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Ceramics International 38 (2012) 6621–6628

CERAMICS INTERNATIONAL

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

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Received 23 March 2012; received in revised form 27 April 2012; accepted 16 May 2012
Available online 27 May 2012

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.

☆ Grants: Zhejiang Provincial Medical Research Foundation (2007A170), Science and Technology Development Program of Hangzhou City (20080333B23).
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http://dx.doi.org/10.1016/j.ceramint.2012.05.048

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.

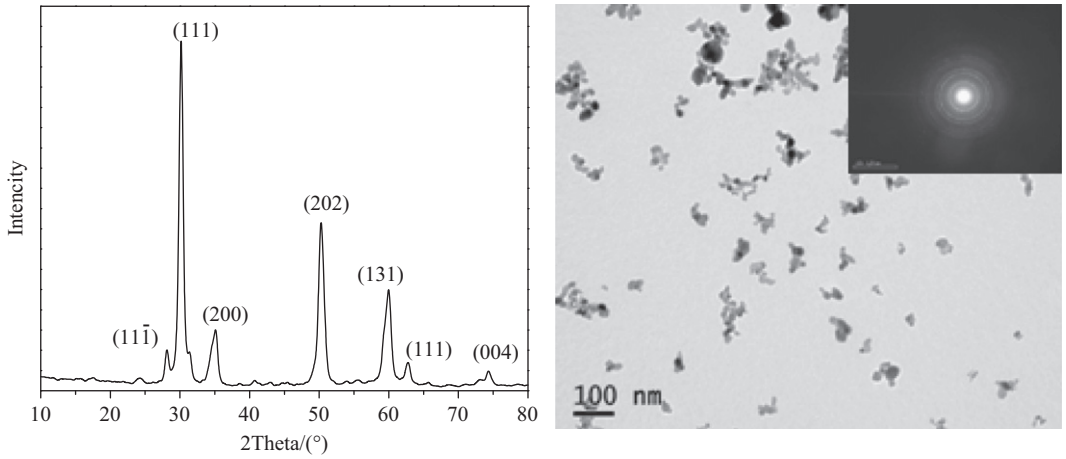

Fig. 1. Nano-ZrO_2 powder, (a) XRD pattern and (b) TEM photo.

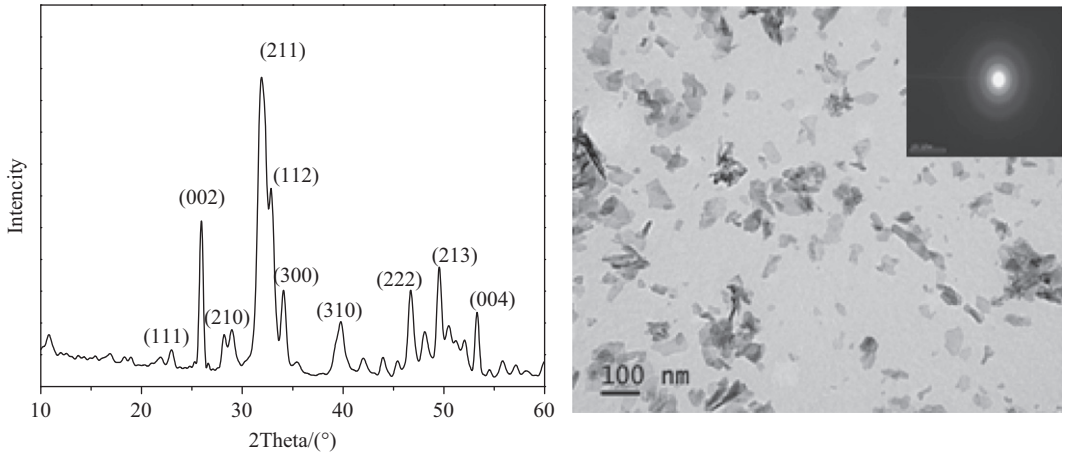

Fig. 2. Nano-HA powder, (a) XRD pattern and (b) TEM photo.

