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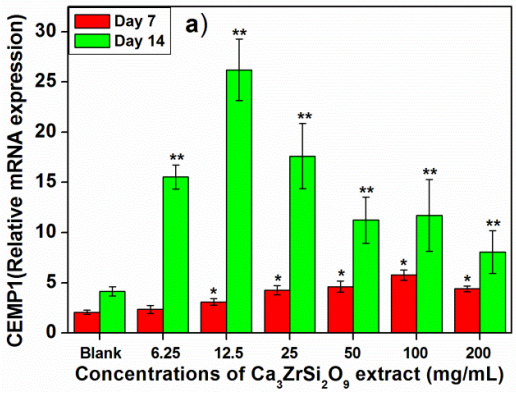
Graphic of contents: $Ca_3ZrSi_2O_9$ bioceramics promote the cementogenic/osteogenic differentiation of PDLCs.



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Stimulatory effect of $Ca_3ZrSi_2O_9$ bioceramics on cementogenic/osteogenic differentiation of periodontal ligament cells

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Abstract

The regeneration of periodontal tissues to cure periodontitis remains a medical challenge. Therefore, it is of great importance to develop a novel biomaterial that could induce cementogenesis and osteogenesis in periodontal tissue engineering. Calcium silicates (Ca-Si) based ceramics have been found to be a potential bioactive material due to their osteostimulatory effect. Recently, it is reported that zirconium modified calcium-silicate-based ($Ca_3ZrSi_2O_9$) ceramics stimulate cell proliferation and osteogenic differentiation of osteoblasts. However, it is unknown whether $Ca_3ZrSi_2O_9$ ceramics possess specific cementogenic stimulation for human periodontal ligament cells (hPDLCs) in periodontal tissue regeneration *in vitro*. The purpose of this study was to investigate whether $Ca_3ZrSi_2O_9$ ceramic disks and their ionic extracts could stimulate cell growth and cementogenic/osteogenic differentiation of hPDLCs; the possible molecular mechanism involved in this process was also explored by investigating Wnt/β-catenin signalling pathway of hPDLCs. Our results showed that $Ca_3ZrSi_2O_9$ ceramic disks supported cell adhesion, proliferation and significantly up-regulated relative alkaline phosphatase (ALP) activity, cementogenic/osteogenic gene expression (CEMP1, CAP, ALP and OPN) and Wnt/β-catenin signalling pathway-related genes (AXIN2 and CTNNB) for hPDLCs, compared to that of β-tricalcium phosphate (β-TCP) bioceramic disks and blank controls. The ionic extracts from $Ca_3ZrSi_2O_9$ powders also significantly enhanced relative ALP activity, cementogenic/ osteogenic and Wnt/β-catenin-related gene expression of hPDLCs. The present results demonstrate that $Ca_3ZrSi_2O_9$ ceramic is capable of stimulating cementogenic/osteogenic differentiation of hPDLCs possibly via activation of the Wnt/β-catenin signalling pathway, suggesting that $Ca_3ZrSi_2O_9$ ceramics have the potential to be used for periodontal tissue regeneration.

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Key words: *Bioceramics; Cementogenic stimulation; Gene expression; Wnt/ß-catenin signalling pathway*

1. Introduction

Bioceramics have been shown great potential for bone regeneration by stimulating cell differentiation and mineralized tissue formation.[1-3] Among of them, calcium phosphate and calcium silicate (Ca-Si) based bioactive ceramics have been intensively investigated for bone regeneration.[4-8] Although conventional β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP) have been widely used as bone defect filling materials due to its favourable biocompatibility, its osteostimulation/osseointegration property is still far from optimal.[9-11] In the past several years, silicate-based bioceramics have been developed as potential bioactive material for bone engineering, in which some of them exhibit excellent enhanced *in vitro* and *in vivo* osteogenesis.[12] Previous reports showed that Ca-Si based bioceramics had the ability to enhance osteogenic differentiation of several stem cells, such as bone marrow stromal cells, adipose-derived stem cells, human dental pulp cells and periodontal ligament cells.[7, 13-18]

Meanwhile, other findings found that incorporation of bioactive ions (e.g. Zinc, Strontium, Titanium) into Ca-Si ceramics significantly improved their bioactivity, which inspired us that bioactive ions modified bioceramics may be an alternative method to enhance cell differentiation for tissue regeneration.[19-21] Zirconium (Zr) ions are quadrivalent that resembles titanium. Zirconium-related materials, such as zirconia ceramics (ZrO_2), have been widely used in dentistry and dental implantology since they possess excellent mechanical properties and biocompatibility.[22, 23] Zr implants showed favourable osseointegration property *in vivo*.[24] To improve stability of Ca-Si system, Ramaswamy *et al.* incorporated Zr ions to modify Ca-Si based ceramic and generated the $Ca_3ZrSi_2O_9$ ceramic (Baghdadite), and

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the findings demonstrated that $Ca_3ZrSi_2O_9$ ceramics induced cell proliferation and osteogenic differentiation of osteoblasts by upregulation of bone-related genes mRNA expression of Collagen type 1, ALP and Bone Sialoprotein.[25] Due to the advantages of $Ca_3ZrSi_2O_9$ ceramics for bone regeneration, it is inspired that they may be used for periodontal tissue regeneration by stimulation of cementogenic/osteogenic differentiation of human periodontal ligament cells (hPDLCs). Periodontal tissue regeneration remains a medical challenge for dentists, as it is challenging to reconstitute the periodontium complex including alveolar bone, periodontal ligament (PDL) and root cementum. PDLCs have been regarded as potential candidate for periodontal tissue engineering and showed to differentiate into osteoblastic, fibroblastic and cementoblastic lineages.[26-29] In addition, PDLCs have also shown to potentially form cementum-like tissue *in vivo*.[26] The differentiation of PDLCs is regulated by cell signalling cascades, biomaterial composition and ion concentration in the cultured medium.[13, 29] However, it is unknown whether $Ca_3ZrSi_2O_9$ ceramics possess cementogenic/osteogenic stimulation of hPDLCs for periodontal tissue regeneration. The involved mechanism of $Ca_3ZrSi_2O_9$ stimulating cementogenic/osteogenic differentiation of hPDLCs is completely unknown. The aim of this study was to investigate *in vitro* cementogenic/osteogenic stimulation of $Ca_3ZrSi_2O_9$ ceramics for hPDLCs as well as the possible mechanism underlying this process. Therefore, the interaction of both $Ca_3ZrSi_2O_9$ ceramic disks and their ionic products with hPDLCs has been systematically investigated in this study, including cell proliferation, cementogenic/osteogenic differentiation and Wnt/β-catenin signalling pathway gene expression of hPDLCs.

