observed a network structure, indicating the adhesion and growth of BMSCs on the material surface. We also observed the formation of

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