2.5. Effects of the HA/ZrO_2 composite powder on MSCs mineralization

The third generation of mesenchymal stem cells were seeded to 24-well plates at a density of $1 \times 10^4/cm^2$. Composite powder was suspended in regular culture medium and in the osteogenic medium that contained the osteogenic agents, 10 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and 100 nM dexamethasone 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 triplicates for each concentration. Culture media and powder were removed three weeks later by washing in PBS. Cells were treated with Vonkossa staining.

2.6. MTT assay

The third generation of mesenchymal stem cells were seeded to 24-well plates at a density of $1 \times 10^4/cm^2$. The HA/ZrO_2 composite powder were 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 medium and powder were removed five days later by washing in PBS. 100 μl of 20% MTT was added to each well. Supernatants were removed by suction after incubating at 37 °C for 4 h. 150 μl of dimethyl sulfoxide (DMSO) was added to each well. After fully mixed by shaking for 10 min, OD492 was measured by a microplate reader.

2.7. Measurement of alkaline phosphatase (ALP) activity

Cells were lysed as previously described. ALP activity was measured using the p-nitrophenyl phosphate method. Cell lysate reacted with the colorless p-nitrophenyl phosphate, which generated the yellow colored p-nitrophenol. Absorbance (A) was measured at 405 nm. The amount of phenol produced from the reaction was determined by comparing with the standard. Alkaline phosphatase activity was defined as the ratio of the amount of phenol (mg) to total protein content. Procedures were performed according to the kit instruction.

2.8. Expression of Collagen I, osteocalcin, and osteopontin mRNAs

The third generation of cells were seeded into 25 cm^2 culture flasks with a density of $1 \times 10^4/cm^2$. Composite powder was suspended in regular culture medium and in the osteogenic medium that contained the osteogenic agents, 10 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid, and 100 nM dexamethasone to prepare different concentrations of the powder suspensions (0, 50, 200 μg/ml). 4 ml of suspensions with different concentrations of powder were added to each flask. Three weeks later, culture media and powder were removed by washing in PBS. Expression of Collagen I, osteopontin, and osteocalcin mRNAs in cell samples was evaluated by RT-PCR. Total RNA extraction and RT-PCR were performed as previously described. Size and gray scale of the bands were analyzed using the Image Pro Plus software. The product of the two was shown as IOD (Integrated Optical Density).

2.9. Vonkossa staining

Cells to be tested were washed twice with PBS, fixed in a fixative solution containing formaldehyde–acetone–citric acid for 10 min, and washed once with single distilled water. Appropriate amounts of 5% silver nitrate were added to the samples to fully cover the cell surface, followed by exposure in the sun or UV light for 1 h. Images were taken under light microscope. For each sample, four areas with more than 80% coverage of cells were randomly chosen and imaged under 4 × magnification lens. Size and optical density of positive stained areas were evaluated by the Image Pro Plus software. The product of the two was presented as IOD (Integrated Optical Density).

2.10. Statistical analysis

All data were analyzed using the SPSS statistical software. All measurements were expressed as mean ± SD ($\overline{x} \pm s$). Differences between two groups were analyzed using the independent samples *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. XRD analysis and SEM observation of HA/ZrO_2 composite powder

Fig. 3 is the SEM image of HA/ZrO_2 composite powder. It can be seen that the diameter of ZrO_2 particles with spherical shape was less than 1 μm and the particles combined closely after sintered at high temperature. Rare voids or cracks can be observed from the image. Although the partially nano-ZrO_2 grew up as a result of 1600 °C high temperature, in which the diameter was less than 10 μm. These grains bonded well with normal grains.

