Biomaterials 30 (2009) 5041–5048

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

In vitro and in vivo evaluation of akermanite bioceramics for bone regeneration

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ARTICLE INFO

Article history:
Received 28 March 2009
Accepted 30 May 2009
Available online 9 July 2009

Keywords:
Akermanite
β-Tricalcium phosphate
Extract
Mesenchymal stem cell
Rabbit femur defect models

ABSTRACT

This study investigated the effects of a calcium magnesium silicate bioceramic (akermanite) for bone regeneration in vitro and in vivo, with β-tricalcium phosphate (β-TCP) as a control. In vitro, the human bone marrow-derived mesenchymal stromal cells (hBMSCs) were cultured in an osteogenic medium supplemented with a certain concentration of two bioceramics' extracts for 20 days. An MTT assay showed that akermanite extract promoted proliferation of hBMSC significantly more than did β-TCP extract. The results of alkaline phosphatase (ALP) activity test and the expression of osteogenic marker genes such as ALP, osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP) demonstrated that the osteogenic differentiation of hBMSC was enhanced more by akermanite extract than by β-TCP extract. In vivo, a histomorphology analysis and histomorphometry of the two porous bioceramics implants in rabbit femur defect models indicated that both in early- and late-stage implantations, akermanite promoted more osteogenesis and biodegradation than did β-TCP; and in late-stage implantations, the rate of new bone formation was faster in akermanite than in β-TCP. These results suggest that akermanite might be a potential and attractive bioceramic for tissue engineering.

1. Introduction

Bioceramics, especially calcium phosphate ceramics, due to their good ability of biocompatibility and osteoconductivity, have been widely used for bone tissue repair in orthopedic and dental applications [1–5]. Representative of these ceramic scaffolds is β-tricalcium phosphate (β-TCP), which is a remarkable scaffold that has been extensively investigated for its bone regeneration and bone-engineering applications [6–8]. Because of its similar molecular composition to human bone, β-TCP has been widely used in clinic as bone substitute materials. However, the drawbacks of β-TCP, such as the low compressive strength and fracture toughness in mechanical properties [9] and high resorption rate [10], have hindered its wider applications to bone tissue repair.

Recently, akermanite ($Ca_2MgSi_2O_7$), as a Ca-, Mg- and Si-containing bioceramic, has received more attention due to its more controllable mechanical properties [11] and degradation rate [12]. In previous studies, cells such as marrow-derived or adipose-derived stem cells [13,14] and osteoblasts [15] have displayed better activities of proliferation and osteogenesis on akermanite than on β-TCP. All these findings suggest that this Mg containing silicate ceramic as a bone graft material may meet the requirement of bone regeneration than β-TCP. However, the mechanism of akermanite's bioactivity is still unknown.

The materials chemistry of biomaterials is one of the main factors in the proliferation and differentiation of various cells. Previous studies have shown that degradable bioceramics may regulate the growth and metabolism of various cells by releasing ions such as Si, Ca, P, and Na into the culture medium or body fluid, which also activates the expression of related genes to enhance the formation and mineralization of extracellular matrices [11,16,17]. Studies on the proliferation and osteogenesis of cells cultured in medium supplemented by ceramic extracts could help us to understand more about the superiority of akermanite, in comparison with β-TCP, as a scaffold

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doi:10.1016/j.biomaterials.2009.05.077

