# PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF HUMAN PERIODONTAL LIGAMENT CELLS ON AKERMANITE AND β-TCP BIOCERAMICS

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## Abstract

The purpose of this study was to investigate the effects of akermanite as compared to β-TCP on attachment, proliferation, and osteogenic differentiation of human periodontal ligament cells (hPDLCs). Scanning electron microscopy (SEM) and actin filament labeling were used to reveal attachment and growth of hPDLCs seeded on β-TCP and akermanite ceramic. Cell proliferation was tested by lactic acid production and MTT analysis, while osteogenic differentiation was assayed by alkaline phosphatase (ALP) expression and real-time polymerase chain reaction (PCR) analysis on markers of osteopontin (OPN), dentin matrix acidic phosphoprotein-1 (DMP-1), and osteocalcin (OCN), and further detected by enzyme-linked immunosorbent analysis (ELISA) analysis for OCN expression. Besides, the ions released from akermanite and their effect on hPDLCs was also measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES), MTT analysis, ALP expression and real-time PCR analysis. hPDLCs attached well on both ceramics, but showed better spreading on akermanite. hPDLCs proliferated more rapidly on akermanite than β-TCP. Importantly, osteogenic differentiation of hPDLCs was enhanced on akermanite compared to β-TCP. Besides, Ca, Mg and Si ions were released from akermanite, while only Ca ions were released from β-TCP. Moreover, more pronounced proliferation and higher osteogenic gene expression for hPDLCs cultured with akermanite extract were detected as compared to cells cultured on akermanite. Therefore, akermanite ceramic showed an enhanced effect on proliferation and osteogenic differentiation of hPDLCs, which might be attributed to the release of ions containing Ca, Mg and Si from the material. It is suggested that akermanite ceramics may serve as a potential material for periodontal bone regeneration.

**Keywords:** Akermanite, human periodontal ligament cells, bone regeneration.

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## Introduction

Alveolar bone loss/defect resulting from inflammation, trauma and surgical resection, (Pihlstrom *et al*., 2005; Shang *et al*., 2010), would usually fail to provide sufficient support and retention for dentures or dental implants during prosthodontics treatment (Cawood *et al*., 1991). Reconstruction of alveolar bone is an important part of therapeutic procedures in periodontal tissues regeneration therapy and pre-prosthetic, pre-implantology surgery (Orciani *et al*., 2009; Wang *et al*., 2009). Several approaches have been developed for repairing alveolar bone loss/defect, including autologous bone grafts (Schliephake *et al*., 1997; Cordaro *et al*., 2002), distraction osteogenesis (Chiapasco *et al*., 2001), and guided bone regeneration (GBR) (Buser *et al*., 1990). These methods may achieve certain effects on bone regeneration, but also present limitations for clinical application, such as extensive harvest of healthy tissue for autologous bone grafts (Wang *et al*., 2009), intraoral distraction devices for distraction osteogenesis, and secondary infections for GBR. The development of biomedical material has made this an attractive alternative with great potential for alveolar bone regeneration (Yuan *et al*., 2010).

As a material for bone regeneration, bioceramics usually have good osteoconductivity and bioactivity with a similar mineral phase of natural bone tissue. Among bioceramics, β-TCP serves as a typical representative that has been extensively studied and clinically used for bone regeneration (Tanimoto *et al*., 2008). However, its shortcomings, e.g., improper degradation rate, insufficient mechanical properties such as low fracture toughness, and lack of properties for enhancing cell differentiation *in vitro* and stimulating bone regeneration *in vivo* limit its further application (Bouler *et al*., 2000; Miranda *et al*., 2008). Therefore, there is a requirement for development of new bioceramics with suitable degradation rate, superior mechanical properties, sufficient bioactivity or osteoinductivity for bone regeneration.

Akermanite ($Ca_2MgSi_2O_7$) as a novel bioceramic containing Ca, Mg and Si ions, has relatively more controllable degradation rate and mechanical properties such as fracture toughness, bending strength and Young's modulus (Kokubo *et al*., 1991; Wu and Chang, 2007). More importantly, it obviously stimulated more proliferation and osteogenic differentiation of osteoblasts, bone marrow stromal cells (BMSCs) and adipose-derived stem cells (ADSCs) as compared to β-TCP (Wu *et al*., 2005; Sun *et al*., 2006; Wu and Chang, 2006; Wu *et al*., 2006; Liu *et al*., 2008). All these preliminary studies indicated that akermanite bioceramic might serve as a suitable biomaterial for bone regeneration.

Periodontal ligament cells (PDLCs) are considered as a key cell type for periodontal tissues regeneration including regeneration of alveolar bone, periodontal ligament (PDL) and cementum, due to their unique localization and mesenchymal stem cell-like properties with osteogenic and fibrogenic capacity. It was confirmed that PDLCs could regenerate cementum and periodontal ligament in rat and canine models (Akizuki *et al*., 2005; Hasegawa *et al*., 2005; Flores *et al*., 2008a; Flores *et al*., 2008b). As for alveolar bone regeneration, PDLCs shared many osteoblast-like properties, such as high alkaline phosphatase (ALP) activity, expression of bone-associated proteins, and formation of mineralized nodule in *in vitro* studies (Arceo *et al*., 1991; Wescott *et al*., 2007). *In vivo*, the repair of periodontal intrabony defects with autologous PDLCs/CALCITITE 4060-2 implantation was reported in three clinical cases (Feng *et al*., 2010). Besides, compared to BMSCs which have osteogenic capacity with intrinsic alterations to aging, PDLCs appeared to have no differences between those derived from young and older people (Zhou *et al*., 2008). Based on the above factors, our hypothesis is that akermanite may be able to induce osteogenic differentiation of the PDLCs, which have good prospects as seed cells for periodontal bone regeneration.

In order to explore the effects of akermanite bioceramic on human PDLCs (hPDLCs), and the potential of akermanite as an osteogenic biomaterial for periodontal tissues regeneration, the attachment, proliferation and osteogenic differentiation of hPDLCs on akermanite were investigated for the first time and compared with β-TCP ceramics *in vitro*. More importantly, the direct effect of Ca, Mg, Si ions released from akermanite on hPDLCs was also explored in this study to determine whether these ions have played a role on these effects.

## Materials and Methods

### Isolation, culture and identification of hPDLCs

hPDLCs were prepared from adult healthy premolars extracted for orthodontic reasons. The use of hPDLCs in this study was approved by the Institutional Review Board, following informed consent taken from the patients. Teeth were washed with phosphate buffered saline (PBS) three times, and periodontal ligament tissue was dissected from the mid-third portion of premolar roots with a sharp surgical scalpel. Then, tissue explants were minced into small pieces and placed onto six-well tissue culture plates. The explants were kept in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 mg/L streptomycin (Invitrogen, Carlsbad, CA, USA), and cultured in a humidified 37 ºC/5 % $CO_2$ incubator. Cells were allowed to grow out of the explants and reached confluence. hPDLCs were then passaged, expanded, and identified for future use. In the present study, cells after passage 3 were used as previously described (Hou *et al*., 2007; Wescott *et al*., 2007).

hPDLCs were identified with hematoxylin-eosin (HE) staining, and immunocytochemical staining for vimentin and cytokeratin. HE staining was performed with the HE Color Development Kit (Beyotime, Jiangsu, China) for cell morphology observation. For immunocytochemical staining, hPDLCs were fixed with 4 % paraformaldehyde for 30 min, and then permeabilized with 0.1 % Triton X-100 for 20 min followed by blocking with 1 % bovine serum albumin (BSA, Sigma, St. Louis, MO, USA). Cells were incubated with primary antibody to vimentin or cytokeratin overnight at 4 °C, followed by HRP-conjugated secondary antibody at room temperature for 1 h. Finally, the diaminobenzidine (DAB) kit was used to develop the color and the slides counterstained with hematoxylin. Secondary antibody was incubated in the absence of primary antibody as negative controls.

### Preparation of ceramic discs and cell seeding

β-TCP and akermanite bioceramic discs with a dimensions of 10 mm diameter × 0.8 mm were fabricated as previously described (Wu *et al*., 2006; Liu *et al*., 2008). Briefly, β-TCP powders were synthesized by a precipitation method using calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and ammonium phosphate dibasic ($(NH_4)_2HPO_4$), while the β-TCP ceramic discs were prepared by uniaxial pressing of the β-TCP powders at 4 MPa and sintering at 1100 °C for 3 h with a heating rate of 2 °C/min. Akermanite powders were synthesized by a sol-gel process using tetraethyl orthosilicate ($(C_2H_5O)_4Si$, TEOS), magnesium nitrate hexahydrate ($Mg(NO_3)_2{\cdot}6H_2O$) and calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), while the akermanite ceramic discs were prepared by uniaxial pressing of the akermanite powders under 10 MPa and sintering at 1370 °C for 6 h. These two ceramics were identified by X-ray diffraction (XRD, Geigerflex, Rigaku, Japan), Scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS, JEOL, Tokyo, Japan). Before being used for the following cell seeding studies, the ceramic discs were rinsed in distilled water three times and autoclaved at 125 ºC/0.14 MPa for 30 min. All discs were incubated in culture medium for 24 h before use.

hPDLCs used for seeding were enzymatically lifted from culture dishes with trypsin/EDTA (0.25 % and 0.53 mM, respectively), and centrifuged for 5 min at 1000 rpm. The cells were resuspended in fresh culture medium, and then loaded on β-TCP and akermanite ceramic discs, respectively. A seeding density of $5 \times 10^3$ cells/disc was applied for studies on attachment and proliferation, while a higher density of $2 \times 10^4$ cells/disc (near confluence) was used for osteogenic differentiation assays as previously described (Sun *et al*., 2006).