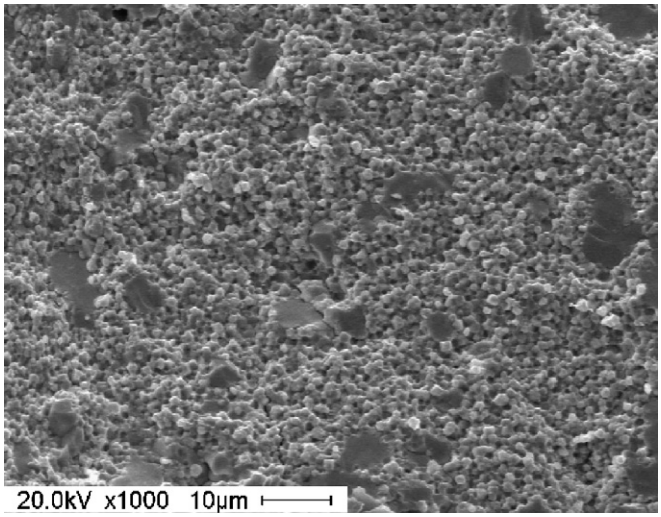

Fig. 3. SEM of 1600 °C sintered composites.

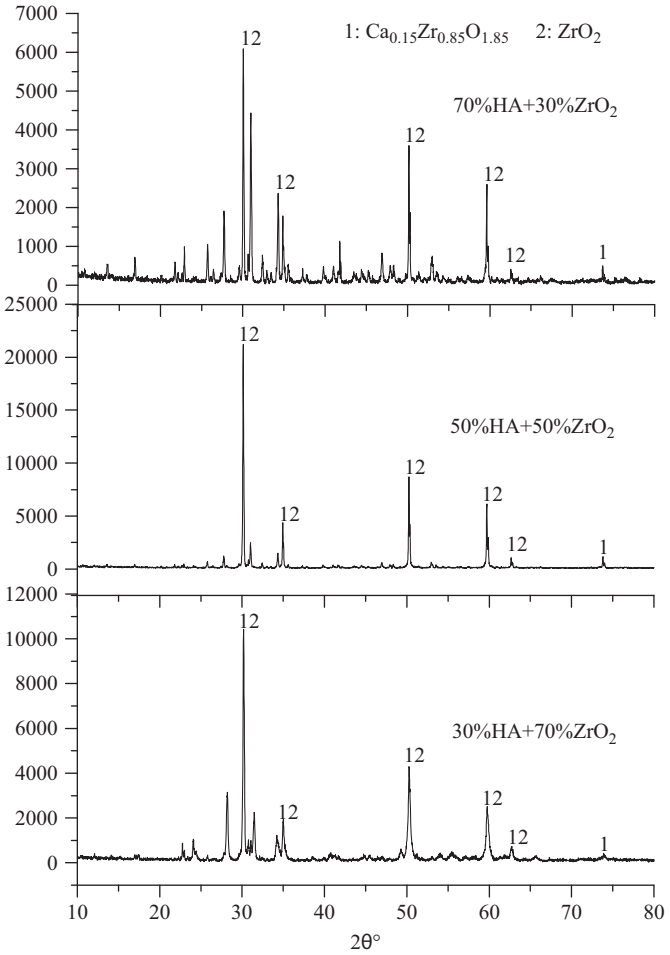

Fig. 4. XRD analysis of HA/ZrO_2 composites with different proportions.

Fig. 4 is the typical XRD pattern of sintered composites with different proportions of HA and ZrO_2 powders, in which those unmarked peaks were $Ca_3(PO4)_2$ compound. Obviously, the ZrO_2 phase still existed in composite after

sintering at 1600 °C and the HA phase was not detected, which transformed into the β-$Ca_3(PO_4)_2$ (β-TCP), α-$Ca_3(PO_4)_2$ (α-TCP) and $CaZrO_3$ phases. The contents of β-TCP, α-TCP and $CaZrO_3$ gradually decreased with eliminated percentage of HA. This is because CaO released during the process in which HA transformed into β-$Ca_3(PO_4)_2$. X-ray diffraction did not detect CaO on the surface of the composite material since CaO dissolved completely in the ZrO_2 phase and reacted with ZrO_2 to form a stable $CaZrO_3$ phase. The appearance of the β-TCP phase in sintered composites is due to a structural synergistic effect of hexagonal HA. The α-$Ca_3(PO_4)_2$ phase with small amount transformed from β-$Ca_3(PO_4)_2$.