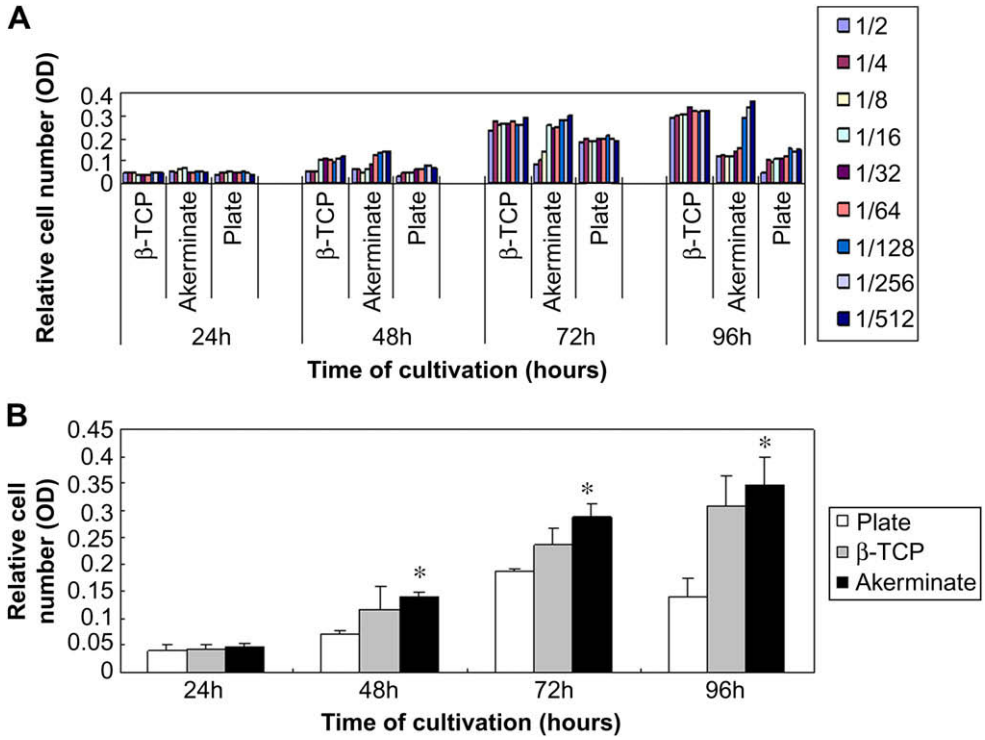

Fig. 1. Cell proliferation time course: MTT assay of hBMSC cultured in growth medium supplemented with different concentrations of akermanite and β-TCP extracts (A) and in the optimal concentration (1/256 of 200 mg/ml) extracts of akermanite and β-TCP (B). The plate represents the hBMSC cultured in the growth medium without any biomaterial extracts. The results represent the mean ± SD of triplicate cultures of one representative experiment of the three performed. Data for these measurements were analyzed using a Student's two-tailed *t*-test, assuming equal variance with Microsoft Excel software (*$p < 0.05$).

biomaterial. In addition, in vivo experiments are critical to evaluating the biocompatibility of materials for their applications as bone grafting or bone tissue engineering materials.

Bone marrow-derived stromal cells (BMSCs), known as a kind of adult stem cells for their ability to differentiate into various types of cells, are widely used as "seed" cells in bone tissue engineering [18–20]. In the present study, the human bone marrow-derived stromal cell (hBMSC) was used as an in vitro model for culturing, using osteogenic mediums supplemented with various bioceramic extracts. In addition, an rabbit femoral condyle model [21,22] was used to investigate the osteogenic ability of akermanite ceramic implants, for comparison with β-TCP ceramics, in vivo.

2. Materials and methods

2.1. Materials

The powders and porous scaffolds of the two bioceramics (Akermanite and β-TCP) were prepared as described previously [23,24]. Both the akermanite and β-TCP cylindrical implants had the same size (5.5 mm in diameter × 8 mm in length) and were sterilized for animal surgery.

2.2. Preparation of biomaterial extracts

1 g each of ceramic akermanite and β-TCP powder was soaked in 5 ml Serum-Free α-MEM (Sigma) and incubated in a humidified 37 °C/5% CO_2 incubator for 24 h. The mixture was then centrifuged for 10 min at 2000*g* at room temperature, and the supernatant was sterilized through a filter (Millipore, 0.22 μm) and stored at 4 °C (ISO10993-1) [25].

2.3. Isolation and culture of hBMSC

The hBMSCs were isolated and cultured as previously described [13,26]. All the cells were cultured in a humidified 37 °C/5% CO_2 incubator and the culture medium was changed every 3 days. To investigate hBMSC proliferation, a growth medium was prepared (α-MEM (Sigma) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin and 100 mg/l streptomycin (Hyclone)). To investigate hBMSC differentiation, an osteogenic medium was prepared (α-MEM supplemented with 10% fetal bovine serum (FBS, Hyclone), 50 mg/ml L-ascorbic acid, 10 mM glycerophosphate, 100 nM dexamethasone (Sigma) and antibiotics (Hyclone)).

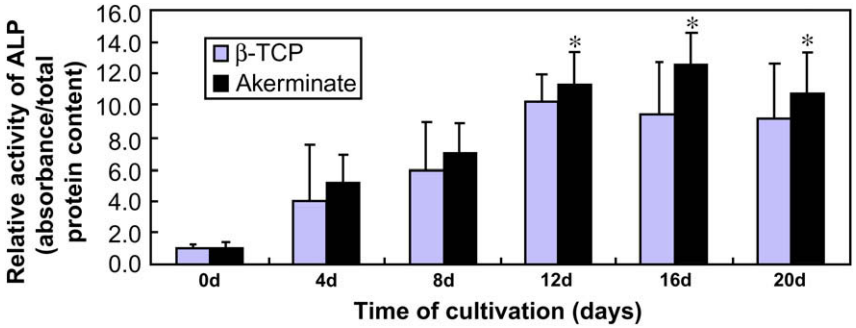

Fig. 2. Measured with the *p*NPP assay, the ALP activity of hBMSC cultured in an osteogenic medium supplemented with ceramic extracts at a concentration of 1/256 dilution of 200 mg/ml. Cells cultured in medium with akermanite extract represented higher ALP activity than did those cultured in medium with the same concentration of β-TCP extract (*$p < 0.05$).