**Adhesion and growth of seeded hPDLCs**

hPDLCs were cultured on β-TCP and akermanite discs for 1 and 7 d, and fixed in 2.5 % glutaraldehyde overnight at 4 ºC. These samples were washed three times with PBS for 5 min, and then dehydrated by increasing the concentration of ethanol. Finally the samples were dried by hexamethyldisilazane, sputter-coated with gold and examined by SEM (JEOL) to observe the adhesion and growth of hPDLCs on the two ceramics.

**Morphology of seeded hPDLCs**

The hPDLCs were cultured on β-TCP and akermanite discs for 24 h, fixed in 4 % paraformaldehyde for 30 min, and then rinsed with PBS. These samples were treated with 0.1 % Triton X-100 in PBS to permeabilize the cells for 20 min and then blocked with 1 % BSA for 20 min. The actin cytoskeletons were labeled by incubating with Phalloidia-TRITC (Sigma) for 30 min. After rinsing with PBS, the cell nuclei was contrast-labeled in blue by 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma), then mounted on glass slides using Fluoromount (Sigma). The actin cytoskeletons of cells were visualized with a confocal laser scanning microscope (Leica, Wetzlar, Germany).

**Cytotoxicity of hPDLCs seeded on two ceramics**

The potential cytotoxicity of hPDLCs seeded on the two ceramics was evaluated using the Live/Dead Double Staining Kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions at 24 h after seeding. Live cells were stained with polyanionic dye calcein (fluorescing green), while dead cells with damaged membranes for EthDIII entering and binding to nucleic acids fluoresced red in the view of a fluorescence microscope (Leica).

**Cell metabolism and proliferation assay**

For the cell metabolism assay, the content of lactate in the culture medium was measured using the L-lactate kit (EQUL Inc, Houston, Texas, USA) (Sun *et al*., 2006). Briefly, 15 μL of the culture medium from both groups at 0, 48 and 72 h after seeding were measured according to the manufacturer's instructions. The total production of lactate was quantified spectrophotometrically at 340 nm by ELX Ultra Microplate Reader (Bio-tek, Winooski, VT, USA). In addition, the cells cultured on discs were detached using trypsin/EDTA (0.25 % w/v trypsin, 0.02 % EDTA), and centrifuged for 5 min at 1000 rpm after being washed twice with PBS. Finally, the cells were resuspended in lysis buffer with 0.2 % NP-40, and total cellular protein content was determined with the Bradford method in aliquots of the same samples with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA), read at 630 nm and calculated according to a series of BSA (Sigma) standards. The lactic acid production was expressed as mg/mL per milligram of total cellular proteins. Analysis of blank discs was carried out as a control.

For the cell proliferation assay, hPDLCs were cultured on both ceramic discs in 24-well plates up to 8 d. Three pieces of co-cultured discs for each group were washed twice with PBS. 400 μL DMEM with supplement 40 μL 5 mg/mL MTT (Amresco, Solon, OH, USA) solution was added and incubated at 37 °C for 4 h to form MTT formazan. Then the medium was replaced with 400 μL dimethyl sulfoxide (DMSO, Sigma) and vibrated for 15 min in order to dissolve the formazan. Finally, the absorbance was measured at 490 nm by ELX Ultra Microplate Reader (Bio-tek).

**Alkaline phosphatase (ALP) staining and activity assay**

The hPDLCs were seeded on β-TCP and akermanite discs under culture conditions in either growth medium (DMEM, 10% FBS) or osteogenic medium (DMEM, 10 % FBS, 50 μg/mL L-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone). ALP staining was performed according to the manufacturer's instructions (Rainbow, Shanghai, China) at day 10. For the level of ALP activity, it was determined as previously described at day 4, 7, 10 after cell seeding (Sun *et al*., 2006; Liu *et al*., 2008). Briefly, the cells were detached from discs using trypsin/EDTA, and centrifuged for 5 min at 1000 rpm after being washed twice with PBS. The cells were resuspended in lysis buffer with 0.2 % NP-40. ALP activity was determined by absorbance at 405 nm using p-nitrophenyl phosphate (pNPP, Sigma) as the substrate. Each sample was respectively mixed with pNPP (1 mg/mL) in 1M diethanolamine buffer and incubated at 37 ºC for 15 min. The reaction was stopped by the addition of 3 N NaOH to reaction mixture. Enzyme activity was quantified by absorbance at 405 nm (Bio-tek). Total protein content was determined with the Bradford method in aliquots of the same samples with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA), read at 630 nm and calculated according to a series of BSA (Sigma) standards. ALP activity was expressed as absorbance at 405 nm (OD value) per milligram of total cellular proteins. All experiments were done in triplicate.

**RNA extraction and quantitative real-time PCR assay**

Total RNA was isolated from cells cultured on β-TCP and akermanite discs in either growth medium or osteogenic medium at day 4 and 7, respectively. At each time point, the cells were detached and centrifuged for 5 min at 1000 rpm after being washed twice with PBS, then, the cells were resuspended in Trizol reagent (Invitrogen). Addition of chloroform to the cell extract in Trizol reagent separated the RNA into an aqueous phase, and then recovered and precipitated with isopropanol. The RNA deposition was rinsed by 70 % ethanol treated with the RNase inhibitor diethyl pyrocarbonate (DEPC, Sigma), and then solubilized in sterile DEPC water. The RNA was used for synthesizing complementary DNA (cDNA) with PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) analysis was performed with the Bio-Rad real-time PCR system (Bio-Rad, Hercules, CA, USA) on markers of osteopontin (OPN), Dentin matrix acidic phosphoprotein-1 (DMP-1) and osteocalcin (OCN), with glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as the house-keeping gene for normalization. Primer sequences for OPN, DMP-1, OCN, and GAPDH are listed in Table 1. Finally, the real-time PCR reaction was run at

**Table 1**: Primer sequences for Real-time PCR

| Gene | Primers (F=forward; R=reverse) | Accession numbers | Product size (bp) |
|---|---|---|---|
| OPN | F: 5'CATGAGAATTGCAGTGATTTGCT3'<br>R: 5' CTTGGAAGGGTCTGTGGGG3' | NM_000582.2 | 186 |
| DMP-1 | F: 5'CGAATCGTTGAGGCTCTCACTGGATTCG3'<br>R: 5'CTCGCACACACTCTCCCACTC3' | NM_004407.3 | 82 |
| OCN | F: 5'GAAGCCCAGCGGTGCA3'<br>R: 5'CACTACCTCGCTGCCCTCC3' | NM_199173.3 | 70 |
| GAPDH | F: 5'TTCGACAGTCAGCCGCATCTT3'<br>R: 5'ATCCGTTGACTCCGACCTTCA3' | NM_002046.3 | 90 |

95 °C for 10 min followed by 95 °C for 30 s; 60 °C for 1 min and 72 °C for 1 min for 40 cycles; the dissociation curve at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. All experiments were done in triplicate.

**ELISA analysis for OCN protein**

OCN content released from cells cultured on β-TCP and akermanite discs in either growth medium or osteogenic medium was measured at day 7 and 10 after cell seeding. The human osteocalcin enzyme-linked immunosorbent assay (ELISA) Kit (Bender, Burlingham, CA, USA) was used to determine OCN content following the manufacturer's instruction. The OCN concentration was determined by correlation with a standard curve, and further normalized with the total cellular protein content as described above. Analysis of blank disks was treated as controls. All experiments were done in triplicate.

**The ions released from akermanite and their effect on hPDLCs**

In order to measure Ca, Mg, and Si release from β-TCP and akermanite ceramics, one disc of each ceramic was added in 1 mL growth medium or osteogenic medium separately. Then the medium was collected at day 4, 7 and 10. The concentrations of Ca, Mg, and Si in growth medium or osteogenic medium were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Varian, Palo Alto, CA, USA). More importantly, the effect of Ca, Mg, and Si released from akermanite ceramic was determined by MTT analysis, ALP activity analysis and real-time PCR analysis on osteogenic genes.

To determine the proper ion concentration in extracts to be used in the following studies, a series of dilutions was tested (to 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512) for the solution obtained by soaking β-TCP or akermanite disc in 1 mL medium as described in previous studies (Sahlin-Platt *et al*., 2008; Huang *et al*., 2009; Ni *et al*., 2009). Briefly, the hPDLCs were seeded in 96-well plates at $5 \times 10^3$ cells/well and cultured in the growth medium. After culture for 1 d, the cells were cultured in the medium supplemented with various concentrations of β-TCP or akermanite extract, and then cultured for 1, 4 and 7 d, respectively. The MTT assay (Amresco) was performed, DMSO (Sigma) was used to dissolve the formanzan, and the absorbance was measured at 490 nm by an ELX Ultra Microplate Reader (Bio-tek). Analysis of hPDLCs cultured in the medium alone was treated as control. All experiments were done in triplicate.

