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Comparative study on *in vivo* response of porous calcium carbonate composite ceramic and biphasic calcium phosphate ceramic

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ABSTRACT

In a previous study, robust calcium carbonate composite ceramics (CC/PG) were prepared by using phosphate-based glass (PG) as an additive, which showed good cell response. In the present study the *in vivo* response of porous CC/PG was compared to that of porous biphasic calcium phosphate ceramics (BCP), using a rabbit femoral critical-size grafting model. The materials degradation and bone formation processes were evaluated by general observation, X-ray radiography, micro-computed tomography, and histological examination. The results demonstrated excellent biocompatibility and osteoconductivity, and progressive degradation of CC/PG and BCP. Although the *in vitro* degradation rate of CC/PG was distinctly faster than that of BCP, at 4 week post-implantation, the bone generation and material degradation of CC/PG were less than those of BCP. Nevertheless, at postoperative week 8, the increment of bone formation and material degradation of CC/PG was pronouncedly larger than that of BCP. These results show that CC/PG is a potential resorbable bone graft aside from the traditional synthetic ones.

1. Introduction

Orthopedic surgery commonly deals with bone defects arising from trauma, tumor resection, and infections [1,2]. Small-sized bone defects can heal over time, whereas large bone defects are difficult to repair without bone grafting [1]. Aside from autografts and allografts, synthetic bone grafts are regarded as an effective choice to repair bone defects [3]. In principle, the qualified synthetic bone grafts should be able to support and stimulate tissue regeneration; furthermore, the *in vivo* resorption rate of bone grafts should match the rate of new bone formation [4].

Calcium phosphate (CaP) possesses outstanding biocompatibility and osteoconductivity, due to its chemical similarity to the bone mineral. CaP ceramics, especially hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and HA/β-TCP biphasic calcium phosphate (BCP) ceramics, are nowadays the primary synthetic bone grafts used in clinics [5]. HA ceramics have relatively slow resorption rate but possess higher mechanical strength compared to other CaP ceramics. On the contrary, β-TCP is a more bioresorbable material but has lower mechanical strength. The degradation rate and mechanical strength of BCP can be modulated by varying the ratio of HA to β-TCP, which can meet the requirements of various implantation sites [6].

Calcium carbonate has excellent biocompatibility and osteoconductivity, and its degradation rate is lower than that of CaP [7–9]. Up to now, considerable attention has been paid to developing synthetic calcium carbonate biomaterials as bone grafts. The difficulty of preparing a calcium carbonate ceramic is that it readily decomposes to CaO and CO_2 between 600 °C and 700 °C [10]. Even so, calcium carbonate ceramics have been successfully prepared with various approaches; these include compacting of calcium carbonate powders, carbonation of $Ca(OH)_2$, adding a sintering agent with lower melting temperature, and sintering under a CO_2 atmosphere [11–15]. Phosphate-based glass (PG) is nontoxic, degradable, and its physical properties (melting temperature, and solubility) can be readily adjusted by changing the content of P_2O_5 and metal oxides [16–18]. PG has been deemed a bioabsorbable biomaterial; therefore, it is used for several biomedical applications, such as blood contacting materials, nerve guidance channels, and reinforced fillers for biopolymers [19–21].

In a previous study, calcium carbonate composite ceramics (CC/PG) were fabricated using a newly developed method, that is fast sintering calcium carbonate at a low temperature (650 °C) using a biocompatible, biodegradable phosphate glass (PG) with low-melting temperature as a sintering agent. The obtained CC/PG had starkly improved compressive strength compared to the neat calcium carbonate ceramics [22]. The *in vitro* degradation rate of CC/PG was significantly faster than that of CaP ceramics (HA and β-TCP). Moreover, the cell response to CC/PG was excellent. In fact, compared to HA and β-TCP, the rat mesenchymal stem cells (rMSCs) cultured on CC/PG had weaker proliferation and expression of early markers of osteogenic differentiation, but expressed

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higher level of osteopontin [23].In this study, a comparison of the *in vivo* response of CC/PG and BCP (HA/β-TCP) is reported.

2. Materials and methods

2.1. Study design

The first step of the study was the preparation of the porous calcium carbonate composite ceramics (CC/PG) and of the porous biphasic calcium phosphate ceramics (BCP). Subsequently, a study of their phase composition, structure and property was performed. Finally, the *in vivo* response of porous CC/PG and BCP was investigated, by implanting them in critical-size femoral defects of rabbits; the performance of the two ceramics was compared.

2.2. Materials

BCP (HA/β-TCP, <20 μm) powders were kindly provided by South China University of Technology, China. The calcium carbonate powders (<150 μm) were purchased from Guilin Kaiwen Calcium Carbonate Material Co. Ltd., China. $(NH_4)_2HPO_4$, $MgCO_3$, Na_2CO_3, and NaCl were purchased from Tianjin Fuchen Chemical Reagent Factory, China. All the commercial chemicals were analytically pure.