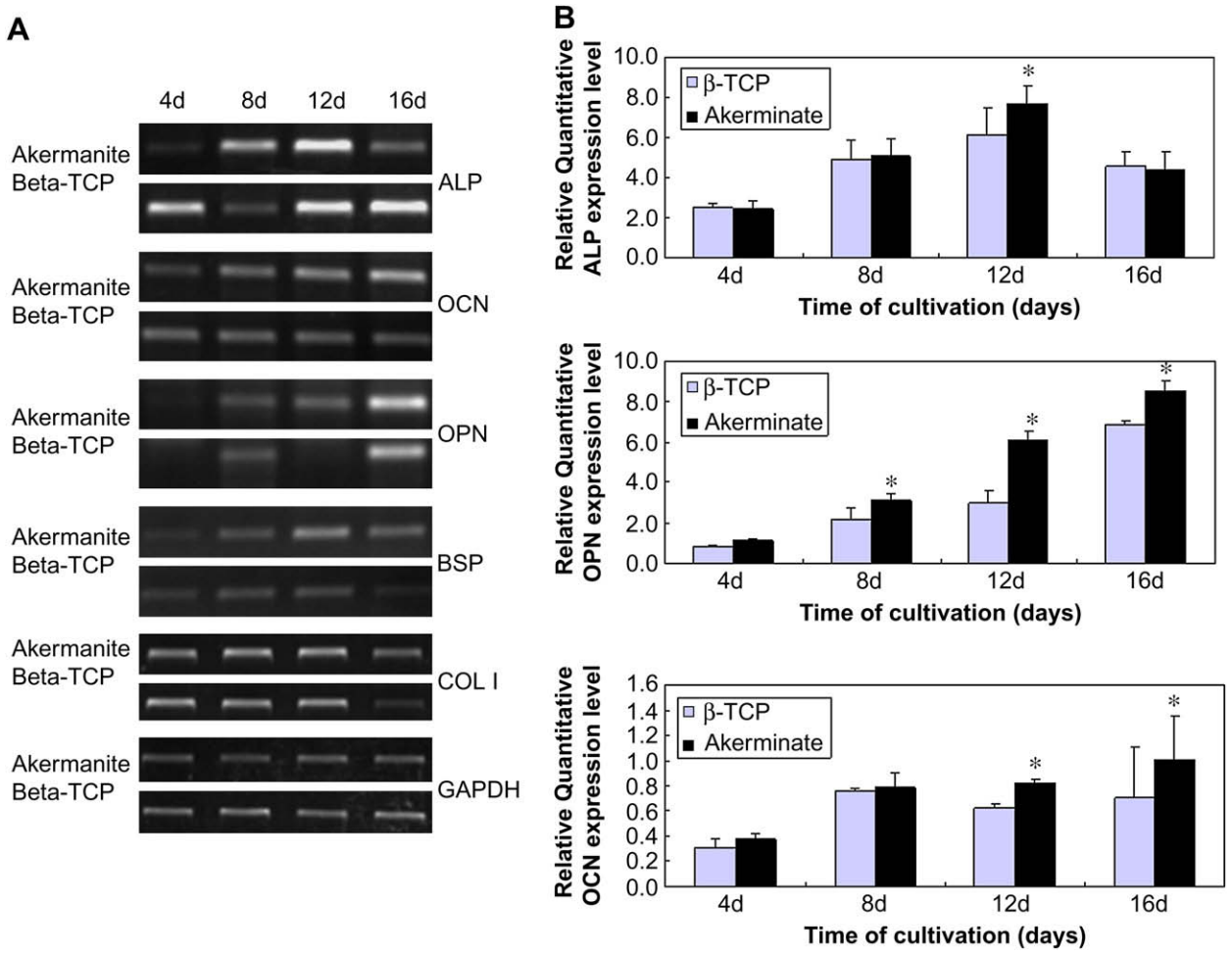

Fig. 3. RT-PCR (A) and Q-PCR (B) for the detection of the expression of osteoblastic marker genes were conducted at 4, 8, 12 and 16 days after culturing hBMSC in osteogenic medium with different ceramic extracts (ALP: alkaline phosphatase, OCN: osteocalcin, OPN: osteopontin, BSP: bone sialoprotein, and COL I: preprocollagen type I). Results were standardized using GAPDH as a housekeeping gene (*$p < 0.05$).

2.4. Cell proliferation assay

To determine the proper concentration of the extracts, a gradient of dilutions was used (to 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512). The hBMSCs were seeded in 96-well plates at 4×10^3 cells/well and cultured in the growth medium described in Section 2.3. After 12 h, the culture medium was replaced by the growth medium supplemented with various concentrations of ceramic extracts then cultured for 24 h, 48 h, 72 h and 96 h. The MTT assay (Sigma) was performed according to the manufacturer's instructions. DMSO (Sigma) was used to stop the reaction, and the absorbance was quantified spectrophotometrically using a microplate reader (Safire^2, TECAN) at wavelengths of 570 nm and 650 nm.

2.5. Alkaline phosphatase (ALP) activity

The hBMSCs were seeded in 6-well plates and cultured for 20 days in the osteogenic medium supplemented with the ceramic extracts described above. The culture medium was changed every 3 days. The alkaline phosphatase (ALP) activity was determined at 405 nm using *p*-nitrophenyl phosphate (*p*NPP) (Sigma) as the substrate and the total protein contents were determined with the BCA method, which is described previously in the literatures [13,27].