The hPDLCs were seeded in 6-well plates and cultured in growth medium or osteogenic medium supplemented with proper concentrations of β-TCP or akermanite extract determined above. ALP staining was performed according to the manufacturer's instructions (Rainbow) at day 10. While ALP activity at day 4, 7 and 10 was determined at 405 nm using pNPP (Sigma) as the substrate and the total cellular protein contents were determined with the Bradford method, as described above. The total RNA of hPDLCs at day 4 and 7 was isolated using the TRIZOL reagent (Invitrogen). The cDNA was synthesized with PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instruction. Real-time PCR analysis was performed with Bio-Rad real-time PCR system (Bio-Rad, Hercules, CA, USA) as described above, on markers of OPN, DMP-1 and OCN, with GAPDH as the house-keeping gene for normalization. Analysis of hPDLCs cultured in growth medium or osteogenic medium alone was used as control. All experiments were done in triplicate.

**Statistical analysis**

All measurements are presented as mean ± standard deviation. Statistical analysis for the assays of hPDLCs seeded on two ceramics was performed by independent-samples t-tests assuming equal variance, while one-way ANOVA tests were processed for the analysis on the effects of Ca, Mg, and Si released from akermanite ceramic using SPSS (Chicago, IL, USA) 11.0 software. A value of $p < 0.05$ was considered statistically significant.

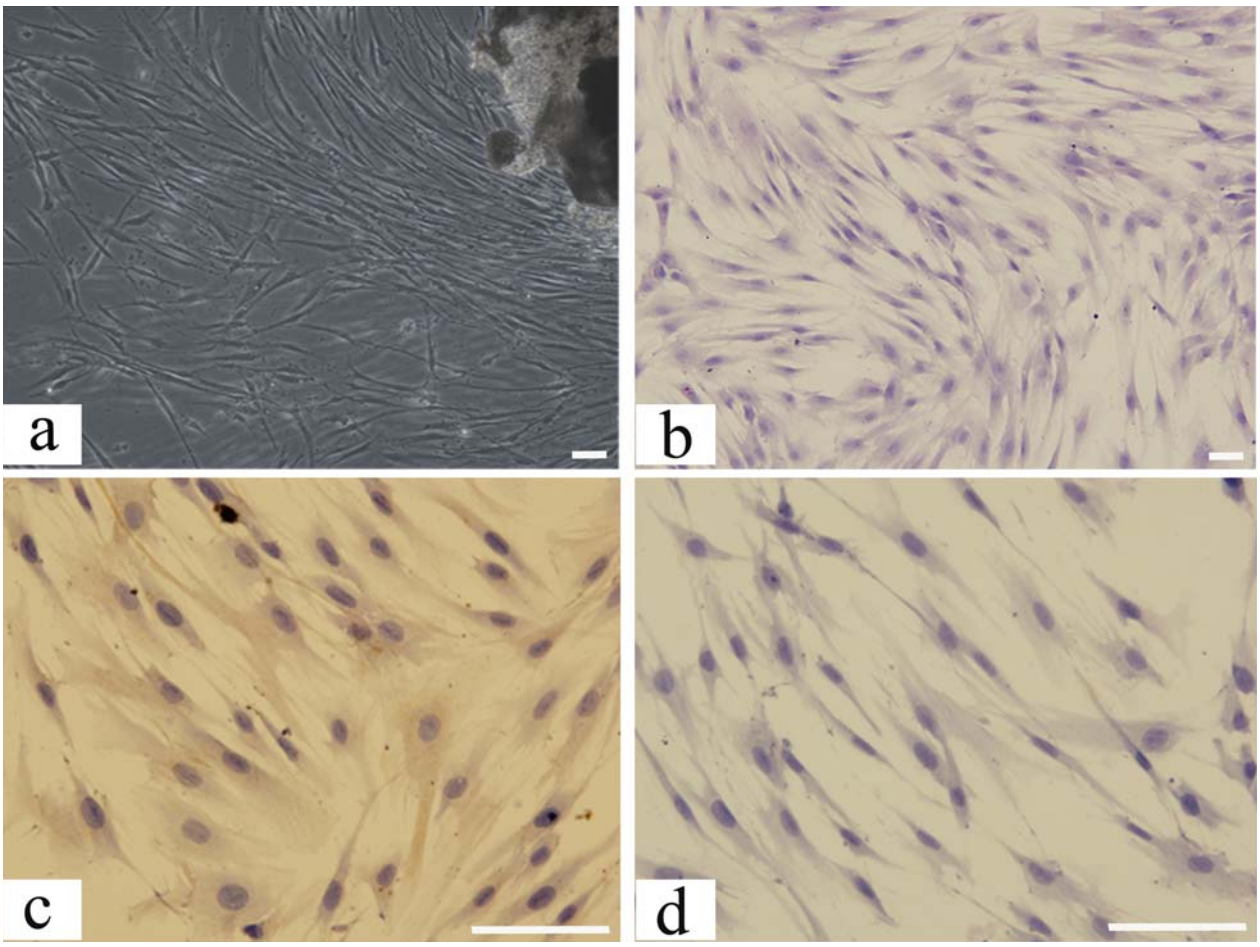


**Fig. 1**. hPDLC culture and identification. The hPDLCs were found growing around the periodontal ligament tissue explants 1-2 weeks after initial incubation **(a)**. The cells after passage 3 illustrated a shape of short fibroblasts with HE staining **(b)**, and showed positive for vimentin **(c)** and negative for cytokeratin **(d)** with immunocytochemical staining. Scale bar = 100 μm.

## Results

### Isolation, culture and identification of hPDLCs

Primary cultured hPDLCs were found growing around the periodontal ligament tissue explants 1-2 weeks after initial incubation (Fig. 1a). Cells that had reached confluence were passaged, and the expanded cells after passage 3 displayed a short fibroblast-like morphology as shown by HE staining (Fig. 1b). The plasma dominant staining for vimentin, and a negative staining for cytokeratin confirmed the mesenchymal origin of the cells (Fig. 1c,d).

### Characterization of β-TCP and akermanite ceramics

The X-ray diffraction (XRD) pattern in Fig. 2a confirmed that the samples were pure β-TCP (PDF card No. 09-0169) and akermanite (PDF card No. 35-0592) phase, respectively. Fig. 2 b,c showed the surface morphologies of β-TCP and akermanite ceramics; most particles were sintered and some micropores were evident both on β-TCP and akermanite ceramics. The corresponding EDS analysis showed that O, Mg, Si, and Ca were detected on the surface of akermanite, while only O, P, and Ca were present on β-TCP (Fig. 2d,e).

### Adhesion and morphology of hPDLCs seeded on ceramic discs

In order to show cell adhesion and morphology on the two ceramic discs, ceramic discs cultured with hPDLCs were examined by SEM 1 and 7 d after cell culture. At day 1 after seeding, cells attached on the surface of both ceramic discs, while the cells on akermanite appeared much flatter and spread out as compared to those on β-TCP (Fig. 3a,c). Cells grew well on both β-TCP and akermanite ceramic discs at day 7 (Fig. 3b,d).

Actin cytoskeletons were labeled to observe cell morphology at 24 h after seeding on two ceramic discs. On both β-TCP and akermanite discs, the cells maintained their typical fibroblastic morphology, while the actin filaments were more fully spread on akermanite discs compared to β-TCP discs. More importantly, actin filaments with regular directions on akermanite discs were well-defined by the actin microfilament system ranging parallel to the long axis of the cells (Fig. 4d-f), while the actin filaments had irregular directions were visualized on β-TCP discs (Fig. 4a-c).