2.3. Preparation of porous CC/PG and BCP ceramics

The method for preparing PG ($50P_2O_5 \cdot 18CaO \cdot 12MgO \cdot 20Na_2O$) was previously described in detail [22]. Briefly, the mixtures of $(NH_4)_2HPO_4$, $CaCO_3$, $MgCO_3$, and Na_2CO_3 were heated at 1000 °C to form glass liquid. The glass liquid was then poured into deionized water. The collected glass pellets were dried at 85 °C, milled for 15 h. The PG powders with a median diameter of 4.5 μm were obtained. The calcium carbonate and PG powders, and NaCl porogen (100–400 μm) were uniformly mixed at a mass ratio of 3: 2: 5. The mixtures were placed into the molds, uniaxially pressed at 10 MPa by a powder pressing machine (769YP-24B, Tianjin Keqi, China), then demolded. The obtained rectangular samples (7 mm × 7 mm × 45 mm) were cold-isostatically pressed using a cold isostatic pressing machine (LDJ100/320–300, Western Sichuan Machinery Co., Ltd., China) under a pressure of 200 MPa for 2 min. The alumina crucibles containing green bodies were heated in air at 650 °C, using a heating rate of 5 °C min^{-1}; they were held at 650 °C for 20 min, and then cooled down to room temperature naturally.

BCP powders were uniformly mixed with NaCl porogens at a mass ratio of 55: 45. The green bodies of BCP were obtained using the same protocol described for the CC/PG; they were then sintered in air at 1100 °C for 2 h (heating rate: 2 °C min^{-1}), and finally cooled down to room temperature naturally.

The obtained rectangular samples of CC/PG and BCP were sectioned into 5 separate parts (7 mm × 7 mm × 9 mm), and then polished to produce cylindrical samples (Φ 6 mm × 7 mm). Subsequently, the CC/PG and BCP samples were placed into boiled deionized water, ultrasonically treated to eliminate the NaCl porogens, and then dried at 85 °C. Finally, the porous CC/PG and BCP ceramics for *in vivo* implantation were obtained.

2.4. Materials characterization

2.4.1. Phase analysis

The CC/PG and BCP ceramic samples were crushed into powders. The phase composition of powders was analyzed by an X-ray diffractometer (XRD, X'Pert PRO, PANalytical Co., the Netherlands) employing CuKα radiation (40 kV, 40 mA). Data were collected for 2θ from 10° to 70° with a step size of 0.0166°.

2.4.2. Compressive strength test

The compressive strength of the samples (6 mm × 6 mm × 12 mm) was determined by a universal material testing machine (Instron 5567, Instron, Britain) at a crosshead speed of 0.5 mm min^{-1}. Each measurement was performed six times.

2.4.3. Pore structure characterization

The morphology of pore architecture of CC/PG and BCP was imaged using a micro-computed tomographic (micro-CT) imaging system (Skyscan1172, Bruker, Germany). The voltage and current of the scanner were set at 80 kV and 100 mA, respectively. Isotropic slice data were acquired by the system and reconstructed to two-dimensional (2D) images by the equipment software. Subsequently, the 2D images were reconstructed to three-dimensional (3D) images. The macroporosity of porous CC/PG and BCP ceramics was obtained by subtracting the higher grey thresholds which corresponded to the materials.

2.5. In vitro degradation profiles

The *in vitro* degradation profiles of CC/PG and BCP ceramics were assessed by measuring the weight loss of samples during immersion in the Tris–HCl solution (pH 7.4, 37 °C). The samples were immersed in the solution with a surface area to solution volume ratio of 0.1 cm^{-1}. The solutions were renewed every week. At selected times, the samples were taken out of solution, rinsed with deionized water three times, dried at 85 °C for 16 h and then weighed. The weight loss (WL) was calculated as follows:

$$WL\% = [(W_0 - W_d)/W_0] \times 100\%$$

where W_0 and W_d denote the weight of the samples before and after the scheduled immersion time, respectively. Each measurement was performed six times.

2.6. In vivo assessments

2.6.1. Surgical procedure

The experimental protocol followed was approved by the Ethics Committee of Animal Research at the Guangzhou Medical University. Twenty-four female New Zealand white rabbits (Guangdong Medical Lab Animal Center, China) between four and five months old, with a weight of 2.5–3.0 kg, were used. The rabbits were randomly divided into two groups: Group I was implanted with BCP; Group II was implanted with CC/PG. The whole surgical procedure was performed under general aseptic conditions. The rabbits were anesthetized with an intramuscular injection of midazolam (0.10 mg per kg body weight) and subsequent ear-intravenous injection of 3% pentobarbital sodium solution (15 mg per kg body weight). A longitudinal skin incision of 2.5 cm was made on the distal end of right rabbit femur, then the subcutaneous tissue, anadesma, muscle and periosteum were dissected layer by layer, until the bone was exposed. A critical size defect (Φ6 mm × 7 mm) in the femur was created with a drill. After the bleeding stopped, either CC/PG or BCP cylindrical samples were placed into the defects. The surgical incision was closed layer by layer. Postoperatively, the rabbits moved freely. The rabbits were intramuscularly injected with cefradine (25 mg per kg body weight) once a day, for three days. The general health condition of the rabbits was examined daily throughout the study.

2.6.2. Radiographic analysis

At 4 and 8 weeks postoperatively, the rabbits were sacrificed by injecting an overdose of pentobarbital sodium solution, whose concentration was over 10 times the regular anesthetic dose. The femora were harvested and imaged by camera (D5100, Nikon, Japan). Afterwards, the harvested femora were examined by an imaging radiographic system (ZU-L3TY, Yoshida, Japan) at 41 kV and 100 mA. Finally, the