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative-polymerase chain reaction (Q-PCR)

The total RNA of hBMSC cultured in media with different concentrations of the ceramic extracts was isolated using the TRIZOL reagent (Invitrogen). Equivalent amount of RNA samples was reverse transcribed for first strand cDNA synthesis (RevertAid ™ M-MuLV, Fermentas) using oligo (dT) as a reverse transcription primer. Then the cDNA was used in gene-specific PCR for GAPDH, ALP, COL I, OPN, BSP and OC. Details of primers and reaction temperatures referencing to the literature [13]. PCR results on 1% agarose gel with 0.5 mg/ml ethidium bromide were photographed under a ultra-violet illumination (Gel Documentation System, UVP, Upland, CA). Quantitative-PCR (Q-PCR) performed by a Real-time PCR machine (ABI, 7900), using a real-time PCR kit (SYBR Premix EX Taq, TaKaRa), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene to normalize.

2.7. Animals and implantation

Thirty-two male New Zealand rabbits (mean body weight = 2.5 kg) were divided into four groups (two for akermanite implantation of 8 weeks and 16 weeks, two for β-TCP implantation of 8 weeks and 16 weeks). The rabbits were operated on while under anesthesia from intramuscular injection of sodium pentobarbital (20 mg/kg, Sigma) under rigorous aseptic conditions. Akermanite and β-TCP porous ceramic scaffolds were implanted into the cavities of the rabbits, which orientated perpendicular to the longitudinal and sagittal axes of the femur [21,22].

All the rabbits were obtained from the Laboratory Animal Center of Shanghai Institute for Biological Science (Shanghai, China, Certificate number SCXK 2003-0003). Handling of the animals was in accordance with policies of Shanghai Jiao Tong University School of Medicine and the National Institute of Health.

2.8. Double fluorescence labeling and harvesting of bone samples

The first label was performed 17 days before sacrificing the rabbits by an intravenous injection, and the second label was performed 2 days before sacrificing. For the 8-week implantations, alizarin red (15 mg/kg, Sigma) was used as the first label and calcein (30 mg/kg, Sigma) as the second label; for the 16-week implantations, tetracycline (20 mg/kg, Sigma) was used as both the first and second labels. The animals were sacrificed after either 8 weeks or 16 weeks of implantation by an

