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Effects of HA/ZrO_2 composite powder on mesenchymal stem cells proliferation and osteogenic differentiation ☆

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Abstract

To evaluate the effects of hydroxyapatite/Zirconiumoxide (HA/ZrO_2) composite powder on proliferation and osteogenic differentiation of rabbit mesenchymal stem cells (MSCs) by using molecular biology methods in vitro. HA/ZrO_2 composite powder prepared by using powder of HA and ZrO_2 with different proportions sintered at 1600 °C were compared with pure HA powder and pure ZrO_2 powder. The effects of the composite powder suspensions on the proliferation of the rabbit MSCs were measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay), alkaline phosphatase (ALP) activities were measured by the ALP colorimetric assay, and the cellular expression levels of Collagen I, osteocalcin and osteopontin mRNAs were determined by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The HA phase was transformed into the β-$Ca_3(PO_4)_2$(β-TCP), α-$Ca_3(PO_4)_2$ and $CaZrO_3$ phases in the composite powder after sintering at 1600 °C, with a positive correlation between the contents of the HA phase and the new phases. The MTT assay showed that both pure HA powder and HA containing composite powder were able to promote cellular proliferation, but pure ZrO_2 powder had no effect in stimulating cell proliferation ($P < 0.05$). Vonkossa staining revealed that the composites and pure HA powder were capable of reducing the percentages of positively stained cells. The ALP colorimetric assay demonstrated that the ALP activities of cells maintained in culture media with HA and HA containing composite powder were significantly higher than that of cells cultured in regular media or media containing pure ZrO_2 ($P < 0.05$). RT-*P*CR results showed that the composite powder were able to stimulate the expression of Collagen I and osteocalcin. The HA/ZrO_2 composite powder can also facilitate osteogenic differentiation.

Keywords: HA/ZrO_2 composite; MSCs; Proliferation; Osteogenic

1. Introduction

Hydroxyapatite (HA) has been used as scaffolds for bone tissue engineering due to its good biocompatibility and bone conductivity [1]. Compared with traditional implants, engineering bone substitutes made from scaffold materials and seed cells show superior performance in many aspects, such as the repair extent of the injury site, as well as the morphology of the newly formed bone [2–4]. Furthermore, the use of fibrin as a cell carrier to impose seed cells growth has been demonstrated effectively.The influence of scaffold materials on the biological behaviors and functions of seed cells are critical for the appliance of the engineering bone and have often been investigated recently. Adult bone marrow derived mesenchymal stem cells are multipotential differentiate cells and have been used in bone tissue engineering [5]. We have developed a novel graded HA/ZrO_2 bio-composite material. The aim of this study was to investigate the effects of the HA/ZrO_2 composite powder on MSCs proliferation and osteogenic differentiation using molecular biology methods.

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2. Materials and methods

2.1. Preparation of HA/ZrO_2 composite powder

The following procedures were carried out at Shanghai University. All raw materials in the present experiment were the analytical reagents. (a) Preparation of HA powders: $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_3PO_4 \cdot 3H_2O$ were used to prepare nano-HA powder. $Ca(NO_3)_2$ was dissolved in 95% ethanol to make a 0.5 mol/L solution. $(NH_4)_2HPO_4$ was dissolved in deionized water to make a 0.5 mol/L solution. NH_4OH was employed to adjust the pH values of the solutions to 10. Five drops of ethanolamine were added to the solution. Under vigorous stirring, a small amount of $(NH_4)_2HPO_4$ was first added to the Ca solution to produce HA crystal nuclei. The rest of the $(NH_4)_2HPO_4$ solution was then added to the Ca solution. The reaction temperature was 25 °C and the pH value was controlled at 10. After stirring for a moment, the reaction was left to age for 12 h. Reaction products were then washed three times with ethanol, dried rapidly using a microwave oven, and finally baked at 700 °C for 1 h in order to obtain HA powders. (b) Preparation of ZrO_2 powders: A mixed solution with a fixed Y^{3+}/Zr^{4+} ratio was prepared from yttrium chloride (Y_2O_3+HCl) and zirconium oxychloride solutions. The concentration of hydrochloric acid was 0.2 mol/L, and that of zirconium salt was 1.0 mol%. The mixed solution was added slowly to ammonia with constant stirring to keep the pH at the range of 9–10. The above solution was allowed to sit still to obtain co-precipitate. Co-precipitate was filtered and washed with distilled water to remove chloride ions (< 10 ppm). It was then washed with ethanol for at least six times to remove H_2O so that the water content was less than 4 vol%. Loose hydroxide precursor was obtained after drying. And the stable ultra-fine 3 mol% ZrO_2 (Y_2O_3) powder was produced after baking at 750 °C for 2 h. (c) Preparation of HA/ZrO_2 composite powders: Composites were prepared by mixing HA and ZrO_2 powders with different proportions (specific formulations shown in Table 1). Mixed powder were placed into a corundum crucible, and sintered at 1600 °C for 3 h under normal pressure in a vertical silicon–molybdenum rod furnace. Sintered products were slowly cooled inside the furnace to room temperature.

