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In vitro and in vivo biocompatibility of graded hydroxyapatite–zirconia composite bioceramic

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Abstract To obtain bioceramics with good osteoinductive ability and mechanical strength, graded hydroxyapatite–zirconia ($HA–ZrO_2$) composite bioceramics were prepared in this work. The biocompatibility of the bioceramics was investigated in vitro based on acute toxicity and cytotoxicity tests and hemolysis assay. Results showed the studied graded $HA–ZrO_2$ had little toxicity to mouse and L929 mouse fibroblasts. Also, hemolysis assay indicated a good blood compatibility of the bioceramics. Based on the results of in vitro tests, animal experiments were performed on white New Zealand rabbits by implantation into hip muscles and femur. It was found that the graded $HA–ZrO_2$ composite bioceramics exhibited superior osteoinductive ability, which may be a promising bioceramics implant.

Introduction

Hydroxyapatite (HA) ceramics have long been recognized as substitute materials for bones due to their chemical and biological similarity to human hard tissues [1–20]. Moreover, HA has been recognized as a bioactive material having the direct bonding capability to the surrounding tissues. However, HA lacks the required strength and toughness for practical applications. Zirconia (ZrO_2) has high toughness and shows wide applications in bone surgery [3–10, 21]. It has been found that $HA–ZrO_2$ composites may show improved fatigue resistance and high strength as well as biocompatibility and bioactivity [22].

Implant fixation to the host tissue is seriously hindered if the HA dissolution rate is two fast. And, the implant-tissue osseointegration is impossible if the coating HA layer is not sufficiently adhered to the substrate. Therefore, as pointed by Kim et al. [3], the stability and integrity of the coating layer should be carefully considered prior to actual clinical applications. In this work, graded $HA–ZrO_2$ composite bioceramics were prepared and the biocompatibility was carefully evaluated by acute toxicity, cytotoxicity to L929 mouse fibroblasts, hemolysis, and animal experiments.

Materials and methods

Sample preparation and characterization

Partially stabilized ultrafine powders of ZrO_2 containing 3.0 mol% Y_2O_3 as a stabilizer were prepared by chemical co-precipitation method. The powders were granulated through spray drying with 1.5 wt% poly(vinyl alcohol) as agglomerant and then consolidated into ZrO_2 green body. Powders of HA were also prepared by chemical precipitation method in water–acetic acid system. HA was composited onto the surface of the ZrO_2 green body by the following method. To fabricate a graded sample, HA/ZrO_2

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blends with different contents of HA were coated onto the surface of ZrO_2 green body in the sequence of 30 wt% HA + ZrO_2, 50 wt% HA + ZrO_2, 70 wt% HA + ZrO_2 and pure HA. The composite green body was then fabricated into the due material in a high temperature furnace through non-pressure sintering at 1,600 °C. Scanning electron microscopy (SEM, Hitachi S-570) was used to observe the morphologies of the composite bioceramics.

Preparation of the extracts

Sample was first disinfected with ethylene oxide and then put into physiological saline solution or RPMI-1640 culture medium (GIBCO, USA) at the ratio of 1.0 g sample/5 ml solution. After the sample was soaked for extraction for 72 h in a 37 °C thermostated container, the solution was disinfected and reserved in 4 °C for further use.

Acute toxicity

Animal test was performed with compliance of the local ethics committee. Twenty healthy male mice of about 20 g weight were randomly divided into three groups: sample and negative/positive groups. Each group had 10 mice. The sample group was administered a dose of 50 ml/kg body weight the extracts by injection, and the control group was injected with physiological saline solution at the same dose. All animals were observed daily for 21 days and the gaining of body weights were recorded at 24, 48 and 72 h. Microscopic examination was performed for examples at the 21 days.

Cytotoxicity test

In the present work, the cytotoxicity of graded HA–ZrO_2 composite bioceramics was investigated using L929 mouse fibroblasts. For each experiment, the cells were incubated into each well of a 96-well chambers (0.1 ml/well) at an initial concentration of 6×10^4 cells/ml. All cells were grown in RPMI-1640 medium for 24 h at 37 °C with an atmosphere of 5% CO_2 in air. Then, the cells were divided into three groups and each group includes eight wells. The old cultured medium was replaced with the extracts of the graded HA/ZrO_2 sample for the sample group. For positive group, the cultured medium was changed to that contains 0.64% phenol while fresh RPMI-1640 medium is for negative group. After incubation of 48 h, a 20 μl of 5% MTT was added into each well. The cells were cultured for another 4 h and then the liquid in the well was let off. After that, 0.1 ml of DMSO was added into each group, and after complete mixture, the absorption value, A, was recorded at 590 nm on an enzyme analyzer. The proliferation ratio was determined by the A value and the proliferation ratio of the control at 24 h was set as 100%. The morphologies of cells were observed by a phase contrast optical microscopy.