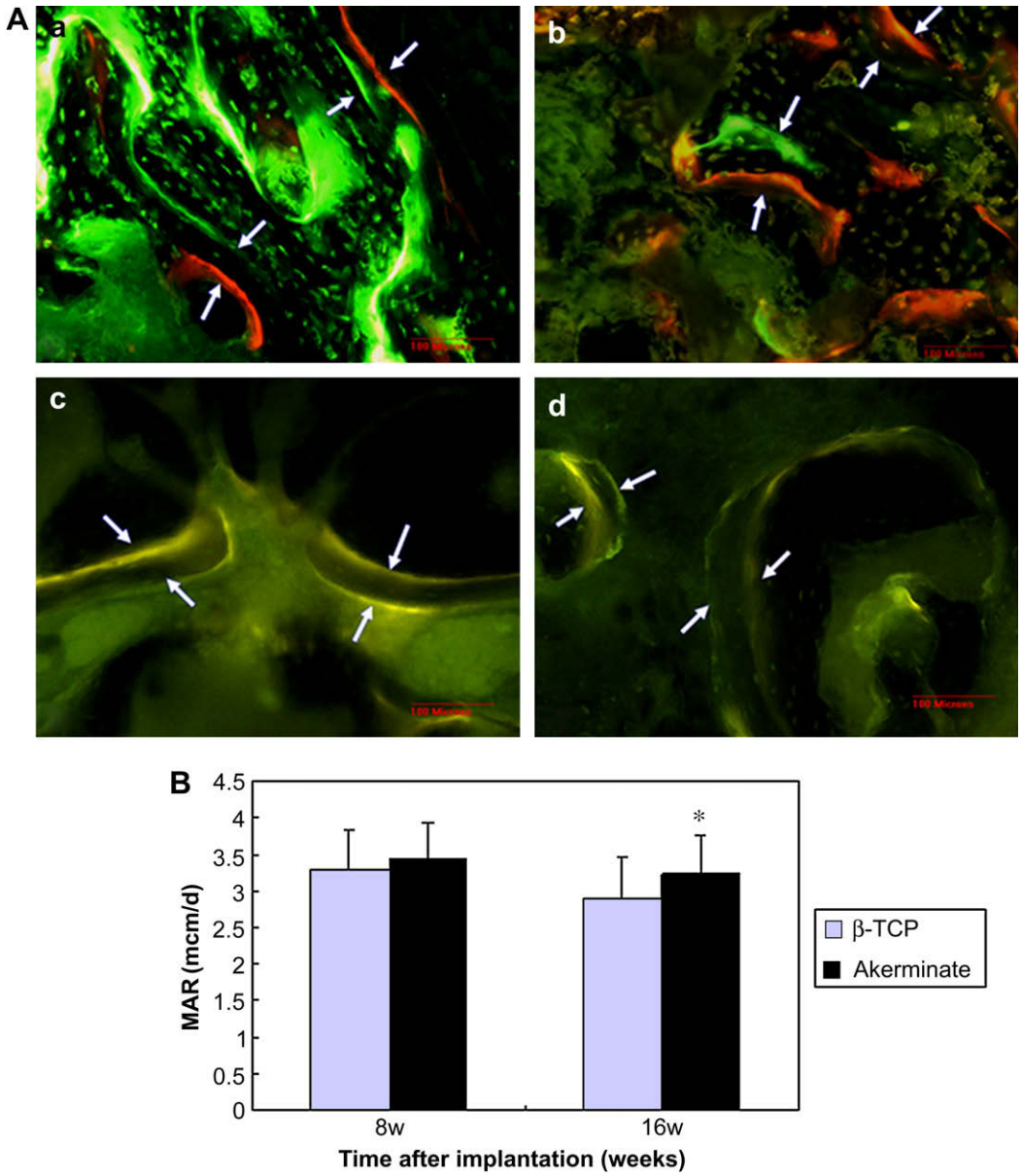

Fig. 4. Distance between two fluorescence labelings (the arrow pointing in A) was used to represent the mineral apposition rate of new bone formation. Double fluorescence labeling was shown in the implants of akermanite (a, c) and β-TCP (b, d) after 8 and 16 weeks, respectively. The quantitation was processed with the BIOQUANT software, and all the measurements were made on ten different double fluorescence labeling sites of each section. (MAR: mineral apposition rate). Original magnification: 10× (*$p < 0.05$).

overdose of pentobarbital sodium, with the femoral condyles and diaphyses removed.

2.9. Histomorphology

The samples, with soft tissue cleaned, were fixed in 10% neutral buffered formaldehyde (pH 7.2) for 10 days, and then rinsed in tap water for 12 h. The fixed samples were then dehydrated in successive alcohol concentrations, cleared with xylene, and embedded in polymethylmethacrylate. After hardening, the samples were cut into 100 μm-thick sections perpendicular to the implants, under cooling water with a sawing microtome (Germany, Leica SP600). The sections then were glued onto a plastic support and polished to 50 μm in thickness and finally were stained with Van Gieson's picric–fuchsine staining.

2.10. Histomorphometry

Different histomorphometric parameters were measured with a microscope (LEICA DM 4000B, German). A semiautomatic image analysis system (BIOQUANT) was used to measure the surfaces of sections. All measurements were made on two sections of each sample.

2.11. Statistical analysis

Statistical analysis was performed with a Student's two-tailed *t*-test, assuming equal variance, with Microsoft Excel software. The significant difference was indicated at *$p < 0.05$ and **$p < 0.01$.

3. Results

3.1. Proliferation of hBMSCs cultured with various ceramics extracts

An MTT assay was used to define an optimal concentration of the two ceramic extracts. Culture media supplemented with different concentrations of ceramic extracts were used to culture the hBMSC. The MTT assay data showed that proliferation proceeded more significantly when the hBMSC was cultured in lower concentrations of ceramic extracts (Fig. 1A). Therefore, a 1/256 dilution of the extracts (200 mg/ml) was chosen as the appropriate