2. Experimental section

2.1. Preparation and characterization of $Ca_3ZrSi_2O_9$ and β–TCP ceramics

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$Ca_3ZrSi_2O_9$ powders were synthesized using zirconia oxide nitrate ($ZrO(NO_3)_2$), $Ca(NO_3)_2{\cdot}4H_2O$, and TEOS.[25] The obtained powders were ground and sieved to 300 meshes for the further preparation of ceramic disks. The sintered $Ca_3ZrSi_2O_9$ ceramic disks were prepared by sintering at 1400ºC according to our previous study, and further confirmed their pure crystal phase by X-ray differaction. Conventional β-TCP bioceramic disks with same size were prepared according to our previous publication for the controls materials.[30]

2.2. Cell morphology of hPDLCs on $Ca_3ZrSi_2O_9$ and β–TCP ceramic disks

hPDLCs were isolated and cultured according to our previous protocols.[31] The ethics approval of hPDLCs in this study was granted by the Human Ethics Committee of Queensland University of Technology, following informed consents taken from all the participants. hPDLCs were seeded on $Ca_3ZrSi_2O_9$ or β–TCP ceramic disks in 48-well plates at the initial seeding density of 10^5cells/disk and cultured in growth medium containing low glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Life Technologies Pty Ltd., Australia) with the supplement of 10% (v/v) fetal bovine serum (FBS; In Vitro Technologies, Australia) and 1% (v/v) penicillin/streptomycin (P/S; Gibco®, Life Technologies Pty Ltd., Australia). hPDLCs seeded on 48-well cell culture plates were used as blank controls.

For scanning electron microscopy (SEM) observation of cell morphology on the disks, hPDLCs were cultured for 1 and 7 days, and were then fixed in 2.5% glutaraldehyde solution, washed three times with the buffer containing 4% (w/v) sucrose and post fixed in 1% osmium tetroxide in PBS. Then the samples were dehydrated by graded ethanol series (30, 50, 70, 90, and 100%) before drying in hexamethyldisilizane (HMDS). Finally, the disks were sputter-coated by gold and observe by SEM (Quanta™ 200) for cell attachment and morphological characteristics of hPDLCs on both bioceramics.

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2.3. Proliferation of hPDLCs on $Ca_3ZrSi_2O_9$ and β–TCP ceramic disks

Cell proliferation was quantitatively assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described in our previous study protocol.[7, 29] In brief, hPDLCs were cultured on the surface of $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks for 1, 3 and 7 days. 0.5 mg/mL of MTT solution (Sigma-Aldrich, Australia) was added to each ceramic disk to form formazan crystals and then 100 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Australia) were used to dissolve the formazan. The absorbance was assessed by Spectra Max Microplate Reader (Molecular Devices, Inc., USA) at 495 nm.

2.4. Relative alkaline phosphatase (ALP) activity of hPDLCs on $Ca_3ZrSi_2O_9$ and β–TCP ceramic disks

hPDLCs were cultured on $Ca_3ZrSi_2O_9$ and β–TCP ceramic disks for 7 and 14 days, and then cells were lysed in 200 μL of 0.2% Triton X-100 for ALP assay. Fifty microliter of cell lysate was mixed with 150uL ALP working solution according to the manufacturer's protocol (QuantiChrom™ Alkaline Phosphatase Assay Kit, BioAssay Systems, USA). The optical density (OD) was measured at 405 nm on spectrophotometer. The ALP activity was obtained as the changed OD values divided by the reaction time and the total protein content measured by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Australia).

2.5. Cemetogenic/osteogenic gene expression of hPDLCs on $Ca_3ZrSi_2O_9$ ceramic disks

Total RNA was extracted from the cells cultured on $Ca_3ZrSi_2O_9$, β-TCP ceramic disks and blank groups using TRIzol® Reagent (Ambion®, Life Technologies Pty Ltd., Australia). qRT-PCR was performed to detect cementum-specific markers of cementum protein 1 (CEMP1) and cementum attachment protein (CAP), osteogenic genes of alkaline phosphatase (ALP) and osteopontin (OPN). Briefly, complementary DNA was synthesized from 1 μg of total

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RNA using DyNAmo™ cDNA Synthesis Kit (Finnzymes, Genesearch Pty Ltd., Australia) following the manufacturer's protocol. qRT-PCR was performed on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Australia) using SYBR® Green detection reagent (Applied Biosystems, Australia). The sequences of primers are shown in Table 1. All reactions were performed in triplicate from three independent experiments. The mean cycle threshold (Ct) value of each target gene was normalized against Ct value of house-keeping gene of 18s ribosomal RNA (18s rRNA) and the relative expression calculated using the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$.

2.6. Wnt/β-catenin signalling pathway of hPDLCs on $Ca_3ZrSi_2O_9$ ceramic disks

To further investigate the effect of $Ca_3ZrSi_2O_9$ ceramic disks on Wnt/β-catenin signalling pathway for hPDLCs, gene expression of axis inhibition protein 2 (AXIN2) and beta-catenin (CTNNB) was analysed by qRT-PCR as described in Section 2.5.

2.7. The effects of $Ca_3ZrSi_2O_9$ extracts on cell proliferation and ALP activity of hPDLCs

To further investigate the effect of ionic extracts from $Ca_3ZrSi_2O_9$ powders on cell proliferation and ALP activity, the dissolution extracts were prepared by soaking $Ca_3ZrSi_2O_9$ powders in serum-free DMEM at the concentration of 200 mg/mL according to International Standard Organization (ISO/EN) 10993-5.[32] After incubation at 37ºC for 24 h, the mixture was centrifuged and the supernatant was sterilized using a 0.2 μm filter. Serial dilutions of extracts (100, 50, 25, 12.5 and 6.25 mg/mL) were prepared using serum-free DMEM to optimize the proper concentration used in the following studies.[20]

For the MTT assay, hPDLCs were cultured in each concentration of $Ca_3ZrSi_2O_9$ extracts supplemented with 10% FBS and 1% P/S in the 96-well plates for 1, 3 and 7 days. MTT assay was performed as described in Section 2.3. For relative ALP activity assay, cells were

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lysed at 7 and 14 days after cultured in $Ca_3ZrSi_2O_9$ extracts and ALP activity were measured as the method in Section 2.4. hPDLCs cultured in the normal growth medium was analysed as blank controls.

2.8. The effects of $Ca_3ZrSi_2O_9$ extracts on cementogenic/osteogenic and Wnt/β-catenin signalling pathway-related gene expression of hPDLCs

To further investigate the effect of $Ca_3ZrSi_2O_9$ extracts on cemetogenic, osteogenic and Wnt/β-catenin-related gene expression of hPDLCs, qRT-PCR was carried out to detect the gene expression of CEMP1, CAP, ALP, OPN, AXIN2 and CTNNB using the method described in Section 2.5.

2.9. The ionic concentrations analysis

The ionic concentrations of Ca, Si, Zr and P ions in the cell culture medium after culturing with $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks for 1 and 7 days were measured by inductive coupled plasma atomic emission spectrometry (ICP-AES).

2.10. Statistical analysis

All the data were presented as means ± standard deviation (SD) and were analysed using one-way ANOVA. Analysis was performed using SPSS software (SPSS Inc., Chicago, Il, USA). p-value <0.05 were considered to be statistically significant difference.

3. Results

3.1. Characterization of $Ca_3ZrSi_2O_9$ ceramic disks

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SEM analysis showed that $Ca_3ZrSi_2O_9$ ceramics disks were sintered with obvious crystal boundary, and the surface of $Ca_3ZrSi_2O_9$ ceramics was much rougher than that of β-TCP ceramic disks (Fig. 1).