The above reactions can be shown as follows:

$$Ca_{10}(PO_4)_6(OH)_2 \Leftrightarrow 3\beta\text{-}Ca_3(PO_4)_2 + CaO + H_2O$$
$$CaO + ZrO_2 \Leftrightarrow CaZrO_3$$
$$\beta\text{-}Ca_3(PO_4)_2 \Leftrightarrow \alpha\text{-}Ca_3(PO_4)_2$$

After sintering at 1600 °C, the HA phase was completely transformed into the new β-$Ca_3(PO_4)_2$ (β-TCP), α-$Ca_3(PO_4)_2$ (α-TCP) and $CaZrO_3$ phases. Biocompatibility of composite powder can be achieved because the unhydrated high temperature calcium phosphate (TCP) phases interact with water or body fluids at 37 °C to form HA [2].

The reaction that forms HA on the surface of TCP is as follows:

$$4Ca_3(PO_4)_2(s) + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2(surface) + 2Ca^{2+} + 2HPO_4^{2-}$$

The stability of calcium phosphate ceramics depend on the temperature and the presence of water, either during material process or in the in-service environment. At body temperature, only two calcium phosphates are stable when in contact with aqueous media such as body fluids. At PH < 4.2, the stable phase is $CaHPO_4 \cdot 2\,H_2O$, while at pH ≥ 4.2, the stable phase is $Ca_{10}(PO_4)_6(OH)_2$(HA). Other phases, such as β-$Ca_3(PO_4)_2$ (β-TCP) and $Ca_4P_2O_9$, are present at higher temperatures. Therefore, when the solubility of the TCP surface is similar to that of HA inside human body, pH of the aqueous media further decreases, thereby improves the resorbability of TCP by further increasing the solubility of TCP.

As compared to pure HA, β-TCP has better solubility in human body fluid, good biodegradability, biocompatibility and osteo-conductivity [2]. It has been already used for bone repair.

3.2. Effects of HA/ZrO_2 composite powder on MSCs proliferation

Fig. 5 illustrated the results from the MTT (cytotoxic) assay for the HA/ZrO_2 composite powder. The 70% HA composite powder shown in this figure were generated by sintering a mixture containing 70% HA and 30% ZrO_2; the 50% HA composite powder was generated by sintering a mixture containing 50% HA and 50% ZrO_2; the 30% HA composite powder was generated by sintering a mixture containing 30% HA and 70% ZrO_2. Our results showed that HA and composite powder with certain HA percentage both played a positive role in stimulating cell proliferation, whereas pure ZrO_2 powder had no such effect. The difference was statistically significant ($P < 0.05$).

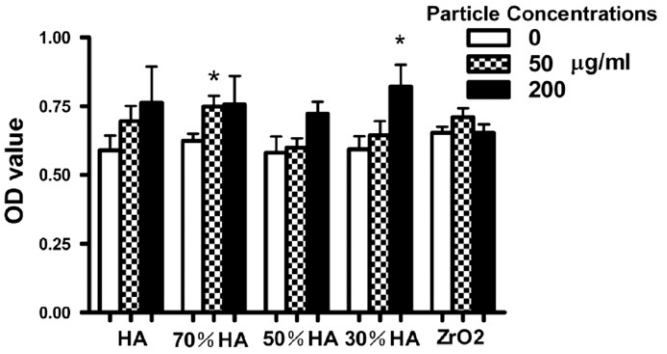

Fig. 5. Effects of HA/ZrO_2 composite particles on MSCs proliferation. Comment: 70% HA composite was sintered by mixture of 70 wt% HA + 30 wt% ZrO_2; 50% HA composite was sintered by mixture of 50 wt% HA + 50 wt% ZrO_2; 30% HA composite was sintered by mixture of 30 wt% HA + 70 wt% ZrO_2.*$P < 0.05$, $n = 6$.

