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Osteoclastic cell behaviors affected by the α-tricalcium phosphate based bone cements

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Abstract Calcium phosphate cements (CPCs) have recently gained great interest as injectable bone substitutes for use in dentistry and orthopedics. α-tricalcium phosphate (α-TCP) is a popularly used precursor powder for CPCs. When mixed with appropriate content of liquid and kept under aqueous conditions, α-TCP dissolves to form a calcium-deficient hydroxyapatite and then hardens to cement. In this study, α-TCP based cement (CP) and its composite cement with chitosan (Ch-CP) were prepared and the osteoclastic responses to the cements and their elution products were evaluated. Preliminary evaluation of the cements revealed that the CP and Ch-CP hardened within ~10 min at an appropriate powder-to-liquid ratio (PL) of 3.0. In addition, CP and Ch-CP were transformed into an apatite phase following immersion in a saline solution. Moreover, the osteoblastic cells were viable on the cements for up to 10 days. Mouse-derived bone marrow cells were isolated and activated with osteoclastic differentiation medium, and the effects of the CP and Ch-CP substrates and their ionic eluants on the osteoclastic activity were investigated. Osteoclastic cells were viable for up to 14 days on both types of cements, maintaining a higher cell growth level than the control culture dish. Multi-nucleated osteoclastic cells that were tartrate-resistant acid phosphatase (TRAP)-positive were clearly observed when cultured on the cement substrates as well as treated with the cement eluants. The TRAP activity was found to be significantly higher in cells influenced by the cement substrates and their eluants with respect to the control culture dish (Ch-CP > CP ≫ control). Overall, the osteoclastic differentiation was highly stimulated by the α-TCP based experimental cements in terms of both the substrate interaction and their elution products.

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1 Introduction

Calcium phosphate cements (CPCs) are an attractive choice for the reconstruction of hard tissues [1–3]. The self-setting property under biological fluids enables CPCs to be potentially useful as an injectable device for filling defective sites and healing the fractured bone. Moreover, further hydrolysis transforms the initial calcium phosphate composition into a bone mineral-like hydroxyapatite phase, which is biologically compatible [1, 2]. Some challenges, in terms of improving mechanical properties, control over degradation rate and allowing porous structure, still remain as to the clinical application of CPCs. Therefore, many studies are being conducted in an attempt to develop CPCs that have better properties than those currently available [3].

α-Tricalcium phosphate (α-TCP) is one of the initial precursors of CPCs that has been widely used due to its appropriate setting reaction and ability to hydrolyze into a calcium-deficient hydroxyapatite (CDHA) according to the following reaction [3–6]:

$$3Ca_3(PO_4)_2[\alpha\text{-TCP}] + H_2O = Ca_9(HPO_4)(PO_4)_5OH\ [\text{CDHA}]$$

However, α-TCP based cements are very weak and brittle, which is considered to be one challenge that must be overcome to find more clinical uses [3]. As a result, a range of biopolymers have been introduced to improve the mechanical and biological properties of CPCs. These biopolymers primarily include degradable components such as chitosan, gelatin, collagen, and synthetic polymer phases [7–14]. Among those, chitosan has recently gained great interest as an additive for CPC [12–15]. Chitosan is a kind of polysaccharide obtained from the deacetylation of chitin and its derivatives are known to enhance the affinity of calcium ion binding [16]. Chitosan is largely acid-soluble and compatible with cells and tissues; therefore, it is useful in wound healing and tissue engineering scaffolds [16]. Due to its ionic properties, chitosan has also been extensively studied as gene and drug delivery systems [17].

The addition of chitosan to CPCs has recently been reported in several works [13–15]. The results have shown that the addition of chitosan enhanced the flexibility of brittle CPC as well as the mechanical strength, where the CPC powder used was a mixture of dicalcium phosphate–tetracalcium phosphate [13, 14]. Bone cell growth and differentiation were also shown to be enhanced with the addition of chitosan [15]. Taken together, these results suggest that the use chitosan in concert with CPCs is beneficial in terms of mechanical and biological aspect. Another promising application of chitosan-based CPCs is in the drug delivery systems due to the potential for chitosan to form complexes with biofunctional molecules and genes that are negatively-charged [17].