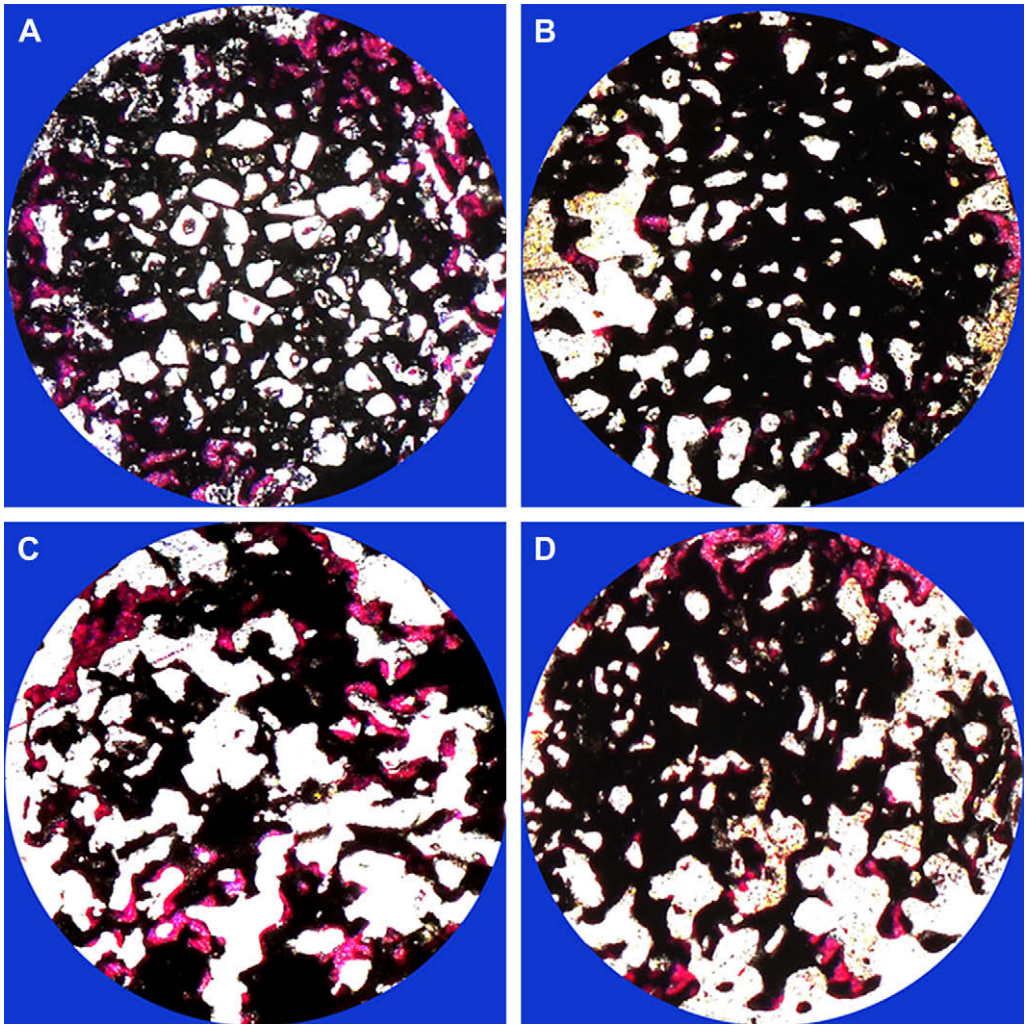

Fig. 5. New bone formation and material degradation of the akermanite (A, C) and β-TCP (B, D) implants after 8 (A, B) and 16 (C, D) weeks (Van Gieson's picric–fuchsine staining of transverse section). Red color indicates newly formed bone. Original magnification: 10×.

concentration for further cell culture experiments. Clearly, the proliferation of hBMSC cultured in the medium supplemented with akermanite extract was greater than that cultured in the medium supplemented with the same concentration of β-TCP extract. Whether cultured in a medium with extracts of akermanite or β-TCP, the hBMSC proliferated faster than did hBMSC cultured without either of the two types of ceramic extract (Fig. 1B).

3.2. The activity of ALP of hBMSC cultured in osteogenic medium supplemented with various ceramic extracts

The hBMSCs were cultured in an osteogenic medium supplemented with akermanite or β-TCP extract (the concentration as 1/256 dilution of 200 mg/ml). As the results in Fig. 2, the hBMSC cultured in both ceramic extracts showed a time-dependent increase in ALP activity, and cells in medium with akermanite extract had a higher ALP activity than did hBMSC cultured in medium with β-TCP extract.

3.3. Osteoblastic marker genes expression of hBMSC cultured in an osteogenic medium supplemented with various ceramic extracts

RT-PCR (Fig. 3A) and Q-PCR (Fig. 3B) were used to detect the expression of several osteoblastic differentiation related marker genes when the hBMSC was cultured in osteogenic medium supplemented with akermanite or β-TCP extracts for 4, 8, 12 and 16 days. The expression of alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP) was significantly enhanced in hBMSC cultured in medium supplemented with akermanite extract, compared with those cultured with β-TCP extract. However, there were no noticeable differences in pre-procollagen type I (COLI) expression.

3.4. Double fluorescence labeling of the implant with different porous ceramic scaffolds

The mineral apposition rate of new bone formation was investigated by measuring the distances of double fluorescence labeling (Fig. 4A). After 8 weeks of implantation, the mineral apposition rate showed no noticeable difference between the akermanite and β-TCP scaffolds and after 16 weeks the mineral apposition rate decreased in both types of ceramic implants. However, at the late stage of implantation (16 weeks), the quantitative measurements (Fig. 4B) showed that the mineral apposition rate of new bone formation in implanted akermanite scaffolds was higher than that in the β-TCP implants.

3.5. The new bone formation and material degradation of the implant with the two ceramic scaffolds

Van Gieson's picric–fuchsine staining of transverse sections was used to show the new bone formation and in vivo bioceramic