3.2. Attachment and morphology of hPDLCs on $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks

The attachment and morphology of hPDLCs was detected by SEM after 1 and 7 days culturing on the surface of both $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks (Fig.2). At day 1, cells were attached and spread well with filapodia-like process on the surface of both $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks. At day 7, hPDLCs appeared confluent on the surface of two ceramic disks with close contact with the ceramic disks (Fig. 2).

3.3. Proliferation and ALP activity of hPDLCs on $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks

The MTT assay showed that the proliferation of hPDLCs increased obviously in a time-dependent manner in each group. The cell proliferation rate on $Ca_3ZrSi_2O_9$ was slightly higher than that of β-TCP ceramic disks at day 1, 3 and day 7; however, the cell proliferation on these two disks was lower than that on blank group (Fig. 3). The relative ALP activity of hPDLCs on the $Ca_3ZrSi_2O_9$ ceramics showed a significant higher level compared to other two groups at both 7 and 14 days ($p<0.05$, Fig. 4).

3.4. Cementogenic/osteogenic gene expression of hPDLCs on$Ca_3ZrSi_2O_9$ and β-TCP ceramic disks

qRT-PCR analysis showed that $Ca_3ZrSi_2O_9$ ceramic disk significantly enhanced the cementogenic and osteogenic gene expression of hPDLCs compared to β-TCP ceramics and blank control (Fig. 5). The expression of cementogenic gene of CEMP1 and CAP on $Ca_3ZrSi_2O_9$ ceramics was significantly higher than β-TCP ceramic and blank control group at

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day 14 (Fig. 5a-b). It is noted that osteogenic gene expression of ALP and OCN was also dramatically upregulated for hPDLCs on $Ca_3ZrSi_2O_9$ ceramic disks compared to other two groups at both day 7 and 14 (Fig. 5c-d).

3.5. Wnt/β-catenin signalling pathway-related gene expression of hPDLCs on $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks

The expression of Wnt/β-catenin-related genes of AXIN2 and CTNNB was investigated by qRT-PCR after culturing hPDLCs on two ceramics and control medium for 7 and 14 days. The results showed that the expression of AXIN2 and CTNNB of hPDLCs on $Ca_3ZrSi_2O_9$ ceramics was significantly higher than β-TCP ceramics and blank control group at both day 7 and day 14 (Fig. 6a-b).

3.6. Ionic concentration analysis for the cell culture medium after cultured with $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks

ICP analysis demonstrated that there is no Zr ions release in β-TCP medium, and the concentration of Zr ions from $Ca_3ZrSi_2O_9$ medium was also very low (0.02mg/L) at day 7 and 14 (Table 2). The released Ca and Si ions from hPDLCs cultured on $Ca_3ZrSi_2O_9$ disks were obviously higher than those from β-TCP disks. However, the concentration of P ion from $Ca_3ZrSi_2O_9$ disks was lower than those from β-TCP groups (Table 2).

3.7. The effect of $Ca_3ZrSi_2O_9$ extracts on cell proliferation

To investigate the effect of ionic products from $Ca_3ZrSi_2O_9$ extracts on proliferation of hPDLCs, cells were cultured in different concentrations of extracts for 1, 3, 7 days. MTT assay showed that cell proliferation rate of hPDLCs was comparable between 6.25-100mg/mL range concentration of $Ca_3ZrSi_2O_9$ extract and blank group (Fig.7). However, cell

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proliferation in 200mg/mL extracts decreased significantly on day 3 and day 7 compared to blank control (p<0.05, Fig.7).

3.8. The effects of $Ca_3ZrSi_2O_9$ extracts on cementogenic/osteogenic differentiation of hPDLCs

The relative ALP activity was further investigated for hPDLCs cultured in the extracts of $Ca_3ZrSi_2O_9$ powders at the concentrations of 6.25 to 200 mg/mL after 7 and 14 days. The ALP activity of hPDLCs was significantly elevated in all range concentrations of $Ca_3ZrSi_2O_9$ extracts at day 7, compared to blank control (Fig. 8). $Ca_3ZrSi_2O_9$ extracts at the range concentration of 6.25 to 100 mg/mL significantly enhanced ALP activity of hPDLCs on day 14, while 50mg/mL extracts showed the highest value (Fig. 8).

qRT-PCR results of gene expression showed that $Ca_3ZrSi_2O_9$ extracts enhanced hPDLCs cementogenic/osteogenic differentiation at day 7 and day 14 (Fig. 9). The gene expression of CEMP1, CAP and ALP of hPDLCs was significantly increased at the concentration of 12.5 to 100 mg/mL compare to that in blank group at day 7 and 14 (Fig. 9a-c).

At day 7, the expression of Wnt/β-catenin-related gene of AXIN2 was upregulated by $Ca_3ZrSi_2O_9$ extracts at the concentrations of from 12.5 to 100 mg/mL (Fig. 9e). The expression of CTNNB was enhanced by $Ca_3ZrSi_2O_9$ extracts at the concentration of 50mg/mL at day 7 (Fig. 9f). However, the expression of CTNNB was reduced at all the range concentrations of $Ca_3ZrSi_2O_9$ extracts at day 14 compared to blank group (Fig. 9f).

4. Discussion

We, for the first time, explored the effect of $Ca_3ZrSi_2O_9$ ceramics on hPDLCs by investigating cell attachment, proliferation, cementogenic/osteogenic differentiation and Wnt/β-catenin signalling pathway-related gene expression for hPDLCs cultured with $Ca_3ZrSi_2O_9$ disks and $Ca_3ZrSi_2O_9$ extracts. Our results demonstrated that $Ca_3ZrSi_2O_9$ ceramic

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disks supported hPDLCs adhesion and proliferation, and also significantly promoted cementogenic/osteogenic markers expression of CEMP-1, CAP, ALP and OPN and Wnt/β-catenin-related gene of AXIN2 and CTNNB expression for hPDLCs. Furthermore, the ionic products from $Ca_3ZrSi_2O_9$ powders also significantly stimulated cementogenic/osteogenic differentiation of hPDLCs. The underlying mechanism could be Si, Ca and Zr-containing ionic products from $Ca_3ZrSi_2O_9$ ceramics may be able to activate cementogenic/osteogenic related gene expression of hPDLCs via activation of the Wnt/β-catenin signalling pathway, suggesting that $Ca_3ZrSi_2O_9$ ceramic has the potential to be used for periodontal tissue regeneration.