In this study, we produced α-TCP based CPC and a combination of this CPC with chitosan. In particular, the behaviors of the experimental CPCs in relation with osteoclastic cells were addressed which should also be considered as an important criterion to develop the CPCs for absorbable bone substitutes. Although a majority of works on the CPCs including chitosan-CPC have been carried out on the osteoblastic cells, very little is known about the effects on the osteoclastic cells [18–20]. Herein, the mouse bone marrow derived osteoclastic cells were used to investigate the cell behavior in response to the two types of experimental CPCs.

2 Materials and methods

2.1 Preparation of powders and cements

The experimental α-TCP cement powder was obtained by a solid-state thermal reaction between $CaCO_3$ and dicalcium phosphate anhydrous (DCPA). Briefly, commercially available $CaCO_3$ and DCPA were mixed in a bottle for 3 h and then placed in a Pt crucible and subjected to heat treatment at 1400°C for 5 h and subsequent quenching. The obtained powder was then ball-milled in high purity ethanol for 24 h, after which it was sieved down to 45 μm. The sieved product was then used as the initial CPC powder. The mean particle size of the experimental CPC was analyzed to be 4.79 (±0.037) μm (Saturn DigiSizer 5200, Micromeritics, USA). Hydroxyapatite (Alfa Aesar, USA; specific surface area = 68 m^2/g) powder was added to the CPC powder at 2 wt% to enhance the crystallization of the α-TCP into an apatite. As the liquid phase, 5% Na_2HPO_4 in distilled water was used. Moreover, a chitosan liquid was prepared by dissolving 2% chitosan (85% acetylation, medium molecular weight, Sigma-Aldrich Cat. No. 448877) in 1.5% acetic acid solution.

The cements were induced to set by vigorously mixing the powder with either the 5% Na_2HPO_4 or the chitosan solution, which is hereafter referred to as CP and Ch-CP. The powder to liquid (PL) ratio was determined at 3.0, the point with setting time being relatively short (<10 min) and with good mixing property, based on the results of a pilot study conducted using various PL ranges (2.6–3.5). The mixed cement was then molded, and incubated in a 100% humidity chamber for 24 h. The hardened cement (dimension of ϕ 5 mm × 2 mm) was further immersed in 10 ml phosphate buffered saline (PBS) for various lengths of time to observe any changes in the phase transformation α-TCP into an apatite within a fluid.

2.2 Mechanical tests and characterization

The setting time of the cements was measured by a Gillmore needle test (400 g needle). The effect of ball-milling time (0, 6, 24, and 48 h) and HA addition (1, 2, and 5 wt%) on the setting time was investigated in a pilot study. The optimal ball-milling time and HA addition was determined at 24 h and 2 wt%, respectively, at which the setting time became relatively short within ~10 min. The compressive strength of the samples before and after the immersion in PBS was measured using Instron 3344 at a loading rate of 1 mm/min. The tests were conducted on wet samples. A total of five specimens were tested under each condition. The stress–strain curves were obtained and maximum stress (compressive strength) was determined. The porosity of the samples was measured by Archimedes method. The morphology of the specimens was observed by scanning electron microscopy (SEM, Hitachi). The phase change during immersion in PBS was characterized by X-ray diffraction (XRD, Rigaku).

2.3 Preparation of samples for cellular tests

The cements were prepared in a disc type with dimensions of either ϕ 5 mm × 2 mm or ϕ 10 mm × 2 mm using designed polypropylene moulds. Samples were placed in each well of a 96- or 24-well plate and then sterilized in 70% ethanol for 30 min, after which they were dried under laminar flow. Before cell seeding, the cements were soaked in PBS and then in α-modified eagle medium (α-MEM). To evaluate the effects of the elution products of the cements on cell activity, a Transwell insert containing a cement sample was placed in each well of a 24-well plate and the indirect biological effects on the cells were evaluated.

2.4 Osteoblast cell viability

To determine the osteoblastic cell viability on the experimental cements, MC3T3-E1 cells with passages of 10–15 were used. The cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS, Gibco) containing 1% antibiotic/antimycotic liquid (10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of amphotericin B/m, Gibco) in a humidified atmosphere of 5% CO_2 in air at 37°C. A cell suspension with a density of 2×10^4/ml was then prepared and seeded on each hardened cement sample and subsequently incubated in an atmosphere of 5% CO_2 in air at 37°C. The cell proliferation was then measured using the MTS method. When the MTS reagent (tetrazolium salt) is applied to living cells, it is reduced by cells into a color formazan product which is soluble in culture medium. The quantity of formazan product was measured at an absorbance of 490 nm using a microplate reader which is directly proportional to the number of living cells.