3.3. Effects of the HA/ZrO2 composite powder on MSCs mineralization

As shown in Fig. 6A, there was no positive staining for MSCs maintained in regular culture medium. Significant positive staining can be observed in MSCs maintained in osteogenic medium without any material powder (Fig. 6B). The addition of composite and pure HA powder reduced the percentage of cells stained positive (Fig. 6C,J), whereas adding ZrO_2 powder had no effect (Fig. 6K,L). Changes in the percentage of positively stained cells were not associated with dose.

3.4. Effects of HA/ZrO2 composite powder on MSCs osteogenic differentiation

3.4.1. Effects of different composite powder on ALP expression

Fig. 7 showed the effects of different material powder on ALP expression. Our results demonstrated that ALP expression was significant upregulated (50–100 U/g) in cells cultured in osteogenic media added with HA or HA containing composited powder, suggesting the initiation of osteogenic differentiation. Although the effects of composite powder suspensions on ALP expression did not show a clear positive correlation with the concentration, ALP activities of cells cultured in osteogenic media added with HA and HA containing composited powder were significantly higher than that of cells maintained in regular culture medium or medium with pure ZrO_2 ($P < 0.05$, $P < 0.01$). Our results indicate that HA and HA containing powder may facilitate osteogenic differentiation of MSCs.