The complexity of the tooth supporting apparatus makes periodontal tissue regeneration a challenging field, as it involves the regeneration of cementum, a functionally oriented PDL and alveolar bone in periodontal defect.[33, 34] It has been demonstrated that hPDLCs could be differentiated into osteoblastic, fibroblastic, and cementoblastic cell lineages, indicating that hPDLCs are a desirable cell population for regenerating periodontal tissues.[26, 27] In recent years, increasing researches focus on the interaction of biomaterials and mesenchyme-derived cell lines to accelerate the process of periodontal tissue regeneration[35]. Previously, Ramaswamy *et al.* reported that $Ca_3ZrSi_2O_9$ ceramics stimulated osteogenic differentiation of osteoblasts.[25] However, whether $Ca_3ZrSi_2O_9$ ceramics could promote cementogenic/ osteogenic response of hPDLCs and be used for periodontal tissue regeneration remains unknown. In this study, $Ca_3ZrSi_2O_9$ ceramic disks were prepared and compared to β-TCP bioceramic disks (a gold standard control bioceramic for bone regeneration). CAP has been found as a collagen-like protein and serves as a marker for cementoblastic progenitors of human periodontal ligament.[36, 37] CEMP-1 has been characterized as a novel, cementum-specific protein expressed by periodontal ligament subpopulations and cementoblasts.[38] ALP is known as an early marker for osteoblastic differentiation, and OPN is also an important

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genes related to osteogenesis.[39] It was found that $Ca_3ZrSi_2O_9$ disks induced cell attachment and proliferation of hPDLCs as well as β-TCP bioceramic disks. Interestingly, $Ca_3ZrSi_2O_9$ ceramic disks significantly enhanced relative ALP activity, cementogenic gene (CEMP1 and CAP) and osteogenic gene (ALP and OPN) expression of hPDLCs compared to those on the β-TCP bioceramic disks. The results indicate that $Ca_3ZrSi_2O_9$ ceramic disks possess excellent *in vitro* cementogenic/osteogenic stimulation for hPDLCs.

Previous studies have indicated that the chemical composition and surface microstructure of bioceramics are two important factors to stimulate cell response.[7, 29] ICP analysis for $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks revealed that $Ca_3ZrSi_2O_9$ bioceramics contained higher concentration of Ca, Si and Zr ions in the culture medium compared to β-TCP bioceramic (Table 2). It suggests that Ca, Si and Zr ions released from $Ca_3ZrSi_2O_9$ ceramics may play a major role in up-regulation of cementogenic/osteogenic gene expression of hPDLCs. We further investigate the biological effect of the ionic extracts of $Ca_3ZrSi_2O_9$ powders on cementogenic/osteogenic differentiation of hPDLCs. Similar results have been obtained, the ionic extracts of $Ca_3ZrSi_2O_9$ powders also significantly promoted ALP activity and cementogenic/osteogenic gene (CEMP-1, CAP, ALP and OPN) expression. Previous reports showed that ionic products from Si-containing bioceramics, such as $Ca_2MgSi_2O_7$ and $Ca_7Si_2P_2O_{16}$, could significantly improve osteogenic differentiation of human bone marrow stromal cells (BMSCs), osteoblasts and PDLCs.[7, 13, 15, 18] Moreover, Si themselves play an important role in regulating the proliferation and osteogenic differentiation of BMSCs.[40] In the result, it is also noted that cell proliferation was comparable between 6.25-100mg/mL range concentration of $Ca_3ZrSi_2O_9$ extract and blank group; however, decreased proliferation rate was found on $Ca_3ZrSi_2O_9$ ceramics compared to blank. This inconsistence may be caused by several important factors influencing cell response to the bioceramic disks, such as chemical composition, surface roughness, hydrophilicity and topography. In this study, the

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concentration of Si ions in the ceramic disks medium (0.57-2.12mM) is significantly higher than that of graded $Ca_3ZrSi_2O_9$ powders extracts (0.002-0.091mM). Furthermore, previous findings showed that high concentrations of Si ions (2.17-5.0mM) may inhibits cell proliferation of osteoblasts or BMSCs.[21, 40] In this study, the medium for $Ca_3ZrSi_2O_9$ ceramic disks showed high concentration of Si ions, which may be one of important factor to inhibit cell proliferation compared to blank control. In addition, since the surface roughness of $Ca_3ZrSi_2O_9$ ceramic disks is significantly different with that of cell culture plates, which may be the other factor to influence the cell proliferation on the disks. Although the direct effect of Zr^{4+} ions on osteogenesis is unclear, zirconia implants or zirconium oxide coat-implants showed comparable or even better osseointegration compared to titanium implant in various studies.[24, 41] Considering the trace amount of Zr ion in $Ca_3ZrSi_2O_9$ exacts, it is reasonable to speculate that the Si may play a key role in stimulating osteogenic/cementogenic differentiation of hPDLCs, while Zr^{4+} and Ca^{2+} ions have a synergetic effect with Si on biological performance of hPDLCs. Moreover, incorporation of Zr ions to Ca-Si ceramics may influence its bioactivity in other ways. Our previous study shows that the incorporation of Zr ions into Ca-Si system results in a more stable $Ca_3ZrSi_2O_9$ ceramic with higher surface roughness than pure $CaSiO_3$ bioceramics.[25] Our study also showed higher surface roughness of $Ca_3ZrSi_2O_9$ ceramics than β-TCP, and $Ca_3ZrSi_2O_9$ ceramics could better support cell attachment, proliferation and differentiation of hPDLCs compared to that on β-TCP ceramics. In addition, the incorporation Zr ions to Ca-Si based ceramic may influence other ions release rate, such as Ca or Si by decrease the dissolution of pure calcium-silicate system bioceramics. Our result showed the higher concentration of Ca and Si ions released from $Ca_3ZrSi_2O_9$ ceramic than that from β-TCP ceramics, suggesting $Ca_3ZrSi_2O_9$ ceramic with proper concentration of Ca and Si ions release may promote cell proliferation and differentiation of hPDLCs. Collectively, it is speculated that the Ca, Zr and Si-containing chemical

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compositions of $Ca_3ZrSi_2O_9$ ceramics have a positive effect on cementogenic/osteogenic differentiation of hPDLCs. In addition, we further found that the surface of $Ca_3ZrSi_2O_9$ ceramics disks was much rougher than that that of β-TCP ceramic disks, which may be the other important factor to influence the biological response of hPDLCs on ceramic disks.

The Wnt/β-catenin signalling pathway is a critical regulator in organ developmental process including cell fate specification, proliferation and migration.[42] Wnt/β-catenin signalling is initiated when Wnt ligands binds to their receptors, which results in the inhibition of GSK-3β and the accumulation of cytoplasmic β-catenin.[42] Consequently, β-catenin together with the transcriptional factor LEF/TCF, regulates the transcription of Wnt target genes.[42] Axin2, also known as conductin or axil, is a direct target of the Wnt canonical signalling and regarded as the reporter gene for duration and intensity of Wnt signalling pathway.[43] Previous studies reported that activation of Wnt/β-catenin signalling pathway stimulated cementogenic and osteogenic differentiation when hBMSCs cultured on nagelschmidtite bioceramics and hPDLCs cultured on Li-containing bioactive glass scaffolds.[7, 29] In this study, we investigated Wnt/β-catenin signalling pathway of hPDLCs cultured in $Ca_3ZrSi_2O_9$ ceramics disks and ionic exacts. The results demonstrated that compared to β-TCP bioceramics and blank control, $Ca_3ZrSi_2O_9$ ceramics significantly upregulated the Wnt/β-catenin-related genes (AXIN2 and CTNNB) of hPDLCs. Our study on ionic extracts of $Ca_3ZrSi_2O_9$ powders showed the similar results that the ionic extracts of $Ca_3ZrSi_2O_9$ powders promoted AXIN2 and CTNNB gene expression at early time point (day 7) compared to control. Therefore, the results suggest that Wnt/β-catenin signalling pathway may play an important role in $Ca_3ZrSi_2O_9$ ceramic stimulated cementogenic/osteogenic differentiation of hPDLCs.