2.5 Mouse-derived osteoclastic cells and differentiation

Osteoclast-like cells were obtained from the mouse bone marrow. Briefly, the mouse (6 days old) femora and tibiae were removed, after which the marrow cavities were flushed with α-MEM containing 10% FBS and 1% antibiotic/antimycotic liquid. The tissue was then centrifuged and the supernatant was resuspended in α-MEM and then cultured for up to 14 days. Culture medium was refreshed every 3 days.

Cells were seeded on each sample (culture dish or cements) at 6×10^4 (for 96-well plate) or 3×10^5 (for 24-well plate). At the culturing time of 3 days after seeding on each sample, osteoclastic cell differentiation was stimulated by the addition of 10 ng/ml recombinant mouse macrophage colony-stimulating factor (M-CSF) together with 50 ng/ml receptor activator of nuclear factor kappa B ligand (RANKL) and 1 ng/ml transforming growth factor β1 (TGF-β1) in α-MEM containing 10% FBS. The osteoclastic cell culturing medium was refreshed every 3 days.

2.6 Synthesis of tartrate-resistant acid phosphatase (TRAP)

Osteoclast-like cells were characterized by their multinucleated morphology and positive staining for TRAP. For TRAP staining, aliquots from supernatant solutions were incubated with 1 M sodium citrate solution, 50 mM sodium tartrate (pH 8.2) and 0.1% Triton X-100 (all chemicals from Sigma) as the substrate for 10 min. The level of enzymatic product *p*-nitrophenol was then stopped by the addition of 50 mM NaOH. The light absorbance of these samples was then measured on a spectrophotometer (MR600 spectrophotometric microplate reader; Dynatech) at 405 nm. For the quantification of the total protein, a commercial DC protein assay kit (BioRad) was used, according to the manufacturer's instruction. A 200 μl of each sample was reacted with commercial chemical reagents (working reagents) for 15 min at room temperature, and the color change in the reaction product (5 μl) was read at an absorbance 595 nm using a spectrophotometric microplate reader. Total protein contents were deduced from the protein standard curve which was made using a bovine serum albumin (Sigma). The TRAP activity test was conducted on three replicate samples for each condition ($n = 3$).

2.7 Morphology observation and counts

The actin rings were visualized by staining of F-actin with rhodamine-conjugated phalloidin. Briefly, the cells were fixed with 3.7% formaldehyde in PBS for 10 min and then permeated by treatment with 0.1% Triton X-100 in PBS for 1 min. The cells were then incubated in an Alex Fluor 546 phalloidin (invitrogen A22283) solution diluted in 1% bovine serum albumin at room temperature for 3 h to stain F-actin. Next, the samples were washed with water, after which the actin rings formed by the osteoclasts were observed using a fluorescence microscope (Olympus BX-50, Osaka, Japan). For electron microscopy, the cell-cement construct was fixed with 2.5% glutaraldehyde, dehydrated with a graded series of ethanol, treated with hexamethyldisilazane and sputter coated with gold. The cell morphology was observed by SEM at an accelerating voltage of 15 kV.

The osteoclast-like cells affected by the elution products from the cements were stained for TRAP using a commercially available leukocyte acid phosphatase staining kit, 387 A (Sigma, Saint Quentin Fallavier). Briefly, cells were washed with PBS to remove non-adherent cells and then

fixed in 3.7% formaldehyde in PBS for 5 min. Next, the cells were incubated at 37°C in staining solution for 10 min and then rinsed with PBS. Multinucleated TRAP-positive cells were observed under optical microscopy and those with more than three nuclei were considered as osteoclasts, which were counted based on the microscopic examination of three different areas in triplicate samples.

2.8 Statistical analysis

The data was represented as the mean ± 1 standard deviation. Statistical analysis was carried out using Student *t*-test and significance was considered at P value < 0.05.

3 Results and discussion

3.1 Setting and properties of cements

Table 1 shows the characteristics of the current experimental CPCs based on α-TCP. The setting times of both types of cements were similar (within 10 min). When compared to other studies on α-TCP based CPCs, the currently obtained setting time was relatively short [21, 22]. In practice, the setting reaction largely depends on the initial powder characteristics, such as the powder size, reactivity and impurity, as well as the liquid type used and powder-to-liquid (PL) ratio [3]. The results of a pilot study revealed that the initial powder size had a significant effect on the setting time, i.e., the decrease in powder size accelerated the setting reaction. Moreover, increasing the PL ratio accelerated the setting reaction; however, the addition of the powder was limited to allow proper mixing. In practice, we could shorten the setting time within ~10 min by reducing the powder size by ball milling and increasing the PL ratio to 3, which was determined as the optimal condition for the preparation of cements.