Hemolysis assay

Hemolysis assay was tested to evaluate the blood compatibility of the graded HA–ZrO_2 composite bioceramics. A 0.2 ml of erythrocyte suspension was exposed to 10 ml of the extracts of the bioceramics (sample group), distilled water (positive group), and 0.9% physiological saline solution (negative group). Following incubation at 37 °C for 60 min and centrifugation for 5 min at 1,000 rpm, the absorbance of the supernatant was measured at 545 nm in a spectrophotometer. The percentage of hemolysis was determined by comparing the absorbance of the samples with that of the control totally hemolyzed with distilled water.

Animal model

To further investigate the graded HA–ZrO_2 composite bioceramics, an implant test was performed. Twelve white New Zealand rabbits of 2.5~3.5 weight were randomly divided into four groups, and these groups were investigated the 3, 6, 12, and 24 weeks stability of the implants. These rabbits were premedicated using an intravenous injection of 3% secobarbital. As reported by Simank et al. [23], a direct lateral approach to each distal femur was utilized. The condyle was exposed and a central drill hole about 5 mm proximal to the articular cartilage was made using a aiguille with a diameter of 5 mm. Wafer-like implants of the graded bioceramics were fitted into the cancellous bone. Then the wound was rinsed and closed in layers. Similarly, the bioceramics was also implanted in the hip muscles. The rabbits were allowed unrestricted food and movement in their cages. The animals were euthanized at 3, 6, 12 and 24 weeks. The related tissue was separated and prepared followed by immobilization with 2.5% glutaraldehyde and 1.0% osmic acid. After dehydration by alcohol and gold sputtering, these samples were observed by SEM.

Statistic strategy

All the data were handled by the SPSS10.0 statistical software, and were marked as $\bar{x} \pm s$. The data were given a normal distribution test—to see that they were normally distributed—and a homogeneity test of variance. The differences of the groups were analyzed through one-way analysis of variance.

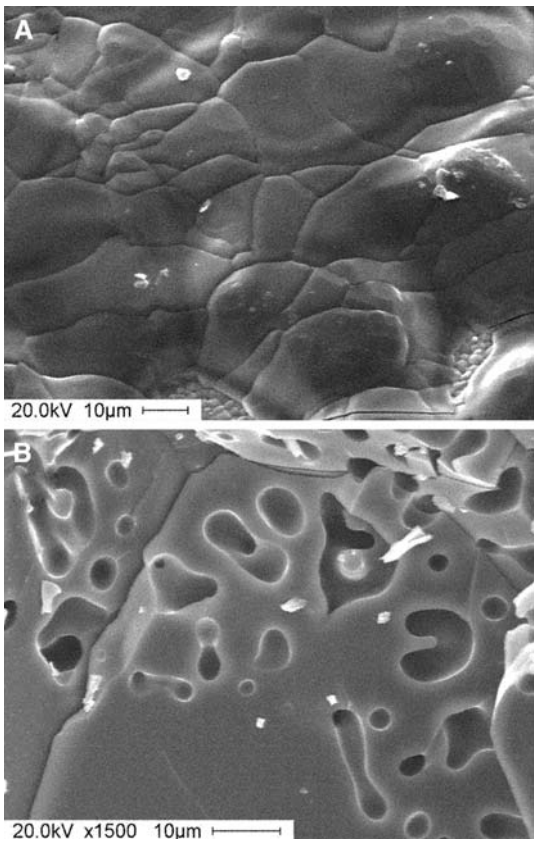

Fig 1 SEM micrographs of surface morphologies of the graded HA–ZrO2 composite bioceramics with (**A**) 3–5 μm and (**B**) 50–80 μm of HA layer

Results and discussions

Graded HA–ZrO_2 composite bioceramics were prepared through sintering at 1,600 °C. In this paper, the biocompatibility evaluations in vitro and in vivo were carried out. Some other points of the resultant bioceramics such as the optimized parameters of preparation, the mechanical strength, the phase structures of HA and ZrO_2 and the chemical composition should also be studied carefully and were described elsewhere due to the limitation of space [24]. Figure 1 is the typical SEM micrographs of the bioceramic surface. It is clear that the thickness of HA layer influences the surface morphology greatly. When the HA layer was thin (Fig. 1A, the thickness is about 3–5 μm), the surface was relatively smooth and composed of granules with diameter of about 10 μm. Furthermore, the HA layer was so thin that some ZrO_2 was exposed. On the other hand, if the thickness of HA layer reached 50–80 mm, as shown in Fig. 1B, the surface was a bit rough with a few