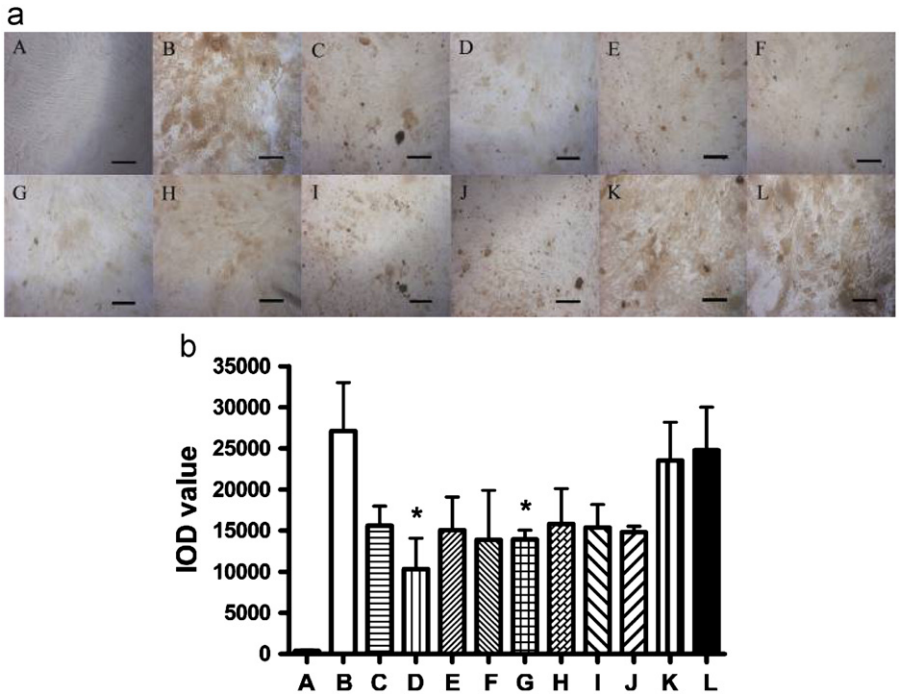

Fig. 6. Effects of the HA/ZrO_2 composite particles on MSCs mineralization. Comment: Unless otherwise noted, all groups of cells went through differentiation inductions. (A) The non-induction control group (cells without induction of differentiation and without the addition of any particles). (B) The induction control group (cells with induction of differentiation and without the addition of any particles). (C) The pure HA group with a low dose of 50 μg/ml. (D) The pure HA group with a high dose of 200 μg/ml. E. 70% HA with a low dose of 50 μg/ml. (F) 70% HA with a high dose of 200 μg/ml. (G) 50% HA with a low dose of 50 μg/ml. (H) 50% HA with a high dose of 200 μg/ml. (I) 30% HA with a low dose of 50 μg/ml. (J) 30% HA with a high dose of 200 ug/ml. (K) The pure ZrO_2 group with a low dose of 50 μg/ml. (L) The pure ZrO_2 group with a high dose of 200 μg/ml. $^*P<0.05$, $n=4$.

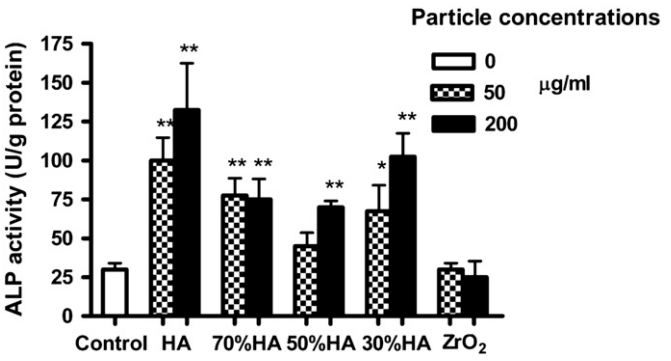

Fig. 7. Effects of HA/ZrO_2 composite particles on ALP expression. Comment: 70% HA composite was sintered by mixture of 70 wt% HA+30 wt% ZrO_2; 50% HA composite was sintered by mixture of 50 wt% HA+50 wt% ZrO_2; 30% HA composite was sintered by mixture of 30 wt% HA+70 wt% ZrO_2. * $P<0.05$, ** $P<0.01$, $n=4$.

3.4.2. Expression of Collagen I and osteocalcin mRNAs in MSCs maintained in culture media containing composite powder

As shown in Fig. 8, MSCs maintained in regular culture medium can express Collagen I, but not osteocalcin. In contrast, MSCs maintained in osteogenic medium can express both Collagen I and osteocalcin. Composite powder can enhance the expression of Collagen I and osteocalcin with varying efficiency (Fig. 8a). IOD values indicated that composite powder were able to stimulate the expression of the two mRNAs. However, when compared with pure HA and ZrO_2, no regularity or gradient was observed for the effects, and no dose dependence was observed (Fig. 8b).

4. Discussion

As the result of recent development in tissue engineering, engineered bone made from scaffold materials and seed cells performs better than traditional filling materials in terms of the extent and speed of repair, and the morphology of new bones. However, the biological biocompatibility and the safety of scaffolds have received increasing attention. The two raw materials, ZrO_2 and HA, which were utilized in this study to produce composite materials, have been used in clinical and basic researches for years. The biocompatibility of the two materials has been widely recognized [6]. Currently, the main method evaluating the biocompatibility of biological scaffolds is to utilize in vitro experiments to investigate the effects of materials or material extracts on cell growth, metabolism and proliferation. The new biocomposite material, a graded HA/ZrO_2 composite, was generated by the dry-laying and sintering process as a collaboration between our institute and the Institute of Polymer Materials at Shanghai University. According to the standards and requirements of the International Standard ISO10993-1: 1992, Biological Evaluation of Medical Devices, our group has carried out a series of in vitro and

