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In vitro degradation and cell response of calcium carbonate composite ceramic in comparison with other synthetic bone substitute materials

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ABSTRACT

The robust calcium carbonate composite ceramics (CC/PG) can be acquired by fast sintering calcium carbonate at a low temperature (650 °C) using a biocompatible, degradable phosphate-based glass (PG) as sintering agent. In the present study, the in vitro degradation and cell response of CC/PG were assessed and compared with 4 synthetic bone substitute materials, calcium carbonate ceramic (CC), PG, hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) ceramics. The degradation rates in decreasing order were as follows: PG, CC, CC/PG, β-TCP, and HA. The proliferation of rat bone mesenchymal stem cells (rMSCs) cultured on the CC/PG was comparable with that on CC and PG, but inferior to HA and β-TCP. The alkaline phosphatase (ALP) activity of rMSCs on CC/PG was lower than PG, comparable with β-TCP, but higher than HA. The rMSCs on CC/PG and PG had enhanced gene expression in specific osteogenic markers, respectively. Compared to HA and β-TCP, the rMSCs on the CC/PG expressed relatively lower level of collagen I and runt-related transcription factor 2, but showed more considerable expression of osteopontin. Although CC, PG, HA, and β-TCP possessed impressive performances in some specific aspects, they faced extant intrinsic drawbacks in either degradation rate or mechanical strength. Based on considerable compressive strength, moderate degradation rate, good cell response, and being free of obvious shortcoming, the CC/PG is promising as another choice for bone substitute materials.

1. Introduction

Bone defects owing to trauma, disease, or congenital defect, etc., are a worldwide problem [1]. Autografts and allografts have been widely used to repair the bone defects in clinic, but they are faced with disadvantages such as donor site morbidity, limited source, and disease transmission, etc. [2,3]. The advancement of bone defect repair is in sore need of developing efficient synthetic bone grafts due to the well-known drawbacks of autografts and allografts. The inorganic component of natural bone is hydroxyapatite (HA). HA and related calcium phosphates, especially β-tricalcium phosphate (β-TCP), possess excellent biocompatibility and osteoconductivity. Calcium phosphate ceramics are the most popular clinical and experimental synthetic bone repair materials [4,5]. However, in general the resorption rate of calcium phosphate ceramics is relatively slow. In particular HA ceramic, which is the most common synthetic bone graft, will remain at the implantation site for several years [6,7]. The slow resorption rate of implants inhibits their replacement by new bone tissues and the ultimate bone defect repair.

Calcium carbonate is one of the most common minerals on the earth. Calcium carbonate crystallizes into three different polymorphs: calcite, aragonite, and vaterite. The aragonite-type calcium carbonate is the main composition of marine coral which has been extensively used to repair bone defects in clinic [8–10]. It was claimed that proliferation and protein expression of human bone marrow cells on the aragonite were comparable with those on the calcite [11]. Calcium carbonate has excellent biocompatibility and faster resorption rate than calcium phosphate [12,13]. Nevertheless, the coral bone grafts are confronted with disadvantages such as severe environmental problems, inflammatory reaction risk, and biological variation, etc. [13]. Hence, development of calcium carbonate ceramic as bone graft is of great significance. The chief obstacle of fabricating calcium carbonate ceramic is the difficulty in sintering calcium carbonate which is liable to decompose to calcium oxide (CaO) and carbon dioxide (CO_2) between 600 °C and 700 °C [14]. Even so, calcium carbonate ceramics can be acquired by various methods such as sintering under the CO_2 atmosphere, carbonation of calcium hydroxide compacts, introducing lithium carbonate as sintering additive, etc. [15–17].

Phosphate-based glass (PG) is nontoxic, hydrolytically degradable and its physical properties (degradation rate, melting temperature, etc.) are facilely adjusted to meet requirements of various applications [18,19]. Absorbable PG can be used as blood contacting materials [20], nerve guidance channel [21], and reinforcement phase for composite materials [22], etc. In the degradation process, PG releases ions such as Ca^{2+}, PO_4^{2-}, et al., benefiting bone regeneration and reconstruction [23].

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Despite the fact that calcium carbonate and PG are endowed with various merits and considered as promising synthetic bone substitutes, the biological evaluation of calcium carbonate ceramic and PG and their comparison with customary synthetic bone grafts are rarely studied. In a previous study, we fabricated calcium carbonate composite ceramic (CC/PG) at a low temperature (650 °C) by introducing PG with low melting temperature as sintering agent [24]. The CC/PG acquired significantly improved compressive strength compared to calcium carbonate ceramic (CC) without introducing a sintering agent. Herein, we aimed to assess the potential of CC/PG as bone graft by conducting in vitro degradation and cell response (cell attachment, viability, proliferation, cytoskeleton, ALP activity, and bone-related gene expression) of CC/PG in comparison to CC, PG, HA and β-TCP ceramics.

2. Materials and methods

2.1. Materials

Hydroxyapatite (HA, <60 μm) and β-tricalcium phosphate (β-TCP, median diameter: 3.7 μm) powders were kindly provided by South China University of Technology, China. The calcium carbonate (calcite) powders (<150 μm) were purchased from Guilin Kaiwen Calcium Carbonate Material Co. Ltd, China. $(NH_4)_2HPO_4$, $MgCO_3$, and Na_2CO_3 were purchased from Tianjin Fuchen Chemical Reagent Factory, China. All the commercial chemicals were analytically pure. The cell culture-related reagents were purchased from Gibco except specialized.