5. Conclusion

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In summary, $Ca_3ZrSi_2O_9$ bioceramics showed the ability to support hPDLCs adhesion and growth, and significantly enhanced cementogenic/osteogenic gene expression of hPDLCs. The possible mechanism for the significantly stimulatory effect of $Ca_3ZrSi_2O_9$ bioceramics on the cementogenic/osteogenic differentiation of hPDLCs may be related to the activation of Wnt/β-catenin signalling pathway by the released Ca, Zr and Si ions. $Ca_3ZrSi_2O_9$ ceramics are promising materials to be used for periodontal tissue regeneration.

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Reference

1. H. Yuan, H. Fernandes, P. Habibovic, J. de Boer, A. M. Barradas, A. de Ruiter, W. R. Walsh, C. A. van Blitterswijk and J. D. de Bruijn, *Proc. Natl. Acad. Sci. U. S. A.* , 2010, 107, 13614-13619.
2. L. L. Hench and I. Thompson, *J. R. Soc. Interface* 2010, 7 Suppl 4, S379-391.
3. C. Wu, W. Fan, Y. Zhou, Y. Luo, M. Gelinsky, J. Chang and Y. Xiao, *J. Mater. Chem.*, 2012, 22, 12288-12295.
4. N. Alves, I. Leonor, H. S. Azevedo, R. Reis and J. Mano, *J. Mater. Chem.*, 2010, 20, 2911-2921.
5. A. El-Ghannam, *Expert Rev. Med. Devices*, 2005, 2, 87-101.
6. M. Zhang, C. Wu, H. Li, J. Yuen, J. Chang and Y. Xiao, *J. Mater. Chem.*, 2012, 22, 21686-21694.
7. C. Wu, P. Han, M. Xu, X. Zhang, Y. Zhou, G. Xue, J. Chang and Y. Xiao, *J. Mater. Chem. B*, 2013, 1, 876-885.
8. A. Singh and J. Elisseeff, *J. Mater. Chem.*, 2010, 20, 8832-8847.
9. A. Ogose, N. Kondo, H. Umezu, T. Hotta, H. Kawashima, K. Tokunaga, T. Ito, N. Kudo, M. Hoshino, W. Gu and N. Endo, *Biomaterials*, 2006, 27, 1542-1549.
10. R. Cancedda, P. Giannoni and M. Mastrogiacomo, *Biomaterials*, 2007, 28, 4240-4250.
11. S. Xu, K. Lin, Z. Wang, J. Chang, L. Wang, J. Lu and C. Ning, *Biomaterials*, 2008, 29, 2588-2596.
12. S. Ni, J. Chang, L. Chou and W. Zhai, *J. Biomed. Mater. Res. Part B: Appl Biomater*, 2007, 80, 174-183.
13. Y. Zhou, C. Wu and Y. Xiao, *Acta Biomater.* , 2012, 8, 2307-2316.
14. C. Wu and J. Chang, *J. Biomed. Mater. Res. Part B: Appl Biomater*, 2007, 83B, 153-160.
15. H. Sun, C. Wu, K. Dai, J. Chang and T. Tang, *Biomaterials*, 2006, 27, 5651-5657.

Journal of Materials Chemistry B Accepted Manuscript

16. W. Peng, W. Liu, W. Zhai, L. Jiang, L. Li, J. Chang and Y. Zhu, *J. Endod.* , 2011, 37, 1240-1246.
17. H. Gu, F. Guo, X. Zhou, L. Gong, Y. Zhang, W. Zhai, L. Chen, L. Cen, S. Yin, J. Chang and L. Cui, *Biomaterials*, 2011, 32, 7023-7033.
18. C. Wu, W. Fan, J. Chang, M. Zhang and Y. Xiao, *J. Am. Ceram. Soc.*, 2013, 96, 928-934.
19. C. Wu, Y. Ramaswamy, J. Chang, J. Woods, Y. Chen and H. Zreiqat, *J. Biomed. Mater. Res. Part B: Appl Biomater*, 2008, 87B, 346-353.
20. C. Wu, Y. Ramaswamy, D. Kwik and H. Zreiqat, *Biomaterials*, 2007, 28, 3171-3181.
21. C. Wu, Y. Ramaswamy, A. Soeparto and H. Zreiqat, *J. Biomed. Mater. Res. Part A*, 2008, 86, 402-410.
22. P. F. Manicone, P. Rossi Iommetti and L. Raffaelli, *J. Dent.*, 2007, 35, 819-826.
23. C. Piconi and G. Maccauro, *Biomaterials*, 1999, 20, 1-25.
24. M. Hisbergues, S. Vendeville and P. Vendeville, *J. Biomed. Mater. Res. Part B: Appl Biomater*, 2009, 88B, 519-529.
25. Y. Ramaswamy, C. Wu, A. Van Hummel, V. Combes, G. Grau and H. Zreiqat, *Biomaterials*, 2008, 29, 4392-4402.
26. B. M. Seo, M. Miura, S. Gronthos, P. M. Bartold, S. Batouli, J. Brahim, M. Young, P. G. Robey, C. Y. Wang and S. Shi, *Lancet*, 2004, 364, 149-155.
27. K. G. Silverio, T. L. Rodrigues, R. D. Coletta, L. Benevides, J. S. Da Silva, M. Z. Casati, E. A. Sallum and F. H. Nociti, Jr., *J. Periodont.*, 2010, 81, 1207-1215.
28. L. Liu, J. Ling, X. Wei, L. Wu and Y. Xiao, *J. Endod.* , 2009, 35, 1368-1376.
29. P. Han, C. Wu, J. Chang and Y. Xiao, *Biomaterials*, 2012, 33, 6370-6379.
30. C. Wu, P. Han, X. Liu, M. Xu, T. Tian, J. Chang and Y. Xiao, *Acta Biomater.* , 2014, 10, 428-438.
31. Y. Zhou, C. Wu, X. Zhang, P. Han and Y. Xiao, *J. Mater. Chem. B*, 2013, DOI: 10.1039/C3TB20445F.
32. *ISO/EN 10993-5. Biological evaluation of medical devices-part 5 tests for cytotoxicity, in vitro methods 8.2 tests on extracts.*, ISO/EN 10993-10995. Biological evaluation of medical devices-part 10995 tests for cytotoxicity, in vitro methods 10998.10992 tests on extracts.
33. F. M. Chen and Y. Jin, *Tissue Eng., Part B Rev*, 2010, 16, 219-255.
34. D. D. Bosshardt and A. Sculean, *Periodontol. 2000*, 2009, 51, 208-219.
35. K. M. Galler, R. N. D'Souza and J. D. Hartgerink, *J. Mater. Chem.*, 2010, 20, 8730-8746.
36. I. Bar-Kana, N. Savion, A. S. Narayanan and S. Pitaru, *Eur. J. Oral Sci.*, 1998, 106, 357-364.
37. H. W. Liu, R. Yacobi, N. Savion, A. S. Narayanan and S. Pitaru, *J. Bone Miner. Res.* , 1997, 12, 1691-1699.
38. M. A. Alvarez-Pérez, S. Narayanan, M. Zeichner-David, B. Rodríguez Carmona and H. Arzate, *Bone*, 2006, 38, 409-419.
39. A. I. Alford and K. D. Hankenson, *Bone*, 2006, 38, 749-757.
40. P. Han, C. Wu and Y. Xiao, *Biomater. Sci.*, 2013, 1, 379-392.
41. V. Sollazzo, F. Pezzetti, A. Scarano, A. Piattelli, C. A. Bignozzi, L. Massari, G. Brunelli and F. Carinci, *Dent. Mater.* , 2008, 24, 357-361.
42. T. Reya and H. Clevers, *Nature*, 2005, 434, 843-850.
43. H.-M. I. Yu, B. Jerchow, T.-J. Sheu, B. Liu, F. Costantini, J. E. Puzas, W. Birchmeier and W. Hsu, *Development*, 2005, 132, 1995-2005.