Table 1
Formulations of composite powders with different proportions of HA/ZrO_2.

Formula	ZrO_2 (wt%)	HA (wt%)
1	0%	100%
2	30%	70%
3	50%	50%
4	70%	30%
5	100%	0%

2.2. Characterization of HA, ZrO_2 and sintered HA/ZrO_2 composite powder

The JEOL-200CX transmission electron microscope (TEM, JEOL Ltd, Japan) was used to analyze the morphology and size of HA, and ZrO_2 powers. The cross-sectional morphology of sintered sample was observed using Hitachi S-570 scanning electronic microscope (SEM) after Au spraying. The D\max-2550 X-ray diffractometer (XRD) with CuKα radiation (200 mA, 40 mV) was employed to detect the components of prepared HA, ZrO_2 powers and sintered composite, in which scanning angle was from 20° to 80° with a speed of 8°/min. Figs. 1 and 2 is the analyzed results of ZrO_2 and HA powers used, which demonstrated that they were pure particles with nano-size.

2.3. Isolation and cultivation of mesenchymal stem cells

New Zealand rabbits were anesthesized using 3% sodium pentobarbital at 1.5 ml/Kg. 8% sodium sulfide was used to remove hairs around the proximal tibia. After disinfecting the surgical field by povidone–iodine and 75% ethanol, the bone marrow needle punctured from the lateral side of tibial tubercle and was rotated to pierce the marrow. A 5 ml syringe containing 2000 U heparin was attached to the needle to aspire 3–4 mL of bone marrow fluid. Aspired bone marrow fluids were slowly added into tubes containing 4 mL lymphocyte separation medium to undergo density gradient centrifugation at 2500 r/min for 30 min. The middle mononuclear cell layer was collected by suction after centrifugation, washed once with PBS, and seeded into 25 cm^2 culture flasks at a density of $3 \times 10^5/cm^2$. 3 mL of DMEM medium plus 15% FBS (100 U/mL penicillin sodium, 100 mg/L streptomycin, pH 7.2–7.4) was added to each flask. Cells were incubated with 5% CO_2 at 37 °C. Half of the culture media were replaced 3–5 days later. Incubation was continued with all culture media replaced twice per week. When cells covered the entire flask bottom, cells were digested with 0.25% trypsin for 5 min to generate the primary MSCs suspension, which was then passed at a density of $1 \times 10^4/cm^2$.

2.4. Effects of the HA/ZrO_2 composite powder on MSCs proliferation under regular culture conditions

The third generation of mesenchymal stem cells were seeded to 24-well plates at a density of $1 \times 10^4/cm^2$. HA/ZrO_2 composite powder was suspended in culture medium to prepare different concentrations of the powder suspensions (0, 50, 200 μg/ml). 0.5 ml suspension with different concentrations of powder was added to each well, with sextuplicates for each concentration. Culture media and powder were removed five days later by washing in PBS. Cells were tested with the MTT assay.