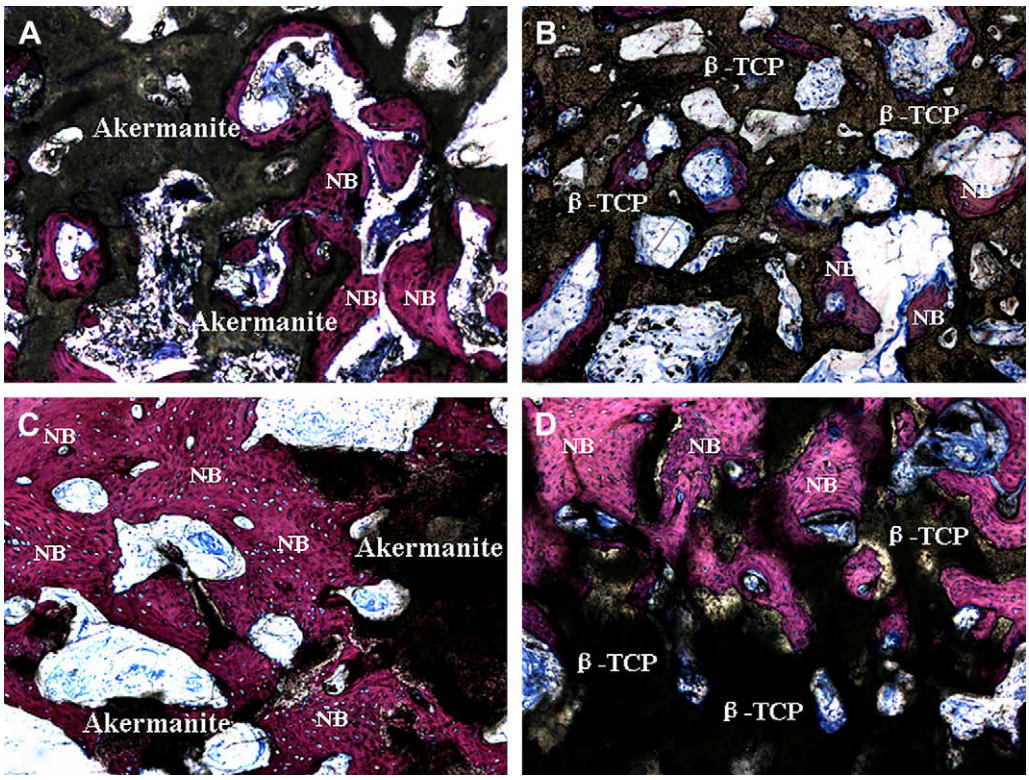

Fig. 6. High magnification images of new bone formation and material degradation of akermanite (A, C) and β-TCP (B, C) implants after 8 (A, B) and 16 (C, D) weeks (Van Gieson's picric–fuchsine staining of transverse section; NB: new bone). Red color indicates newly formed bone. Original magnification: 100×.

degradation. Either with the low (10×, Fig. 5) or the high (100×, Fig. 6) magnification, new bone formation and material degradation were much more evident in the akermanite implantation than in the β-TCP implantation after both 8 and 16 weeks. Compared with β-TCP implants, new bone formation at the edge of the akermanite implants and a slight material degradation were detected after 8 weeks of implantation (Figs. 5A, B, 6A and B). Furthermore, after 16 weeks of implantation (Figs. 5C, D, 6C and D), the newly formed bone tissue penetrated into the center of akermanite implants associated with much more material degradation.

3.6. Histomorphometry of the two different ceramic implants

Different histomorphometric parameters were measured by the semiautomatic image analysis system described above. The results (Fig. 7) showed that the new bone formation volumes were much higher in akermanite implants than in β-TCP implants after both 8 and 16 weeks of implantation. The residual material volumes showed as just the opposite situation: the residual akermanite implant was clearly less than that of the β-TCP implant, indicating a faster degradation of the akermanite ceramics.

4. Discussion

For bone regeneration and bone tissue engineering applications, an ideal biomaterial scaffold should have the properties of favorable biocompatibility, bioconductivity, and biodegradability. An optimal biomaterial used as a bone substitute should not only be a temporary scaffold for supporting the adhesion, growth, proliferation, and differentiation of the 'seed' cells (such as hBMSC), but also be able to degrade into non-toxic products, which can be metabolized via the physiological mechanisms [28].

In the previous studies, it was observed that the proliferation and differentiation of hBMSC cultured on akermanite were significantly enhanced than on β-TCP, which is typically applied clinically as a bone implant bioceramic [13]. As reported in a previous study, when the osteoblasts were treated with the ionic product of

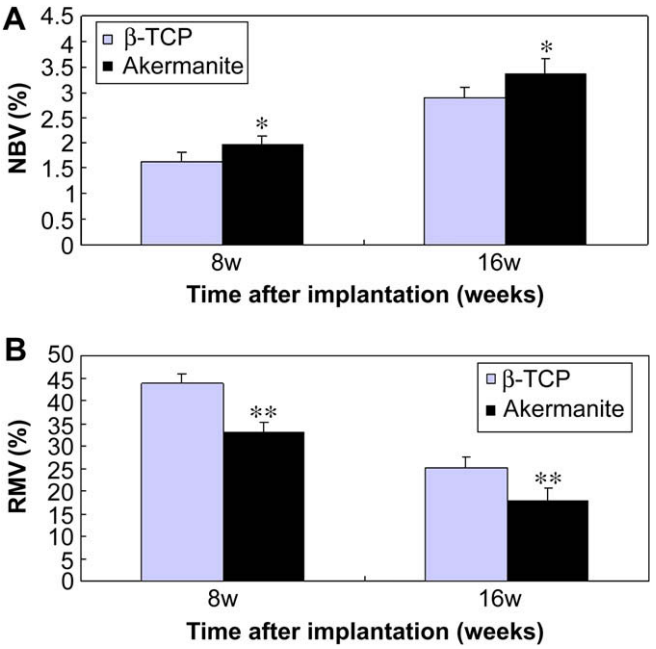

Fig. 7. Histomorphometry showed that the new bone volume (NBV, A) was larger and the residual material volume (RMV, B) was smaller in akermanite implants than β-TCP implants after 8 and 16 weeks implantation. All the measurements were made at 5 different sites for each section ($^{*}p < 0.05$, $^{**}p < 0.01$).