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Table 1.Primer pairs used in qRT-PCR analysis

Gene	Forward primer	Reverse primer
OPN	5'TCACCTGTGCCATACCAGTTAA 3'	5'TGAGATGGGTCAGGGTTTAGC 3'
ALP	5' TCAGAAGCTAACACCAACG 3'	5' TTGTACGTCTTGGAGAGGGC 3'
CEMP1	5' GGGCACATCAAGCACTGACAG 3'	5' CCCTTAGGAAGTGGCTGTCCAG3'
CAP	5' CTGCGCGCTGCACATGG 3'	5' GCGATGTCGTAGAAGGTGAGCC 3'
AXIN2	5' CCCCAAAGCAGCGGTGC 3'	5' GCGTGGACACCTGCCAG 3'
CTNNB	5' GCTACTGTTGGATTGATTCGAAATC 3'	5'CCCTGCTCACGCAAAGGT 3'
18s	5' TTCGGAACTGAGGCCATGAT 3'	5' CGAAC CTCCGACTTCGTTC 3'

Table 2. The ionic concentrations of culture medium after culturing hPDLCs on $Ca_3ZrSi_2O_9$ and β-TCP ceramic disks.

Time		Ionic concentrations (mg/L)	
		$Ca_3ZrSi_2O_9$	β-TCP
Day 1	Ca	92.29±0.71	85.83±0.56
	P	32.89±1.04	45.50±1.29
	Si	16.49±0.11	0.02±0.01
	Zr	0.04±0.004	0.00±0.001
Day 7	Ca	228.71±2.36	81.20±0.59
	P	28.57±0.54	41.34±0.97
	Si	59.56±1.33	0.02±0.01
	Zr	0.04±0.01	0.00±0.001

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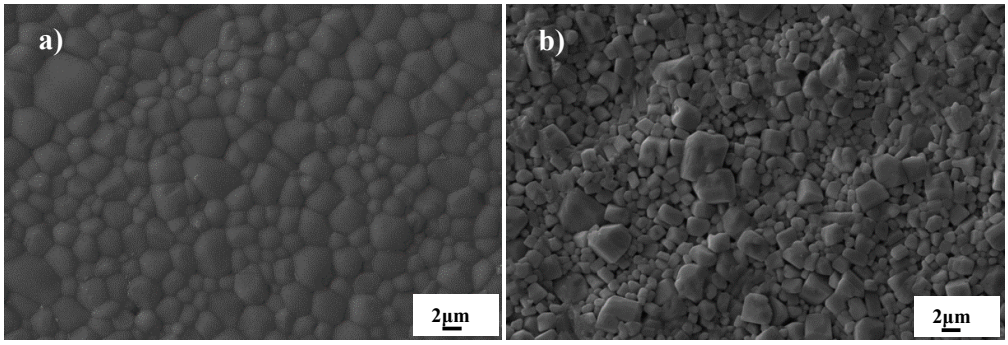

Figure 1. SEM analysis for surface of β-TCP (a) and $Ca_3ZrSi_2O_9$ (b) ceramic disks.

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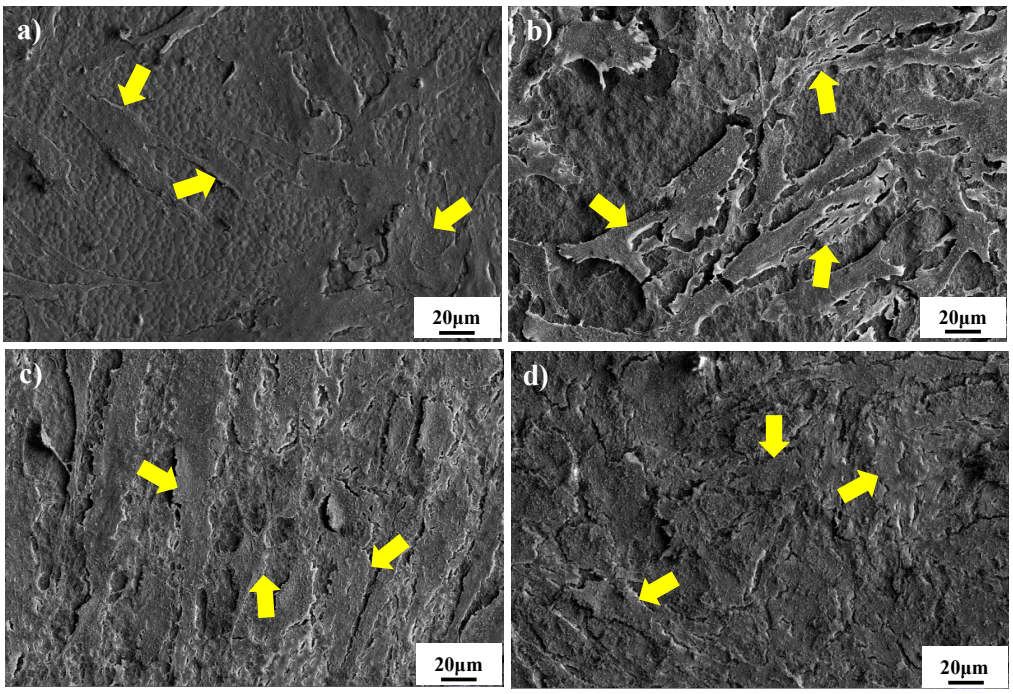

Figure 2. The cell morphology ofhPDLCs on β-TCP (a, c) and $Ca_3ZrSi_2O_9$(b, d) ceramics for 1 day (a, b) and for 7 days (c, d).The yellow arrows indicate the representative cells on the surface of both two ceramic disks.