Table 1 Gaining of the body weights of mice

Group	Gaining weight (g, $\bar{x} \pm s$)		
	24 h	48 h	72 h
Sample	1.09 ± 0.16	2.31 ± 0.45	3.57 ± 0.49
Control	1.20 ± 0.19	2.33 ± 0.44	3.62 ± 0.61

Table 2 Cytotoxicity assay determined as the proliferation ratio of the L929 mouse fibroblasts

Group	The proliferation ratio of cells (%)		
	24 h	48 h	72 h
Sample group	97.7	145.3	174.5
Negative group	100.0	145.9	176.6
Positive group	15.4	30.2	12.6

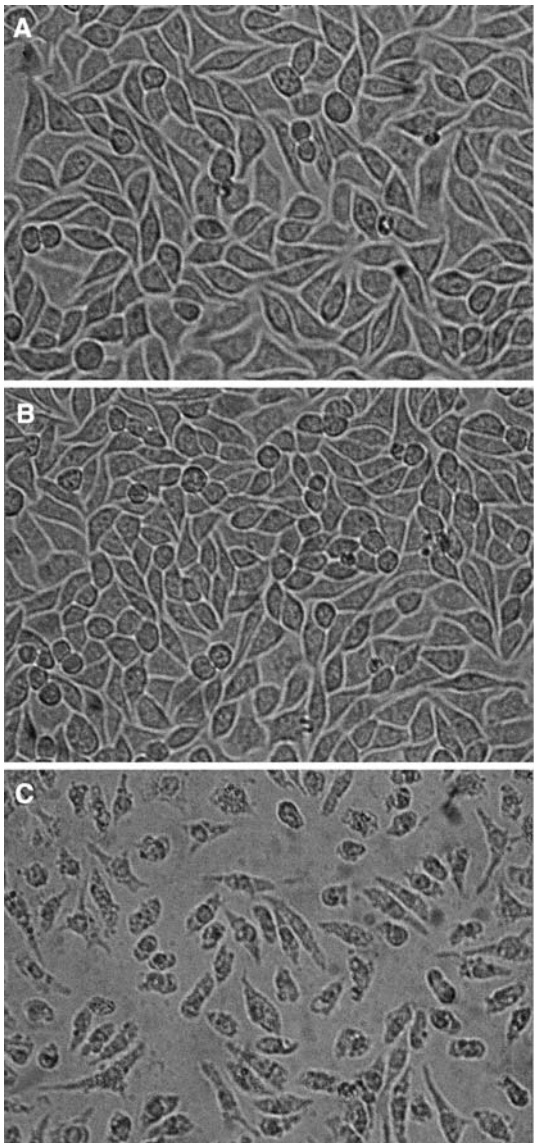

Fig 2 Morphologies of the cultured L929 mouse fibroblasts for 72 h. (**A**) sample, (**B**) negative, and (**C**) positive group (×200)

Table 3 Hemolysis assay results

Group	Absorbance	Hemolysis ratio (%)
Sample group	0.032 ± 0.002	1.7
Negative group	0.019 ± 0.002	0
Positive group	0.803 ± 0.034	100

($n = 8$, $\bar{x} \pm s$)

pores and tiny cracks, which makes its specific surface area larger and easier to bond with surrounding tissues in vivo, thus provides brackets and passages for the growth of new bone tissues, hence better biocompatibility.

To evaluate the biocompatibility of the resultant bioceramics in vitro, the extracts of the bioceramics were prepared. Acute toxicity was performed on white mouse by monitoring the gaining of body weight. The mice administrated with 50 ml/kg body weight the extracts showed no symptoms of lethargy, anorexia, and diarrhea in the period of observation (21 days). Table 1 shows the gaining weight of the mice recorded at 24 h, 48 h, and 72 h. The body weight gain of the mice in the sample group and that of the control mice was of no statistic significance. Thus the graded HA–ZrO_2 has no acute toxicity.

L929 mouse fibroblasts was used to investigate the cytotoxicity of the materials [25]. Table 2 shows the proliferation ratio of the cultured L929 mouse fibroblasts at 24, 48, and 72 h. Fresh RPMI-1640 medium was used to culture the fibroblasts as a negative group while the media with 0.64% phenol addition was used in positive group. It is clear that the proliferation ratios of both sample group and negative group increased steadily with time. It also can be seen that the proliferation ratio of the sample group was very close to that of the negative group. However, the proliferation ratio of the positive is very low and some of the fibroblasts died. Furthermore, the morphologies of the cultured fibroblasts for 72 h could also elucidate the excellent cytocompatibility of the studied bioceramics, as shown in Fig. 2. From these images, the morphologies in sample group were indistinguishable from the negative controls. Fibroblasts in sample group and negative group were of polygon and cell division could be observed. In positive group, dying and dead fibroblasts were significantly smaller.

