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In vitro and *in vivo* evaluation of calcium phosphate composite scaffolds containing BMP-VEGF loaded PLGA microspheres for the treatment of avascular necrosis of the femoral head

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ABSTRACT

Avascular necrosis of the femoral head (ANFH) is difficult to treat due to high pressure and hypoxia, and reduced levels of growth factors such as bone morphogenetic protein (BMP), and vascular endothelial growth factor (VEGF). We generated a novel calcium phosphate (CPC) composite scaffold, which contains BMP-VEGF-loaded poly-lactic-co-glycolic acid (PLGA) microspheres (BMP-VEGF-PLGA-CPC). The BMP-VEGF-loaded microspheres have an encapsulation efficiency of 89.15% for BMP, and 78.55% for VEGF. The BMP-VEGF-PLGA-CPC scaffold also demonstrated a porosity of 62% with interconnected porous structures, and pore sizes of 219 μm and compressive strength of 6.60 MPa. Additionally, bone marrow mesenchymal stem cells (BMSCs) were seeded on scaffolds *in vitro*. Further characterization showed that the BMP-VEGF-PLGA-CPC scaffolds were biocompatible and enhanced osteogenesis and angiogenesis *in vitro*. Using a rabbit model of ANFH, BMP-VEGF-PLGA-CPC scaffolds were implanted into the bone tunnels of core decompression in the femoral head for 6 and 12 weeks. Radiographic and histological analysis demonstrated that the BMP-VEGF-PLGA-CPC scaffolds exhibited good biocompatibility, and osteogenic and angiogenic activity *in vivo*. These results indicate that the BMP-VEGF-PLGA-CPC scaffold may improve the therapeutic effect of core decompression surgery and be used as a treatment for ANFH.

1. Introduction

Avascular necrosis of the femoral head (ANFH) is a pathological disease caused by trauma, hormones, long-term alcohol poisoning, and decompression sickness [1,2]. The disease leads to an impaired microcirculation of the femoral head, resulting in reductions in blood supply and necrosis of the femoral head, and activation of a repair response. This repair response includes vascular regeneration, new bone formation, and dead bone resorption [3]. However, the capacity for vascular regeneration and new bone formation is significantly impaired due to high blood pressure, hypoxia, and lack of growth factors such BMP and VEGF, which leads to weakened mechanical strength of the femoral head [4,5]. Clinical symptoms of AFNH often present as deep-seated groin pain and symptomatic osteonecrosis that collapses in untreated patients.

There are currently a number of unmet medical needs for the treatment of femoral head necrosis [4]. First, additional medical intervention for osteonecrosis is needed to provide biomechanical support and strength due to necrosis of subchondral bone and osteoporosis caused by avascular necrosis. Second, the delivery of exogenous growth factors is needed for bone regeneration, which remains incapacitated because of hypoxia and high blood pressure. Current clinical treatment of femoral head necrosis primarily includes conservative and surgical treatment. The treatment goal of conservative therapy includes decreasing avascular necrosis of femoral head and number of patients requiring hip replacement. Additionally, the treatment aims of surgery include core decompression, bone grafting, osteotomy, femoral head replacement, and total hip replacement. Core decompression is a classical method for the treatment of early ANFH [1,6]. According to a survey of American hip and knee surgeons, core decompression is most commonly used for treatment of femoral head osteonecrosis and intervention before the collapse in the necrosis of the femoral head in younger patients. However, after core decompression, the mechanical strength of femoral head is reduced within a short period which is not conducive in restoring the load function that may cause femur fractures. Surgery on subchondral bone necrosis of the femoral head could provide immediate support, which could improve the success rate of core decompression for patients with femoral head necrosis.

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In recent years, rapid progress has been made in bone tissue engineering, and may provide a new treatment approach for ANFH. Studies [4,5,7,8] have shown that new bone repairing and vascular regeneration in avascular osteonecrosis of the femoral head are related to several growth factors such as BMP and VEGF. In recent years, biodegradable polymers have been used in a variety of biomaterial applications. Calcium phosphate cement (CPC) has good biocompatibility, and osteoconductive, and biodegradable properties, which can serve as an effective carrier of various bioactive factors. Meanwhile, poly(lactic-co-glycolic acid) (PLGA) microspheres have been extensively used for controlled-release drug delivery. Therefore, we propose that a scaffold consisting of growth factors (BMP and VEGF) mixed with CPC bone cement via PLGA microspheres may improve the effectiveness of core decompression, restore the load function instantly, and decrease the progression of osteonecrosis of the femoral head. In this study, such scaffolds were implanted in the bone tunnel via a catheter after core decompression surgery, which can provide immediate support. More importantly, the controlled release of growth factors in the scaffold can adopt roles such as inducing osteogenesis and facilitate angiogenesis, which improve blood supply, and promote the new bone formation in the necrotic intertrabecular spaces of the femoral heads. In this study, we demonstrate whether this composite scaffold could be used for treatment of avascular necrosis of femoral head. Furthermore, studies that assessed the properties of the scaffolding materials, as well as biocompatibility, osteogenesis and angiogenesis, both *in vitro* and *in vivo*, will be performed.