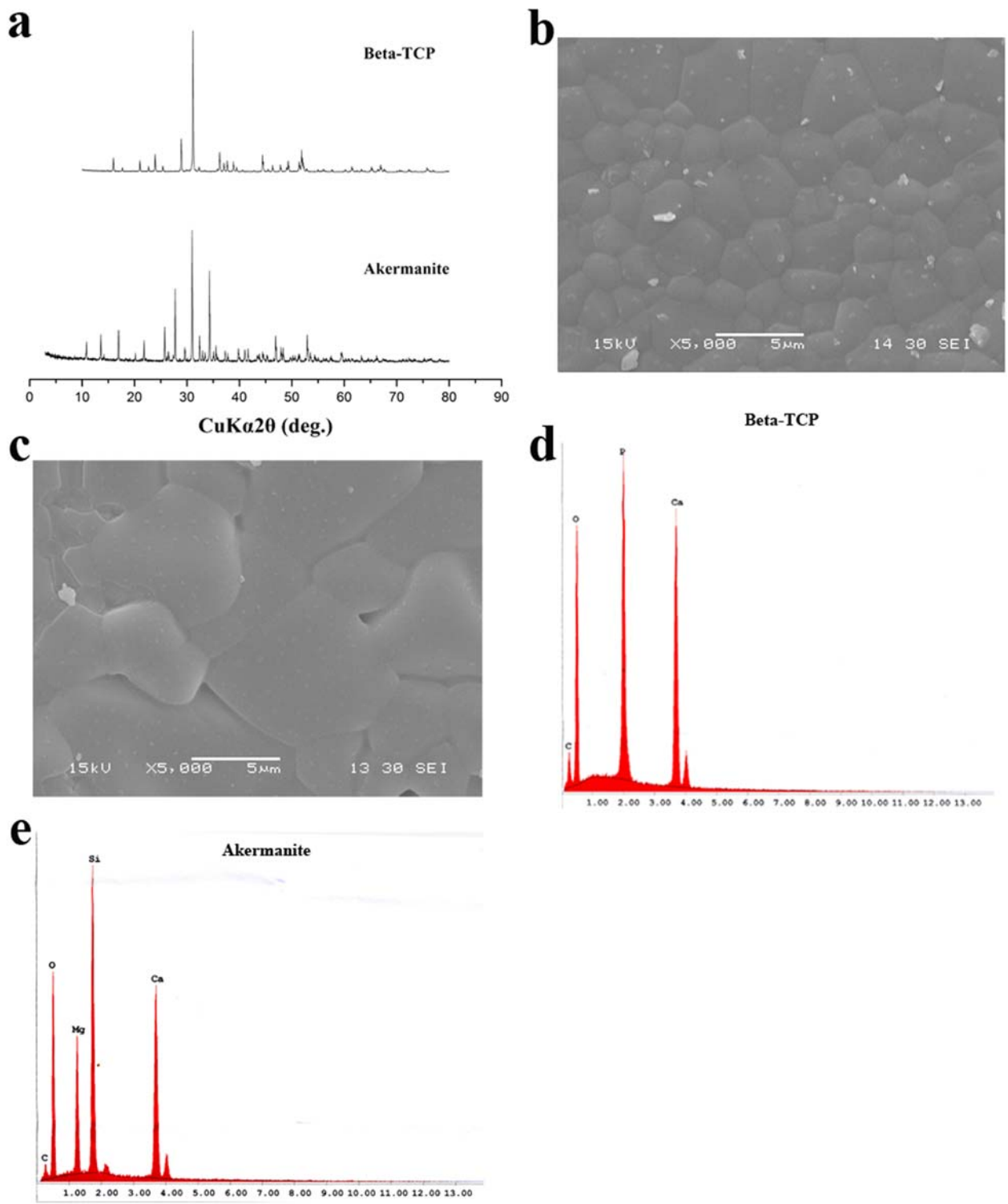


**Fig. 2**. Characterization of β-TCP and akermanite ceramics. XRD patterns of β-TCP and akermanite **(a)**. **(b, c)** represent SEM micrographs of β-TCP and akermanite ceramics, while EDS analysis of these two ceramics is shown in **(d, e)** (**b, d**: β-TCP; **c, e**: akermanite). Scale bar = 5 μm.

**Cytotoxicity, metabolism and proliferation of hPDLCs seeded on ceramic discs**

Live/Dead Double Staining at 24 h after cell seeding on β-TCP or akermanite was conducted to evaluate cytotoxicity of the two ceramic discs. The majority of cells remained viable on both β-TCP and akermanite, indicating that akermanite possessed no obvious cytotoxicity as compared to β-TCP (Fig. 5a-f).

The lactic acid production assay for metabolism showed that lactic acid production of hPDLCs cultured on akermanite was higher than for cells cultured on β-TCP ($p < 0.05$) (Fig. 5g). The MTT assay was performed to continuously compare cell proliferation of hPDLCs cultured on β-TCP or akermanite discs. As shown in Fig. 5h, proliferation of hPDLCs proceeded more significantly on akermanite than on β-TCP.

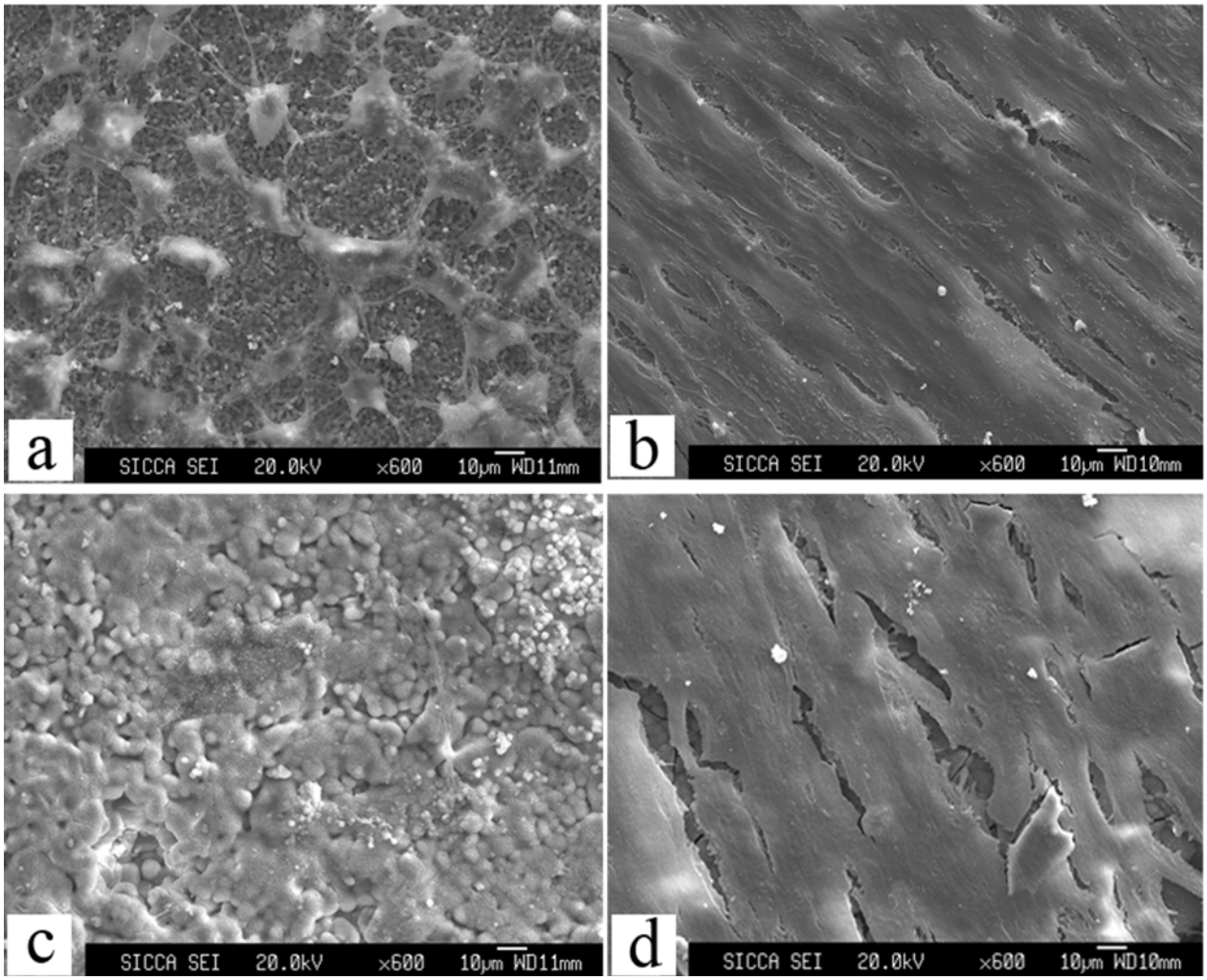


**Fig. 3**. SEM observation of cell adhesion on β-TCP and akermanite surfaces. At 1 d after hPDLCs were seeded on β-TCP **(a)** and akermanite **(c)**, the cells on akermanite appeared much flatter and spread out as compared to β-TCP. At 7 d, cells grew well both on β-TCP **(b)** and akermanite **(d)** ceramic discs. Scale bar = 10 μm.

**Detection of ALP expression for hPDLCs seeded on ceramic discs**

ALP expression of hPDLCs cultured on β-TCP and akermanite discs in both growth and osteogenic medium was examined. As shown in Fig. 6a-d, ALP staining was more intensive for hPDLCs on akermanite discs than those on β-TCP discs in both growth medium and osteogenic medium at day 10. Similarly, the analysis of quantitative examination data showed that the ALP activity of cells cultured on the two types of ceramic disc increased over time throughout the assay period with a higher value in the osteogenic medium. The ALP activity of the hPDLCs seeded on akermanite was higher than that of the cells on β-TCP with a statistically significant difference found at day 10 (Fig. 6e).