The blood compatibility of implants was also very important because the formation of thrombus or the damage of blood cells might induce particularly serious consequences [26, 27]. Thus hemolysis assay was carried out and the results were shown in Table 3. The hemolysis ratio of the sample group was only 1.7%, which indicated the studied materials having little damage on the red blood cells.

Based on the results of in vitro experiments, the graded HA–ZrO_2 composite bioceramics were implanted into the femur and the hip muscles of white New Zealand rabbits. All rabbits tolerated the surgical procedure well. None exhibited infection of surgical site, dislocation of the implants, or adverse reactions such as inflammation or foreign-body reactions on or around the implanted materials. It was found from the implants in muscles that the implants contacted close to the surround muscles and the color the muscle was normal. All these phenomena confirmed the good biocompatibility of the graded HA–ZrO_2 composite bioceramics.

Figure 3 shows the SEM micrographs of materials implanted into the femur for different time. Three weeks after

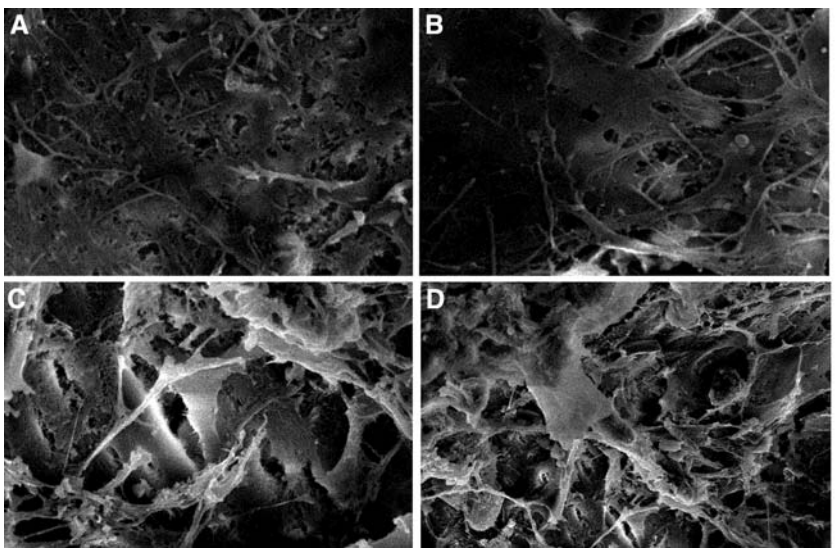

Fig 3 SEM micrographs of graded HA–ZrO_2 composite bioceramics after implanted into femur for different time: (**A**) 3 weeks; (**B**) 6 weeks; (**C**) 12 weeks and (**D**) 24 weeks (×2000)

the operation a little amount of bone was formed on the surface of the implant (Fig. 3A). The amount of bone increased remarkably after another 3 weeks and the bone became sheet-like or island-like structure, which intended to cover the surface of the implant. At 24 weeks, bone increase further and completely covered the implant surface. The graded HA–ZrO_2 composite bioceramics had superior osteoinductive ability, which might due to the existence of a relatively thick layer of HA. It was reported that, for commonly coated HA, thick coating might become unstable before resorption and result in early loosening of the implant by soft tissue reactions and osteolysis, while thin coating might resorb too fast [23]. In our case, the graded HA–ZrO_2 composite bioceramics showed excellent osteoinductive ability and stability. Therefore, it may be a promising bioceramics implant.

Conclusion

Graded hydroxyapatite–zirconia (HA–ZrO_2) composite bioceramics were prepared by chemical co-precipitation method using 3.0 mol% Y_2O_3 as a stabilizer. The bioceramics with thicker HA layer were more rough and might promote the osseointegration. Results from in vitro studies of acute toxicity and cytotoxicity tests and hemolysis assay indicated that the bioceramics had little toxicity to mouse and L929 mouse fibroblasts. Also, hemolysis assay confirmed a good blood compatibility of the bioceramics. Based on the results of in vitro tests, animal experiments were performed on white New Zealand rabbits by implanted into hip muscles and femur. It was found that the graded HA–ZrO_2 composite bioceramics exhibited superior osteoinductive ability, which may be a promising bioceramics implant.

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