bioglass dissolved for 24 h, the expression of several bone formation-related genes increased [16]. Another study also indicated that inorganic ions could accelerate cell proliferation and differentiation by delivering the mitogenic stimuli and enhancing channel sensitivity [25]. In the present study, the hBMSCs were cultured in media supplemented with a certain concentration of akermanite extracts (1/256 dilution of 200 mg/ml) in order to estimate the effect of the ionic products of akermanite bioceramic dissolution on cell proliferation and differentiation.

In the process of hBMSC osteogenesis, alkaline phosphatase (ALP) is an ectoenzyme involved in the degradation of inorganic pyrophosphate to provide a sufficient local concentration of phosphate for mineralization [29–31]. Therefore, ALP, which is expressed during the post-proliferative period of extracellular-matrix maturation, has been widely recognized as a marker for osteoblast differentiation. At the same, osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP), which occurred later [32–34], have also been used as markers of osteoblastic activity. In this study, based on analysis of ALP activity and the expression of those marker genes, it was found that the proliferation and differentiation of hBMSC were enhanced in culture media with a certain concentration of akermanite extracts (as 1/256 dilution of 200 mg/ml), as compared to culturing with β-TCP extracts.

As previous studies have shown, Si and Mg have been found in the dissolution of akermanite ceramics, but not in that of β-TCP [15]. Si as an important element in metabolism [35,36], collagen synthesis [37], bone mineralization [38–41] and connective tissue cross-linking [42], is essential for skeletal and vascular development [43–45]. Si from bioactive glass dissolution could promote osteoblasts proliferation and gene expression, and improve the nodule formation and mineralization of human primary osteoblasts [16,17,46]. Si–CaP scaffolds could also stimulate angiogenesis and bone apposition in the defect site [45]. Likewise, Mg has a close relationship with the bone tissue mineralization [47–49]. Mg-substituted tricalcium phosphate (β-TCMP) has a good biocompatibility to stimulate adhesion and proliferation of human osteoblast cells [50]. Furthermore, the monticellite ceramics ($CaMgSiO_4$) could release soluble ionic products to stimulate osteoblasts proliferation [51]. However, the effect of cell differentiation was not investigated. Our results further confirmed that ionic Si and Mg are factors that play an important role in enhancing bone marrow-derived stem cell proliferation and differentiation.

For bone implant and bone tissue engineering materials, it is critical to investigate the osteogenetic ability of the materials in vivo, using a standardized animal model. The femur defect model of the New Zealand white rabbit has been widely used [22]. In addition, as an ideal scaffold material, the degradation rate of scaffolds must be appropriately coordinated with the growth rate of the new bone tissue, so as to avoid an early stress in the injured site and allow complete bone regeneration of the defects later [52]. The results of the present study demonstrated that, at the late stage of implantation (after 16 weeks), the newly formed bone tissue penetrated into the center of the akermanite, while in the case of β-TCP implants, the newly formed tissue was mainly on the edge of the ceramics, suggesting that the biodegradation and biocompatibility of akermanite is more appropriate for the new bone formation in vivo than is of β-TCP.

5. Conclusion

In the present study, the in vitro results demonstrated that when cultured with the osteogenic medium supplemented with a certain concentration of akermanite extract, the proliferation and differentiation of the hBMSCs were enhanced, as compared with the culture with β-TCP extract. Moreover, in vivo implantation of two porous bioceramics in a rabbit femur defect model demonstrated that the rate of new bone formation was faster in akermanite than in β-TCP after 16 weeks of the implantation. Furthermore, the degradation of akermanite ceramics was also faster than that of β-TCP ceramics. All the in vitro and in vivo results suggest that the akermanite ceramics is more bioactive and thereby better stimulates the proliferation and differentiation of bone marrow-derived stem cells. Also it enhances bone regeneration because of its superior degradation rate and biocompatibility. Therefore, it might be used as a potential and attractive bioceramic for bone regeneration and bone tissue engineering applications.

Acknowledgment

This work is supported by the National Basic Science Research Program of China (973 Program) (Grant Nos. 2005CB522704 and 2007CB936101), the Natural Science Foundation of China (Grant Nos. 30730034 and 30600628), Science and Technology Commission of Shanghai Municipality (Grant No. 08JC1420800) and Program for Shanghai Key Laboratory of Orthopaedic Implant (Grant No. 08DZ2230330).

Appendix

Figures with essential color discrimination. Figures of this article may be difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.05.077.

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