2.2. Preparation of PG, CC/PG, CC, HA, and β-TCP blocks

The method for fabricating PG ($50P_2O_5 \cdot 18CaO \cdot 12MgO \cdot 20Na_2O$) was described in our previous study [24]. In brief, the mixtures of $(NH_4)_2HPO_4$, $CaCO_3$, $MgCO_3$, and Na_2CO_3 were put into an alumina crucible, and placed into a furnace at 1000 °C for 2 h to form glass liquid. The glass liquid was then poured into a stainless steel dish filled with deionized water. The collected glass pellets were dried at 80 °C, then milled using an agate grinding machine for 15 h. The PG powders with a median diameter of 4.5 μm were obtained. The glass transition and melting temperature of the PG were 410 °C and 650 °C, respectively.

The calcium carbonate powders were uniformly mixed with PG powders at a mass ratio of 6:4. The powders of PG, calcium carbonate, HA, and β-TCP, and mixtures of calcium carbonate and PG powders were placed into the molds, respectively, and uniaxially pressed at 10 MPa using a powder pressing machine (769YP-24B, Tianjin Keqi, China), then demolded. The obtained rectangular samples (6 mm × 6 mm × 45 mm) were cold-isostatically pressed employing a cold isostatic pressing machine (Western Sichuan Machinery Co., Ltd, China) with a pressure of 200 MPa for 2 min. The green bodies of calcium carbonate/PG, PG, and calcium carbonate were put into the alumina crucibles and placed into the furnaces in air with a heating rate of 5 °C min^{-1} up to 650 °C, 390 °C, and 650 °C, respectively, held for 20 min, then cooled to room temperature naturally. The calcium carbonate/PG ceramic (CC/PG) and calcium carbonate ceramic (CC), and PG block were obtained. The HA and β-TCP green bodies were sintered at 1200 °C and 1100 °C, respectively, held for 2 h. Finally, the HA and β-TCP ceramics were obtained.

2.3. Material characterization

The blocks of CC/PG, CC, PG, HA, and β-TCP were ground into powders. The powdered samples were analyzed using 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°.

The samples were mounted on an aluminum stub by carbon tape and then sputter-coated with gold. The microstructure of samples was observed under a field emission scanning electron microscope (Nava NanoSEM 430, FEI, The Netherlands) using accelerating voltages of 15 kV.

All the block samples were cut into three sections, then polished. The final dimensions of samples for compressive strength test were 5 mm × 5 mm × 10 mm. The compressive strength of the samples was measured by a universal material testing machine (Instron 5567, Instron, Britain) at a crosshead speed of 0.5 mm min^{-1}. Each measurement was repeated at least six times.

2.4. In vitro degradation

The rectangular samples of CC/PG, CC, PG, HA, and β-TCP were weighed and their surface area was calculated by the dimensions determined by a vernier caliper. The samples were immersed in the Tris–HCl solution (pH 7.4, 37 °C) at a surface area to solution volume ratio of 0.1 cm^{-1}. The solutions were renewed after 1, 3, 5, 7, 14, 21, 28, and 35 days, respectively. The samples were taken out at scheduled time points, rinsed with deionized water 3 times, dried at 85 °C for 16 h, and weighed. The weight loss (WL) was calculated as follows:

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

where W_0 denotes the initial weight of the samples, and W_d represents the weight of the dried samples after the scheduled immersion time. Each measurement was performed six times.

2.5. Evaluation of in vitro cell behaviors

2.5.1. Cell culture and seeding

The cell behaviors on the blocks of CC/PG, CC, PG, HA, and β-TCP were performed with rat bone mesenchymal stem cells (rMSCs, American Type Culture Collection, USA) at passage 5. The samples (5.5 mm × 5.5 mm × 2.5 mm) were sterilized by gamma radiation (15 kGy). High-glucose Dulbecco's modified eagle's medium (H-DMEM, Gibco, USA) with 10 vol.% fetal bovine serum (FBS, Gibco, USA) was used for cell culture. As for evaluations of cell attachment, viability, proliferation, and cytoskeleton, 500 μL of cell suspension (4×10^4 cells/mL) was added onto the samples in 48-well plates. The cell-seeded samples were incubated in a humidified incubator with 5% CO_2 at 37 °C. The culture medium was refreshed every other day.

2.5.2. Cell attachment, viability, and cytoskeleton

After incubation for 12 h, the samples were fetched and washed with phosphate buffered saline (PBS) 3 times, immobilized with 2.5% (v/v) glutaraldehyde solution at 4 °C for 4 h, then dehydrated with graded series of ethanol (30%, 50%, 70%, 90%, 95%, and 100%), and finally dried in air. The morphology of cells attached on various materials was observed by SEM (S-3400 N, Hitachi, Japan).

After cell culture for 2 days, the cell viability on various materials was evaluated using a Live/Dead kit (calcein-AM, Biotium, USA) in accordance to the manufacturer's instructions. The samples were rinsed with PBS 3 times, then incubated in the calcein-AM solution at 37 °C for 45 min. Subsequently, the samples were washed once again with PBS to eliminate the staining solution, and observed under the fluorescence microscope (Zeiss Axioskop 40, Zeiss, Germany).

F-actin is a protein that is relevant to cytoskeletal organization. The cytoskeleton of rMSCs attached on various samples after 2 days of incubation was visualized by staining F-actin with Phalloidin-iFluor™ 488 Conjugate (AAT Bioquest, USA), and staining the cell nuclei with 40,6-diamidino-2-phenylindole (DAPI, AAT Bioquest, USA). Briefly, the samples were rinsed with PBS once, fixed with 4% paraformaldehyde solution for 20 min, then immersed in 0.1 vol.% Triton X-100 solution at room temperature for 5 min. Afterward, cells were labeled with the F-actin staining kit for 1 h and then stained with DAPI for 5 min following the manufacturer's protocols. Finally, fluorescent images of cytoskeleton