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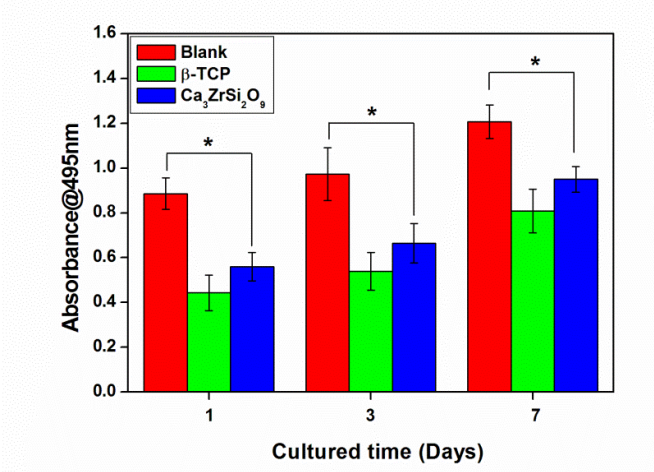

Figure 3. Proliferation of hPDLCson $Ca_3ZrSi_2O_9$, β-TCP ceramic disks and blank groups at day 1, 3 and 7. *: significant difference for $Ca_3ZrSi_2O_9$compared to blank group ($p<0.05$).

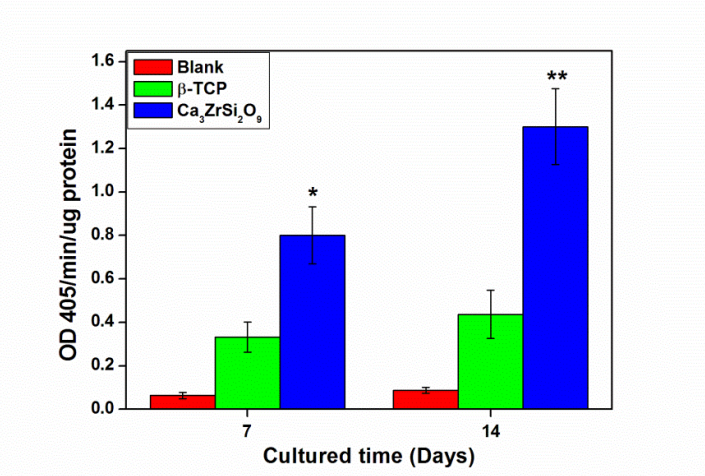

Figure 4. Relative ALP activity of hPDLCs on $Ca_3ZrSi_2O_9$, β-TCP ceramic disks and blank groups at day 7 and 14.*: significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$compared to two other groups at day 7. **:significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$compared to two other groups at day 14.

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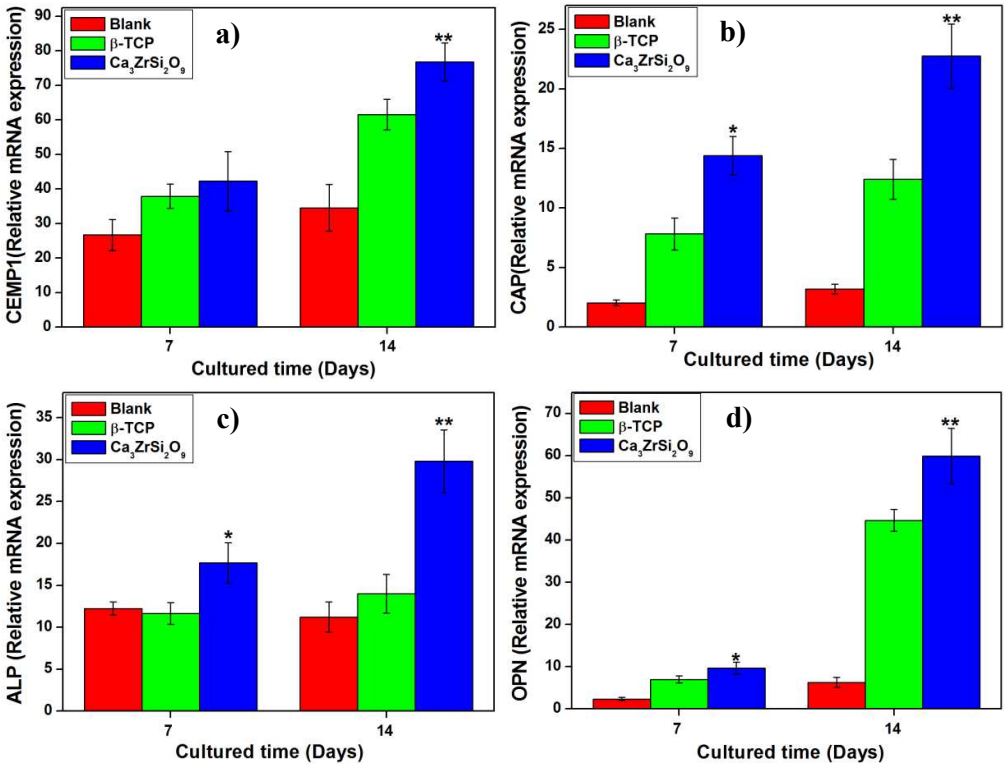

Figure 5. The effect of $Ca_3ZrSi_2O_9$ and β-TCP ceramics on cementogenic genes of CEMP1 (a) and CAP (b), and osteogenic genes of ALP (c) and OPN(d) for hPDLCs. *significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$ group compared to two other groups at day 7.**significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$ group compared to two other groups at day 14.

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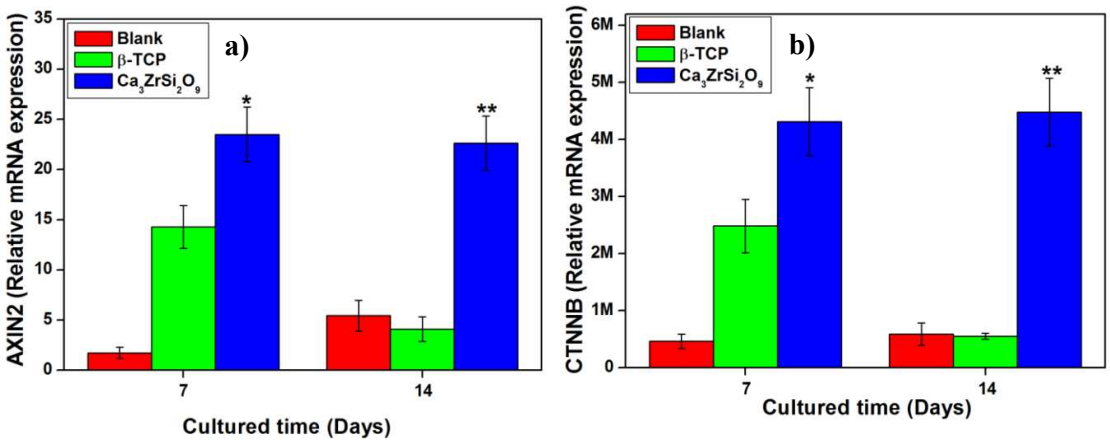

Figure 6. The Wnt/β-catenin signaling pathway-related genes expression of AXIN2 (a) and CTNNB (b)for hPDLCson $Ca_3ZrSi_2O_9$, β-TCP bioceramics and blank group.*significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$ group compared to two other groups at day 7.**significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$ group compared to two other groupsat day 14.