Table 1 Properties of the α-tricalcium phosphate cement (CP) and its composite with chitosan (Ch-CP)

Setting time (min)	CP 8.6 (±2.9)	Ch-CP 7.5 (±2.1)
Porosity (%)		
Before	36.5	38.1
7-day soaking	31.7	45.9
14-day soaking	38.5	47.2
28-day soaking	44.0	51.0
Compressive strength (MPa)		
Before	10.8 (±3.0)	13.1 (±2.1)
7-day soaking	15.6 (±3.3)*	17.4 (±2.0)*
28-day soaking	13.1 (±4.8)	14.5 (±1.9)

The setting time was determined with samples prepared using the PL ratio of 3.0 by the Gilmore needle test. The porosity of the samples before and after soaking in PBS was measured by Archimedes method. The compressive strength was tested on wet samples before and after soaking in PBS using an Instron. Data were presented as mean ± standard deviation for 3 (setting time) and 5 (strength) specimens for each condition. After soaking for 7 days, strength of both CP and Ch-CP was significantly enhanced (* $P < 0.05$)

The initial compressive strength of the wet cements was 10.8 and 13.1 MPa for CP and Ch-CP, respectively. Interestingly, when soaked in PBS for 7 days, the strength of the cements increased significantly for both compositions ($P < 0.05$). However, a prolonged soaking for 28 days decreased the strength values slightly with respect to those at day 7. The trend was similar for both types of CPCs. In fact, the α-TCP based cement followed a hydrolysis reaction under physiological conditions in vitro and in vivo in which α-TCP dissolved to form a calcium-deficient hydroxyapatite [5, 6]. It is believed that the transformed HA phase leads to an increase in strength because the HA nanocrystallites form networks that interconnect the loose interspaces of the α-TCP particles. It was reported that the compressive strength of pure CPC was proportional to its conversion to HA [21]. Moreover, the slight reduction in strength that was observed in response to extended soaking likely results from the hydrolytic degradation of the cements with time, which can create pores and voids that lead to a failure of the product.

The morphological change in the cements with soaking time was observed with SEM as shown in Fig. 1. The formation of HA crystallites was clearly seen. The crystallite formation appeared to increase as the soaking time increased to ~14 days, and was similar thereafter (at ~28 days). There appeared to be no significant morphological differences depending on the cement types.

Mouse-derived osteoblastic cells (MC3T3-E1) were cultured on the hardened cements to assess the cell viability. When cultured for 10 days on the cements, the cell proliferation level was similar to that on the control culture dish (96.7 ± 2.37% for CP and 105.3 ± 2.67% for Ch-CP with respect to control), which confirms that the two types of cements are at least capable of supporting tissue cells.

3.2 Osteoclastic responses to cements

Osteoclast-like cell responses to the experimental cements were evaluated using mouse bone marrow stromal cells (mBMSCs). The cells that initially adhered to the culture dish (Fig. 2a) were shown to undergo osteoclastic differentiation in response to treatment with osteoclastic factors (10 ng/ml M-CSF + 1 ng/ml TGF-β1 + 50 ng/ml RANKL) (Fig. 2b). Almost all cells were stained TRAP-positive, and some multinucleated cells (arrows) were clearly seen. Two different experimental set-ups were designed to investigate