**Osteogenic gene expression for hPDLCs seeded on ceramic discs**

The osteogenic genes OPN, DMP-1 and OCN were assayed by real-time PCR at days 4 and 7, after hPDLCs seeding on β-TCP and akermanite discs (Fig. 7a-c). Compared to those on β-TCP, osteogenic gene expression of hPDLCs on akermanite cultured in growth medium increased, with a statistically significant enhanced expression of DMP-1 and OCN at day 7. Upon incubation in osteogenic medium, increased osteogenic gene expression on akermanite appeared much earlier and stronger. The expression of OPN, DMP-1 and OCN increased for hPDLCs on akermanite with a significant difference compared to those on β-TCP as early as at day 4, and became more pronounced when the culture time was extended to day 7.

**Detection of OCN protein for hPDLCs seeded on ceramic discs**

The OCN protein released from hPDLCs cultured on β-TCP or akermanite discs was detected at day 7 and 10. The amount of OCN protein on the two disc types significantly increased from day 7 to day 10 in both growth medium and osteogenic medium. There was a significant increase of the amount of OCN protein on akermanite discs at day 10 in the growth medium. Interestingly, the OCN secretion by hPDLCs cultured on akermanite increased as much as 22 % at day 7 ($p < 0.05$) and 48 % at day 10 ($p < 0.05$), which was significantly higher than that secreted by cells on β-TCP in osteogenic medium (Fig. 7d).

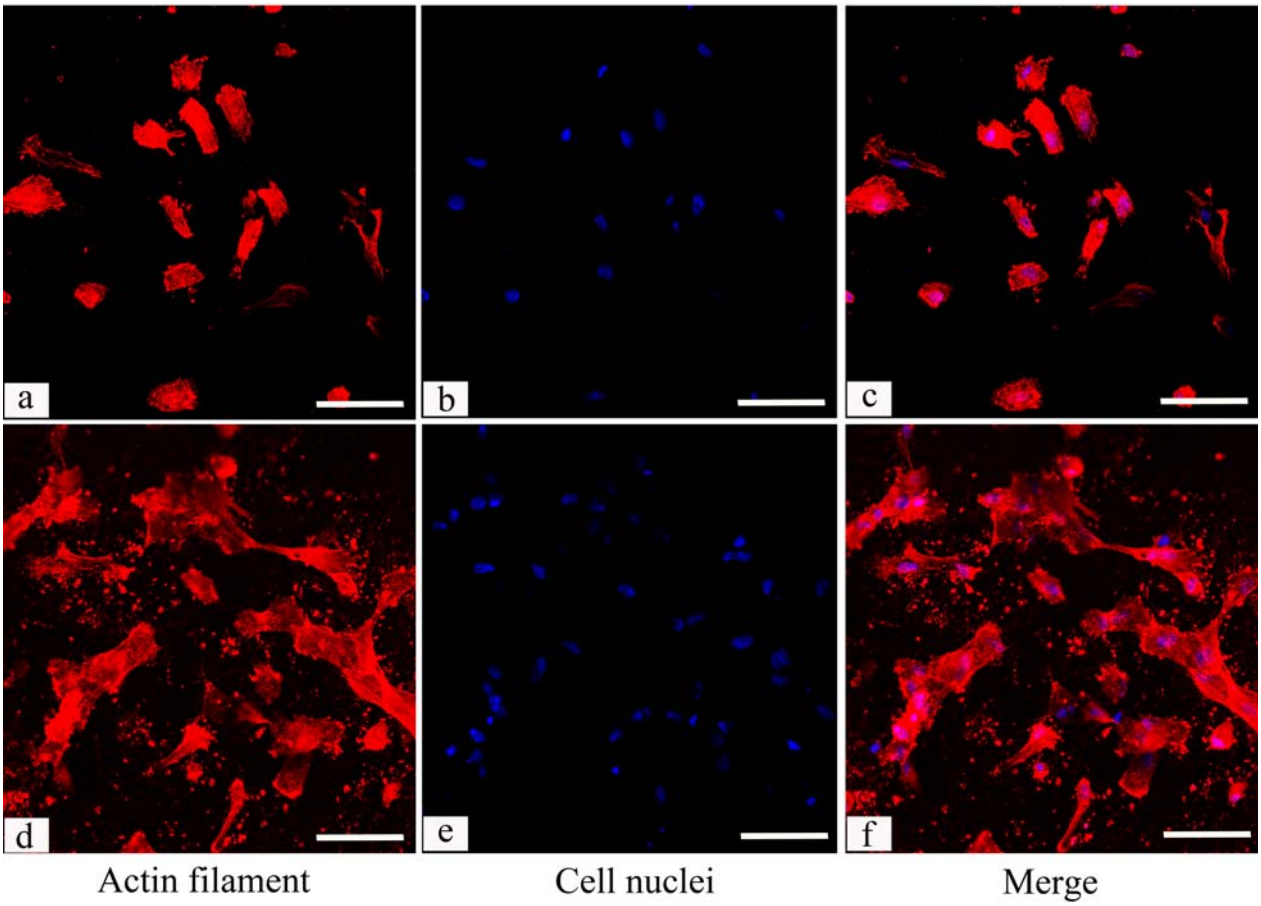


**Fig. 4**. Actin cytoskeletons were labeled to observe cell morphology at 24 h after cells seeding on beta-TCP **(a, b, c)** and akermanite **(d, e, f)**. Actin cytoskeletons stained red (a, d), while the cell nuclei stained blue **(b, e)**. **(c, f)** represent merged images of the two fluorochromes for β-TCP and akermanite. The actin filament distribution was fully spreading with regular directions on akermanite compared to β-TCP. All images were observed by confocal fluorescence microscope. Scale bar = 100 μm.

**The ions released from akermanite and their effects on hPDLCs**

As shown in Fig. 8a-c, a large quantity of Ca, Mg, and Si ions was released from akermanite ceramic throughout the observation period, with a peak at day 4, while only Ca ions were released from the β-TCP ceramic. The ion concentrations in extracts were dynamic through the whole observation period, while different dilutions of the extracts after soaking the two ceramics in medium for 4 d were made in order to cover a range of ion concentrations released from ceramic discs during different culture periods, and to find the best appropriate concentration, which might facilitate the study on the enhancement effect of ions released from the ceramic disc. The MTT assay data showed that proliferation proceeded faster when hPDLCs were cultured in a 1/32 dilution of the extracts, which might be chosen as the appropriate concentration for further study. Besides, the proliferation of hPDLCs cultured in medium supplemented with akermanite extract was greater than for the cells cultured in either β-TCP extract or medium alone throughout the observation period, while there was only a significant difference between hPDLCs in β-TCP extract and medium alone at day 4 (Fig. 8d,e).

As shown in Fig. 9a, ALP staining was more intensive for hPDLCs cultured in akermanite extract than for hPDLCs cultured in β-TCP extract at day 10, respectively. Similarly, the analysis of quantitative examination data showed that the ALP activity of cells cultured in β-TCP and akermanite extracts increased over time throughout the assay period with a higher value in osteogenic medium. ALP activity of hPDLCs cultured in akermanite extract was higher than that in β-TCP extract with a statistically significant difference found at days 7 and 10 (Fig. 9b). The expression of OPN, DMP-1and OCN for hPDLCs cultured in akermanite extract increased, with a statistically significant enhanced expression of OPN at day 7, DMP-1 and OCN at days 4 and 7 in growth medium, but with a statistically significant enhanced expression of OPN, DMP-1 and OCN at day 4, 7 and 10 in osteogenic medium, as compared to β-TCP extract. Besides, there was also a significant difference between hPDLCs cultured in either β-TCP or akermanite extract and the cells cultured in medium alone at day 4 and 7 (Fig. 9c-e).