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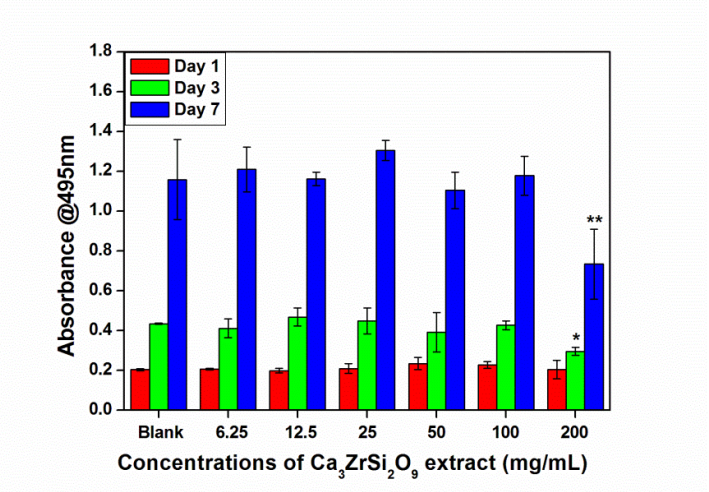

Figure 7. The cell proliferationof hPDLCs in the presence of different concentrations of $Ca_3ZrSi_2O_9$ extracts. *significant difference ($p<0.05$) of cell proliferation rate for hPDLCs on $Ca_3ZrSi_2O_9$ ceramics comparedto blank control at day 3.**significant difference ($p<0.05$) comparedto blank control at day 7.

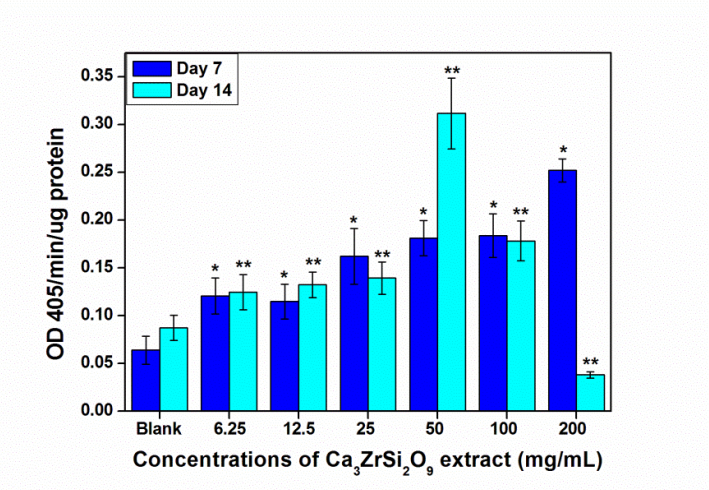

Figure 8. The relative ALP activity of hPDLCs in different concentrations of $Ca_3ZrSi_2O_9$ extracts. *significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$group compared to blank control at day 7. **significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$group compared to blank control at day 14.

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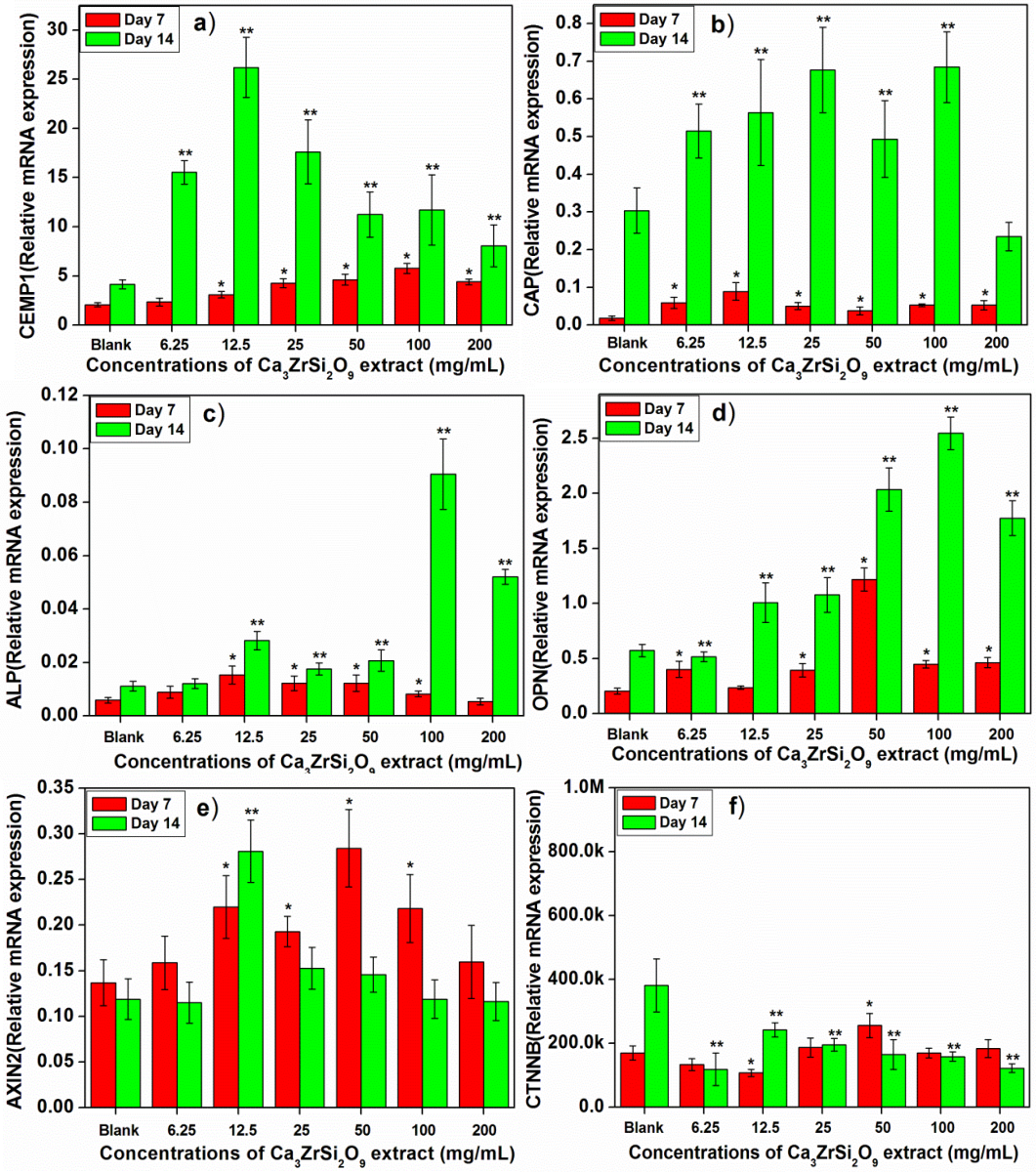

Figure 9. The effect of different concentrations of $Ca_3ZrSi_2O_9$ extracts on cementogenic genes of CEMP1 (a) and CAP (b), osteogenic genes of ALP (c), OPN(d) and Wnt/β-catenin-related genes expression of AXIN2 (e) and CTNNB (f)for hPDLCs. *significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$group compared to blank control at day 7. **significant difference ($p<0.05$) for $Ca_3ZrSi_2O_9$group compared to blank control at day 14.

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