2. Materials and methods

2.1. Materials

PLGA (Medisorb, Lakeshore Biomaterials, Birmingham, AL, USA) with a lactic to glycolic acid ratio of 50:50, and a weight-average molecular weight (Mw) of 22,000 Da, poly vinyl alcohol (PVA) (Mw = 13,000–23,000, Sigma, St. Louis, MO, USA) and isopropanol solution (Sigma) were used for the microsphere preparation. BMP-2 and VEGF-165 were purchased from PeproTech (Rocky Hill, NJ, USA). The CPC powder consisted of a mixture of 80% beta-tricalcium phosphate (β-TCP), 10% dicalcium phosphate anhydrous (DCPA), 5% precipitated hydroxyapatite (pHA) and 5% nanometer hydroxylapatite (nHA).

2.2. Fabrication process of microspheres and scaffolds

The various growth factors loaded and unloaded microspheres were prepared using solid/oil/water (s/o/w) emulsion solvent evaporation method previously reported with slight modification [9,10]. The fabrication process was performed under sterile conditions and all solution was filtrated using a 0.22 μm filter.

Briefly, according to preliminary results of encapsulation efficiency, 100 μl of a bovine serum albumin (BSA, 1 mg/ml) solution, 100 μl of a BSA (1 mg/ml) solution containing 13.88 μg of BMP-2, 100 μl of a BSA (1 mg/ml) solution containing 2.69 μg of VEGF-165, 100 μl of a BMP-2/VEGF-165 BSA (1 mg/ml) solution (containing 13.46 μg of BMP-2 and 2.55 μg of VEGF-165) was emulsified in a solution of 500 mg of PLGA in 1.25 ml of dichloromethane, respectively. The mixture water–oil emulsion was re-emulsified for 30 s in 2 ml of 1% *w*/*v* aqueous poly (vinyl alcohol) solution to create the double emulsion and then added into 100 ml of a 0.3% *w*/*v* aqueous PVA solution and 100 ml of a 2% *w*/*v* aqueous isopropanol solution with stirring at 800–1000 rpm for 5 h to evaporate the solvent. After the dichloromethane was completely eliminated, the composite microspheres were collected by centrifugation, washed three times with double distilled water, and finally were lyophilized on ATR FD 3.0 system (ATR Inc., Saint Charles, MO, USA) and stored until further use at −20 °C.

The BMP-, VEGF-, BMP-VEGF-loaded and unloaded PLGA microspheres and CPC powder composed of solid phase with a volume ratio of 50/50 were mixed with curing liquid by 3:1(g/ml) ratio at room temperature. The solution was mized into a paste and then injected into a mold with diameter of 4 mm and length of 15 mm. All scaffolds (pure PLGA-CPC scaffolds, BMP-PLGA-CPC scaffolds, VEGF-PLGA-CPC scaffolds as well as BMP-VEGF-PLGA-CPC scaffolds) were lyophilized and stored until further use at −20 °C.

2.3. Characterizations of microspheres and scaffolds

2.3.1. Encapsulation efficiency

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the encapsulation efficiency of the BMP-, VEGF-, and BMP-VEGF-loaded PLGA microspheres. The BMP and VEGF ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The supernatants of each loaded microsphere were collected and used to determine the amounts of BMP and VEGF that were not encapsulated in microspheres according to the manufacturer's instructions. The differences between the amount of BMP and VEGF employed for the microsphere preparation and the amount of BMP and VEGF measured in the microsphere supernatant represented the amount of BMP and VEGF loaded into the microspheres. The encapsulation efficiency was presented as the percentage of BMP and VEGF loaded into the microspheres to the total amount of them added during the formulation of the microspheres [11].

2.3.2. Scanning electronic microscopy

The morphology and microstructure of the scaffold were determined by scanning electronic microscopy (SEM, JSM-6390, JEOL, Tokyo, Japan). Prior to observation, scaffolds were soaked in dichloromethane for the dissolve of PLGA microspheres.

2.3.3. Micro-computed tomography

Micro-computed tomography (μCT, μCT 80, Scanco Medical AG, Basserdorf, Swiss) was used to determine the morphological structure of the scaffolds before PLGA microspheres degradation. Scaffolds were scanned at energy of 3.0 kV and intensity of 98 mA at a resolution of 2.26 mm pixel without filter. The data were automatically assessed by a CT analyzer. The region of interest (ROI), in which porosity was measured, was selected as a 2 mm × 2 mm × 2 mm cube.

2.3.4. Mechanical property

The compressive strength of the scaffolds were conducted using an RGD-4005 Test Instruments (Shenzhen, Guangdong, China) at a loading rate of 0.01 mm/s. Five replicates were used for each group, and the load was applied until the scaffold was compressed to approximately 50% of its original length.

2.4. In vitro evaluation of biocompatibility, osteogenesis and angiogenesis of the scaffolds

2.4.1. Cell culture

The study was performed in strict accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care, and approved by the Committee on the Ethics of.

Animal Experiments of the Shandong University (Jinan, Shandong, China). Briefly, bone marrow aspirates were collected under aseptic conditions from the tibia and femur condyle of 4 anesthetized New Zealand rabbits (0.80 kg, 1 month old, male). MSCs were isolated by Percoll (Sigma, St. Louis, MO, USA) density-gradient centrifugation. The cell pellet was resuspended in culture medium, which consisted of 89% DMEM/F12 (HyClone, Logan, Utah, USA), 10% fetal bovine serum (FBS, HyClone), and 1% penicillin/streptomycin/amphotericin B (Sangon Biotech, Shanghai, China).

All cells were cultured at 37 °C, 5% CO_2 and 95% humidity. The culture medium was replaced every 2 days, and the cells were subcultured using trypsin digestion method at 90% confluence. Cells at passage 3 were used for the following experiments.