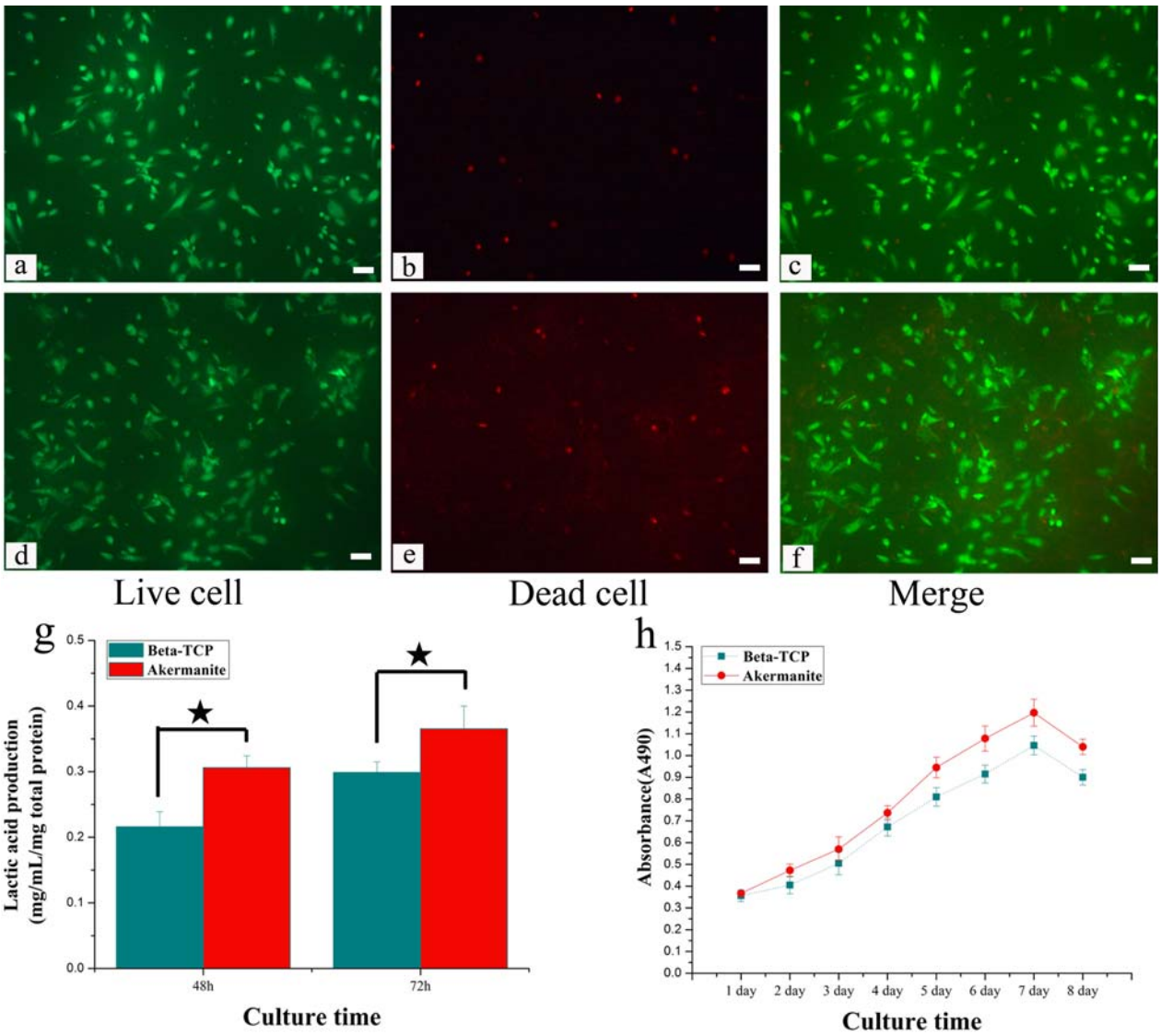


**Fig. 5**. Cytotoxicity and proliferation assay of hPDLCs seeded on β-TCP and akermanite. Live/Dead Double Staining for hPDLCs at 24h after seeding on β-TCP **(a, b, c)** and akermanite **(d, e, f)**. Live cells stained green **(a, d)**, while dead cells appeared red **(b, e)**. The merged images of live /dead cells staining were showed on β-TCP **(c)** and akermanite **(f)**. **(g)** Metabolism analysis of hPDLCs seeded on β-TCP and akermanite was measured with the production of lactic acid after co-cultured for 48 and 72 h. Asterisk indicates significant differences, $p < 0.05$. **(h)** MTT assay of hPDLCs seeded on β-TCP and akermanite from 1 to 8 d for cell viability and proliferation. Scale bar = 100 μm.

## Discussion

The biomaterial for bone regeneration will act as a temporary matrix for cell proliferation, osteogenic differentiation, and extracellular matrix deposition, with consequent bone in-growth until the new bone tissue is fully formed. It is important that the scaffolds have a series of properties that make them suitable for bone regeneration purposes, such as good biocompatibility and osteoinductivity. Adding inorganic elements to scaffolds could significantly improve bioactivity of materials, which has been confirmed in bioglass, glass-ceramic, and bioceramics containing CaO, $SiO_2$ and Mg in previous studies (Zreiqat *et al*., 2002; Wu and Chang, 2007; Abed and Moreau, 2007; Park *et al*., 2010). In view of the effect of these silicate based materials on promoting cell proliferation and osteogenic differentiation, akermanite ($Ca_2MgSi_2O_7$) ceramics were synthesized and used as scaffold for research in bone regeneration (Wu and Chang, 2007). As a novel bioactive ceramic, akermanite possessed a superior property of promoting cell proliferation and osteogenic differentiation for BMSCs and ADSCs when compared to β-TCP, a widely used bioceramic in bone regeneration (Sun *et al*., 2006; Liu *et al*., 2008). However, whether this new ceramic could increase the proliferation and osteogenic differentiation of PDLCs and benefit the periodontal bone regeneration was far from clear.

As for the reconstruction of periodontal tissues including bone, periodontal ligament and cementum, PDLCs seem to be a better cell source possessing some osteogenic and fibrogenic progenitor cells to maintain homoeostasis (Bartold *et al*., 2000; Grzesik *et al*., 2002;

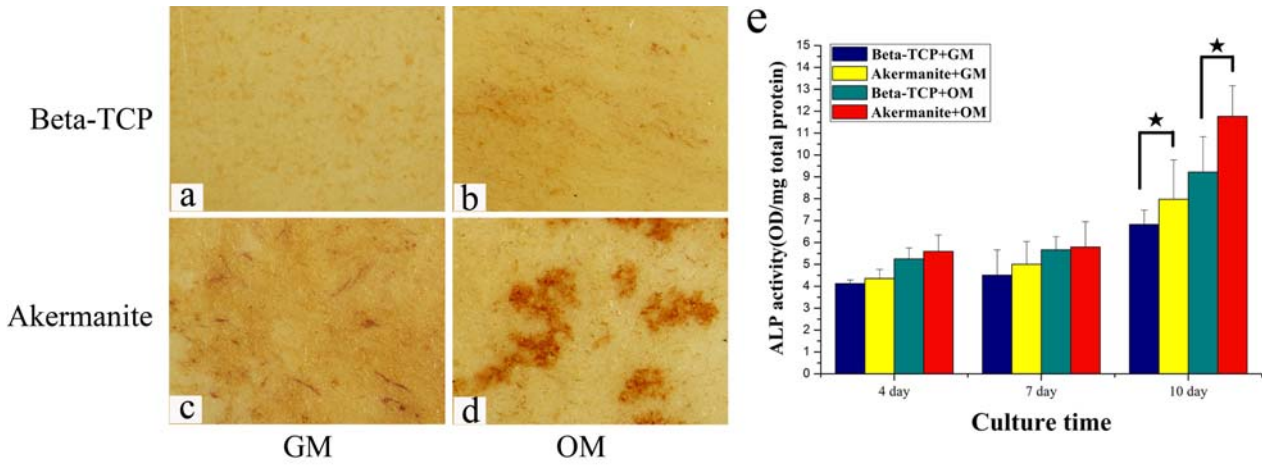


**Fig. 6**. ALP staining and activity analysis of hPDLCs seeded on β-TCP and akermanite. ALP expression of hPDLCs seeded on β-TCP **(a, b)** and akermanite **(c, d)** either in growth medium **(a, c)** or osteogenic medium **(b, d)** was stained by BM Purple. **(e)** The ALP activity of hPDLCs seeded on β-TCP and akermanite was measured with the pNPP assay in growth medium or osteogenic medium at days 4, 7 and 10 after seeding. An asterisk indicates significant differences, $p < 0.05$. GM: growth medium; OM: osteogenic medium.