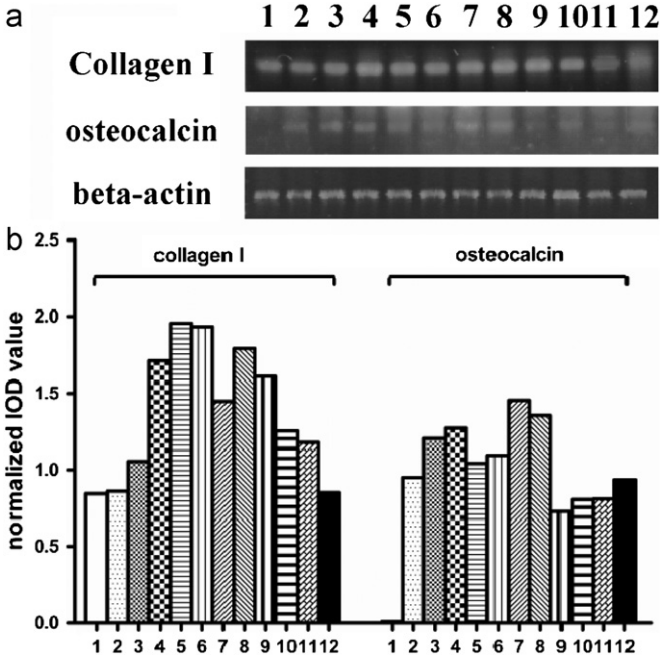

Fig. 8. Expression of Collagen I and osteocalcin mRNAs in MSCs maintained in culture medium containing composite particles. Comment: unless otherwise noted, all groups of cells went through differentiation inductions. (1) The non-induction control group (cells without induction of differentiation and without the addition of any particles). (2) The induction control group (cell with induction of differentiation and without the addition of any particles). (3) The pure HA group with a low dose of 50 μg/ml. (4) The pure HA group with a high dose of 200 μg/ml. (5) 70% HA with a low dose of 50 μg/ml. (6) 70% HA with a high dose of 200 μg/ml. (7) 50% HA with a low dose of 50 μg/ml. (8) 50% HA with a high dose of 200 μg/ml. (9) 30% HA with a low dose of 50 μg/ml. (10) 30% HA with a high dose of 200 μg/ml. (11) The pure ZrO_2 group with a low dose of 50 μg/ml. (12) The pure ZrO_2 group with a high dose of 200 μg/ml.

in vivo studies to examine the graded HA/ZrO_2 composite. Our preliminary study has found that the graded HA/ZrO_2 composite material exhibits good osteo-inductive and osteo-conductive abilities, superior immune compatibility and biocompatibility, excellent osteogenic function, and is capable of forming a biological connection with bone interface [7]. In addition, it shows no cytotoxic effects, no acute in vivo toxicity, and no hemolysis.