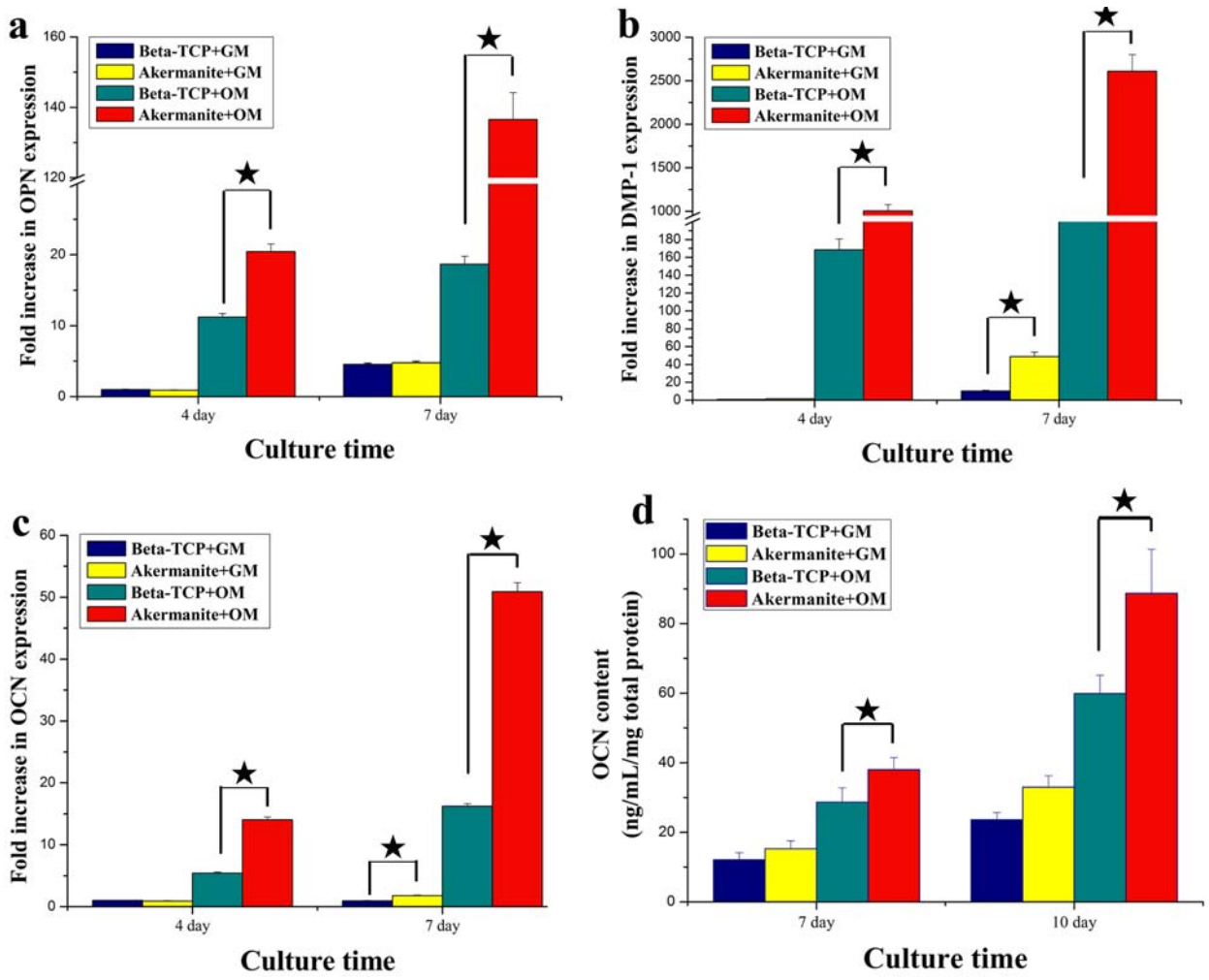


**Fig. 7**. The osteogenic differentiation analysis for hPDLCs seeded on β-TCP and akermanite. **(a, b, c)** Real-time PCR analysis of osteogenic differentiation related gene expression of hPDLCs seeded on β-TCP and akermanite either in growth medium or osteogenic medium at days 4 and 7, respectively. **(a)** OPN; **(b)** DMP-1; **(c)** OCN. **(d)** ELISA analysis for OCN content released from hPDLCs seeded on β-TCP and akermanite in growth medium or osteogenic medium at day 7 and 10, respectively. Asterisk indicates significant differences $p < 0.05$. GM: growth medium; OM: osteogenic medium.

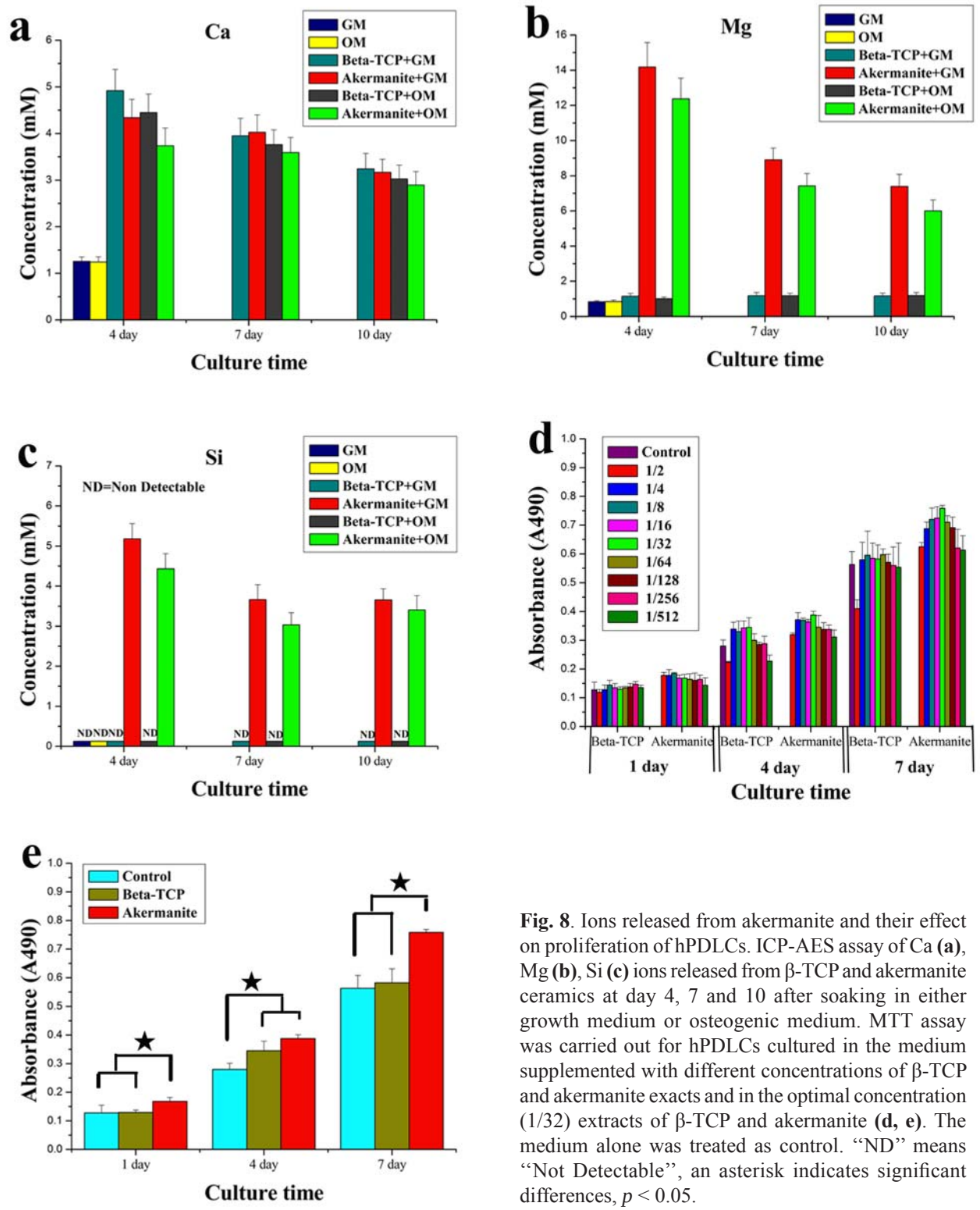


**Fig. 8**. Ions released from akermanite and their effect on proliferation of hPDLCs. ICP-AES assay of Ca **(a)**, Mg **(b)**, Si **(c)** ions released from β-TCP and akermanite ceramics at day 4, 7 and 10 after soaking in either growth medium or osteogenic medium. MTT assay was carried out for hPDLCs cultured in the medium supplemented with different concentrations of β-TCP and akermanite exacts and in the optimal concentration (1/32) extracts of β-TCP and akermanite **(d, e)**. The medium alone was treated as control. ''ND'' means ''Not Detectable'', an asterisk indicates significant differences, $p < 0.05$.

Seo *et al*., 2004). In previous studies, the osteogenic ability of PDLCs was further enhanced by certain hormones (vitamin D3), growth factors (BMP-2) or biomaterials (Hou *et al*., 2007; Kasaj *et al*., 2008; Tang *et al*., 2009; Liao *et al*., 2010). Some studies showed that silicate based materials could promote proliferation and osteogenic differentiation of PDLCs (Maeda *et al*., 2010). The use of inorganic materials for promoting bone formation would eliminate the complexity and side effects of hormones or growth factors, thus Ca, Mg, and Si containing akermanite ceramic might be an effective and safe way to promote osteogenic differentiation of PDLCs.

The adhesion and spreading of cells on the material surface not only manifests interactions between cells and material, but also regulates cellular functions such as proliferation, migration, and extracellular matrix (ECM) production (Anselme *et al*., 2000; Yim and Leong, 2005; Eisenbarth *et al*., 2007). In view of these reasons,

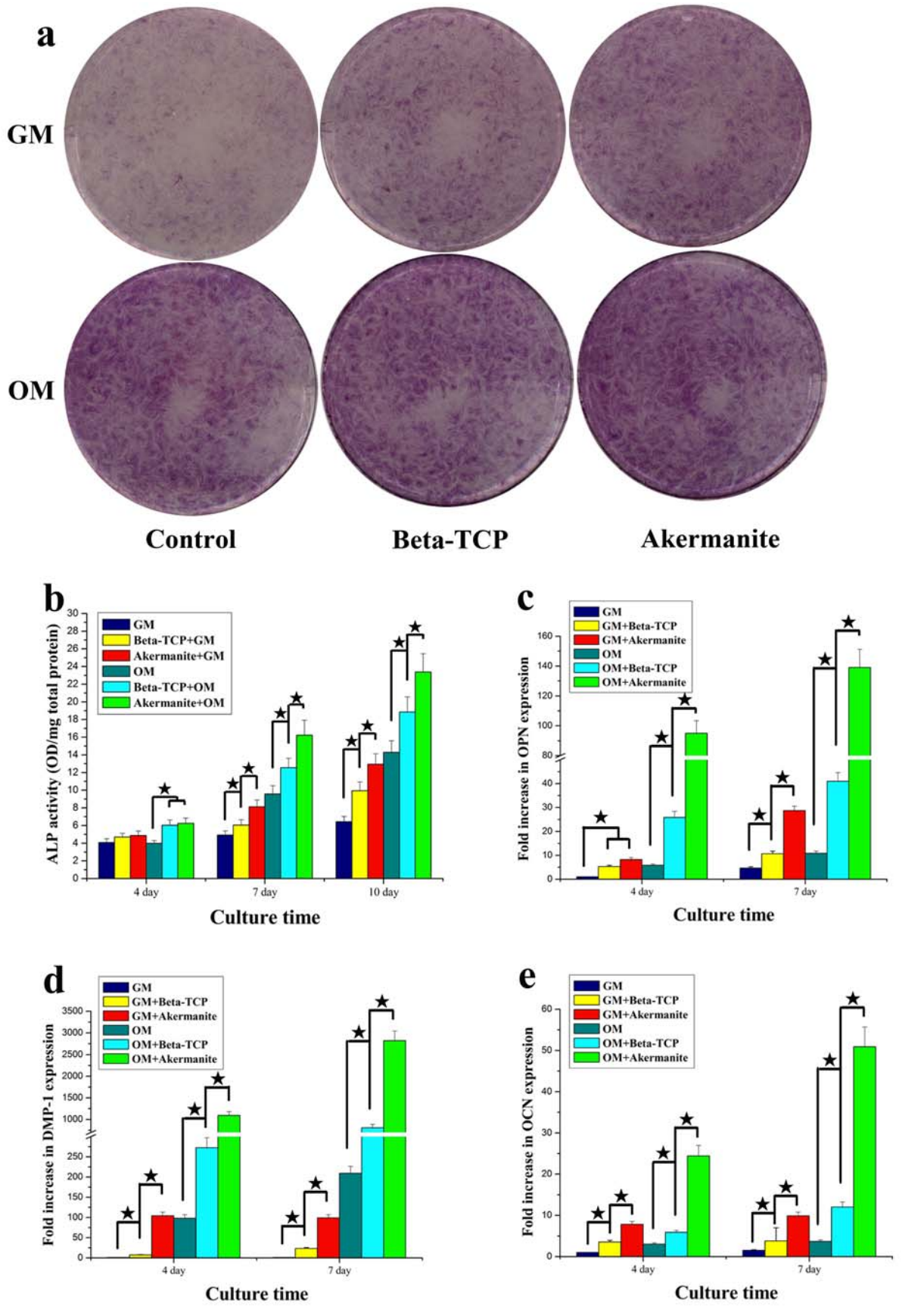


**Fig. 9**. The osteogenic differentiation analysis for hPDLCs cultured in growth medium and osteogenic medium under the optimal extracts concentration of β-TCP and akermanite. ALP expression was stained by BM Purple at day 10 **(a)** and was measured with the pNPP assay at days 4, 7 and 10 **(b)**. Real-time PCR analysis of osteogenic differentiation related gene expression at days 4 and 7. **(c)** OPN; **(d)** DMP-1; **(e)** OCN. The growth medium or osteogenic medium alone was treated as control, respectively. Asterisk indicates significant differences, $p < 0.05$. GM: growth medium; OM: osteogenic medium.

attachment of hPDLCs on the akermanite ceramic disc was observed by SEM in previous studies on BMSCs or ADSCs. It was shown that hPDLCs attached tightly to the underlying akermanite ceramics, while cell morphology on akermanite and β-TCP appeared to be different. hPDLCs seeded on akermanite ceramic appeared much flatter and spread out, resulting in a larger cell extending area as compared to cells seeded on β-TCP as shown in actin cytoskeleton analysis. It was suggested that a flat cell morphology with fully spreading shape and a regular cytoskeleton was better with respect to cell proliferation and differentiation (He *et al*., 2008). The cell metabolism and MTT assay result showed that akermanite ceramics promoted proliferation of hPDLCs as compared with β-TCP ceramics (Sun *et al*., 2006). In addition, these data suggested that akermanite did not display cytotoxicity compared to β-TCP, which was further supported by the result of the Live/Dead Double Staining.

The ability of akermanite ceramic in promoting osteogenic differentiation of hPDLCs was one major focus of this study. As an early marker for osteoblastic cell differentiation, ALP regulated organic or inorganic phosphate metabolism by way of the hydrolyzation of phosphate esters, and acted as a plasma membrane transporter for inorganic phosphates (Liu *et al*., 2008). In this study, the hPDLCs cultured on akermanite ceramic had a higher level of ALP activity than that on β-TCP either in osteogenic or growth medium with a statistical significance detected at day 10. Real-time PCR analysis of markers of OPN, DMP-1 and OCN was carried out for additional evaluation of osteogenic differentiation promoted by akermanite ceramic. OPN is associated with the maturation stage of osteoblasts during attachment and matrix synthesis before mineralization, and is largely considered as an intermediate or relatively earlier marker of osteogenic differentiation (Jiang *et al*., 2009). As a novel marker for osteogenic differentiation, DMP-1 is widely expressed in cartilage, enamel and bone (D'Souza *et al*., 1997; Hirst *et al*., 1997; MacDougall *et al*., 1998). DMP-1 plays important roles in cell attachment, mineralization, dentinogenesis, intramembranous and endochondral ossification (George *et al*., 1993; MacDougall *et al*., 1996; Thotakura *et al*., 2000; Kulkarni *et al*., 2000). Finally, OCN is a later marker of osteogenic differentiation related to matrix deposition and mineralization (Beck *et al*., 2000). Analysis of Real time PCR and ELISA results showed that akermanite alone was sufficient to stimulate osteogenic differentiation of hPDLCs in growth medium, while this promotion would occur much earlier and stronger in osteogenic medium supplemented with L-ascorbic acid, glycerophosphate and dexamethasone.

It was suggested that both physical status (surface morphology) and chemical composition of the biomaterial were responsible for cellular responses to the biomaterial, such as attachment, growth, proliferation, and osteogenic differentiation (Meyer *et al*., 2005; Hoppe *et al*., 2011). In the present study, a large quantity of Ca, Mg and Si was released from akermanite ceramic, while only Ca was released from β-TCP ceramic. Based on these results, it was suggested that the improved osteogenic differentiation of hPDLCs for β-TCP might be associated with the released Ca, which was also proven in a previous study (Dong *et al*., 2002). It was previously confirmed that the Si from bioactive glass dissolved in a certain concentration range could promote cell proliferation (Gough *et al*., 2004; Valerio *et al*., 2004). Supplementation of Mg ions also stimulated adhesion and proliferation of osteoblastic cells (Zreiqat *et al*., 2002; Abed and Moreau, 2007; Park *et al*., 2010). It was also reported in previous studies that Ca and Si ions played an important role in the process of nucleation and hydroxyapatite (Hap) growth, and affected the biological metabolism of osteoblastic cells particularly in the mineralization process and bone-bonding mechanism (Dufrane *et al*., 2003). Mg ions also played a critical role in bone remodeling and skeletal development, and possessed indirect bone healing-promoting effect via integrins transduced signals as shown in some studies (Zreiqat *et al*., 2002; Park *et al*., 2010). In the present study, more pronounced proliferation and higher expression of osteogenic genes for cells in akermanite extract was detected as they were seeded on akermanite bioceramic. Based on the above results, we believe that the release of Ca, Mg, and Si from the akermanite bioceramic plays an important role on the proliferation and differentiation of hPDLCs, although we cannot absolutely rule out the influence of a different microstructure, or different dissolution/reprecipitation on cell behavior. Overall, akermanite ceramic has achieved promising effects on the proliferation and osteogenic differentiation of hPDLCs, which might be attributed to the release of substances containing Ca, Mg, and Si from akermanite bioceramic. However, the detailed mechanism and the corresponding signaling pathway is far from clear at present and needs further investigations.

## Conclusion

In summary, it is suggested that Ca, Mg, and Si containing akermanite ceramic obviously promoted the attachment, proliferation and osteogenic differentiation of hPDLCs as compared with β-TCP ceramics, while the release of substances containing Ca, Mg and Si from akermanite bioceramic plays an important role in this behavior of the cells. Therefore, akermanite bioceramic might act as an excellent candidate biomaterial superior to β-TCP for periodontal bone regeneration.

## Acknowledgements

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**Discussion with Reviewer**

**Reviewer II**: In most cases, ceramics (even TCP ceramic) adsorb calcium ions from the culture medium instead of releasing calcium. What are the special settings in the ion release study to allow calcium release from the ceramics?
**Authors**: We agree that some bioceramics such as β-TCP may adsorb calcium from the cell culture medium. However, based on our previous studies, when we soaked ceramic materials in the culture medium, at the early stage of the soaking, most of the ceramics would first release ions, and with increased soaking time, adsorption of calcium and phosphate ions might occur (Wu *et al*., 2006, text reference). This was also the case in our study. We observed a higher Ca concentration at day 4 as compared to that at days 7 and 10 (Fig. 8a), which suggests adsorption of Ca with increased culture time. Therefore, we prepared ceramic extracts following the same procedure that we used in our previous study.