Our study utilized bone marrow mesenchymal stem cells (MSCs) to investigate the effects of the graded HA/ZrO_2 composites as scaffolds on the biological behaviors of seed cells, including cell growth, proliferation, and osteogenic differentiation. MSCs were chosen for this study because MSCs can be maintained, passed and expanded rapidly in surface-adherent cultures in vitro. In addition, MSCs are multipotent stems cells that under certain conditions can differentiate into a variety of tissue cells, such as osteoblasts, chondrocytes, tendon, adipocytes, fibroblasts, and astrocytes. MSCs have strong self-replication ability and are important seed cells for tissue engineering. And it has been confirmed that there are two types of cells involved in bone generation for in situ repair of bone defects, implanted MSCs and local MSCs that migrated into the implant from the surrounding tissues [8]. When choosing the carrier for seed cells, it is necessary to consider its impact on cell proliferation in addition to its performance in osteo-induction and osteo-conduction. An ideal carrier should have no significant and lasting inhibition on cell proliferation. The MTT assay was introduced by Mosmann in 1983. Initially used in the field of immunology, it has been used to evaluate biocompatibility in recent years. The principle underlying this assay is that succinate dehydrogenase produced from energy metabolism in proliferating cells can reduce MTT to blue crystals deposited inside and surrounding the cells, with the amount of crystals proportional to the number of proliferating cells. Mitochondrial succinate dehydrogenase can catalyze the formation of blue crystals from MTT. The amount of formed crystals is positively related to the number of living cells and their functional status. Its biological end point is an important organelle, mitochondrion, which acts as a powerhouse for cellular processes. Mitochondrial damage is considered to be the most sensitive indicator for cellular damage. Therefore, the absorbance measured under a particular wavelength after dissolving the crystals can reflect the proliferative activity of tested cells. But this type of assay requires a large number of cells. The MTT assay is undoubtedly very sensitive in evaluating cytotoxicity of the test materials. We utilized an indirect contact method, which visualizes the effects of material powder suspensions on cell growth by MTT assay and subsequently determines the conditions of cell growth and proliferation by measuring the differences in absorbance. In this study, we utilized the MTT assay to show that HA and HA containing composites powder play a role in promoting cell proliferation, whereas ZrO_2 powder alone did not stimulate cell proliferation. We propose that the underlying mechanism might be explained by the ability of HA to adsorb serum proteins. It has been confirmed by previous literatures that nHAP can adsorb serum proteins and other macromolecules [9]. In addition, other studies have shown that serum proteins can significantly promote cell proliferation [10]. nHAP powder adsorbed with serum proteins can adhere to cell surface, which might facilitate cell growth. It has been reported previously that nHAP powder can stimulate the proliferation of endothelial cells [1]. Consistent with previous reports, this study showed that both HA and HA containing composite powder play a role in promoting cell proliferation.

Under osteogenic culture conditions, MSCs without composite powder showed significant mineralization, indicating the occurrence of osteogenic differentiation. After adding composite powder, our results demonstrated that composites and pure HA powder were able to reduce to the extent of mineralization. In contrast, the addition of ZrO_2 powder did not affect cellular mineralization. In addition, an association was not found between the extent of mineralization and the dose. Our study suggests that composite and pure HA powder could inhibit mineralization. The underlying reason might be that crystallization of powder in the culture medium adsorbed calcium and phosphorus, resulting in decreased

concentrations of calcium and phosphorus in the culture medium. It has been previously reported that adding calcium and phosphorus to culture systems can promote osteoblast mineralization [11]. It is thus possible that cellular mineralization will be inhibited by decreased concentrations of calcium and phosphorus.

Collagen I, osteocalcin, and ALP are widely used indicators to evaluate MSCs osteogenic differentiation [12]. Undifferentiated MSCs express small amount of Collagen I, but do not express ALP and osteocalcin [13]. After adding osteogenic agent, cells differentiate into osteocytes, which leads to increased expression of Collagen I and the expression of ALP, osteopontin and osteocalcin. The results from the alkaline phosphatase (ALP) activity assay showed that, the ALP activities of MSCs cultured in osteogenic differentiation media containing HA and HA composite powder are significantly higher than those in regular culture medium and in medium with pure ZrO_2. It suggests that HA and HA composite powder may play a role in promoting osteogenic differentiation of MSCs. In addition, we also measured the expression levels of Collagen I and osteocalcin mRNA in MSCs cultured in medium containing composite powder. Our results proved that the cellular expression level of Collagen I was increased and the level of osteocalcin became detectable, indicating the initiation of osteogenic differentiation. Composite powder can stimulate the expression of Collagen I and osteocalcin, suggesting a role in promoting osteogenic differentiation.

5. Conclusion

In summary, this study utilized molecular biology methods to investigate the effects of HA/ZrO_2 composite powder on MSCs proliferation and osteogenic differentiation, which could provide experimental foundations for future clinical applications.

Acknowledgments

The financial support from the Zhejiang Provincial Medical Research Foundation and Science and Technology Development Program of Hangzhou City are gratefully acknowledged.

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