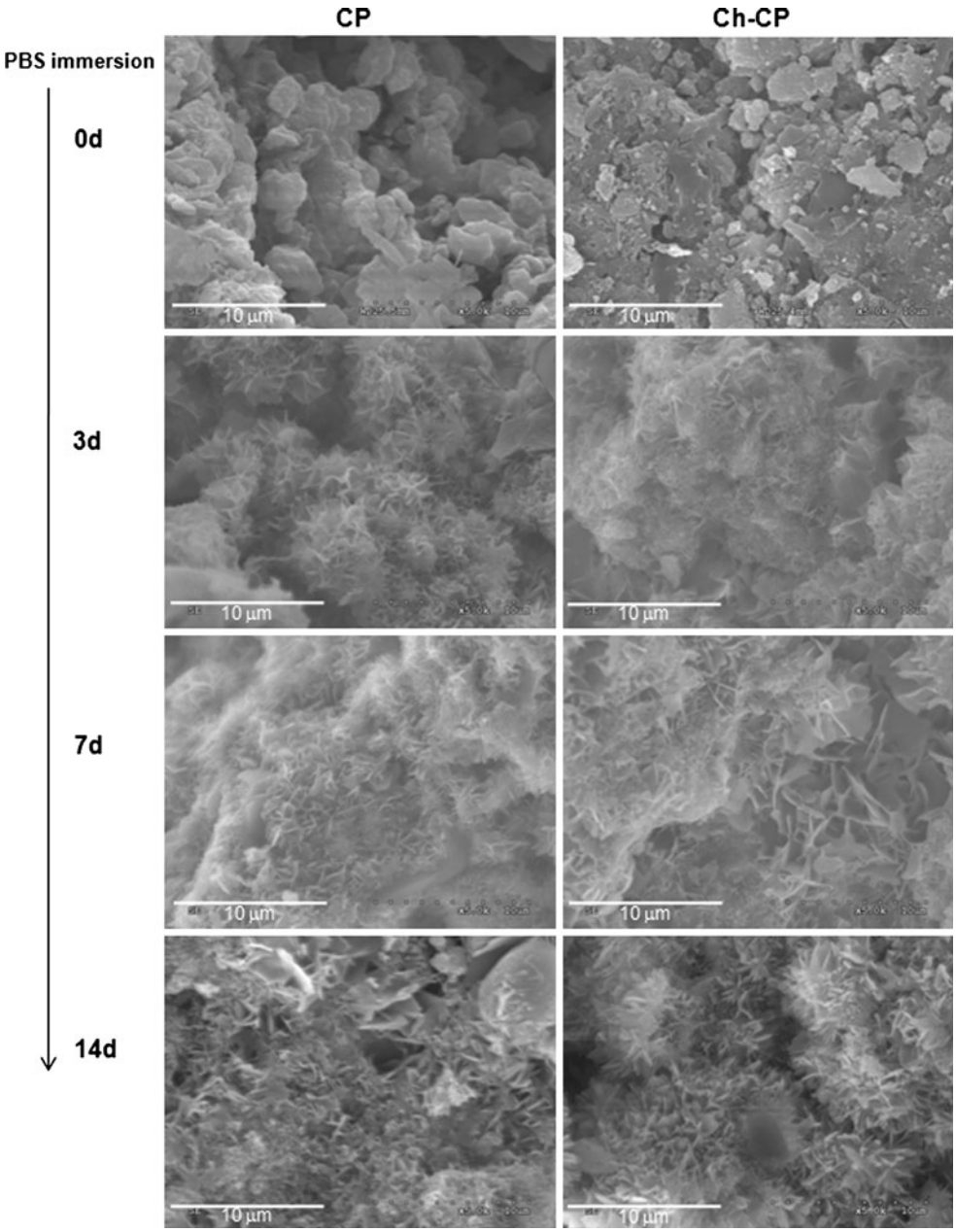

Fig. 1 SEM morphological change in the CP and Ch-CP during immersion in PBS for 3, 7 and 14 days. Images before the PBS-immersion were shown for comparison. The formation of apatite crystallites was noticed during incubation

the effects of either direct substrate or indirect eluants of the cements on the osteoclastic cell behaviors.

For the indirect assessment, cement samples were placed in an insert membrane to elute the products and the mBMSCs were then cultured under the influence of the cement eluants. The viable cells were counted as shown in Fig. 3. MTS assays revealed that the cell viability was significantly higher when cells were influenced by both of the cement eluants as compared to the control ($P < 0.05$). For the specific observation of osteoclastic cells, TRAP-staining was performed, as shown in Fig. 4. Multi-nucleated giant cells were readily visible under the influence of both types of cement (CP in (b) and Ch-CP in (c)), like those in control dish (a). TRAP-positive red staining demonstrated the active osteoclastic development of the cells cultured in the presence of the cements. The TRAP-positive and multi-nucleated cells with nuclei over three were counted as shown in Fig. 5. Significantly higher number of multi-nucleated cells was observed for both types of cements ($P < 0.05$). Moreover, the multi-nucleated cell number was higher in the Ch-CP group than in the CP group at days 5 and 8. The TRAP activity of the osteoclastic cells was also measured, as shown in Fig. 6. At days 8 and 11, the TRAP level was significantly higher in both types of cements than in the control ($P < 0.05$). Based on the results of an indirect assay, osteoclastic

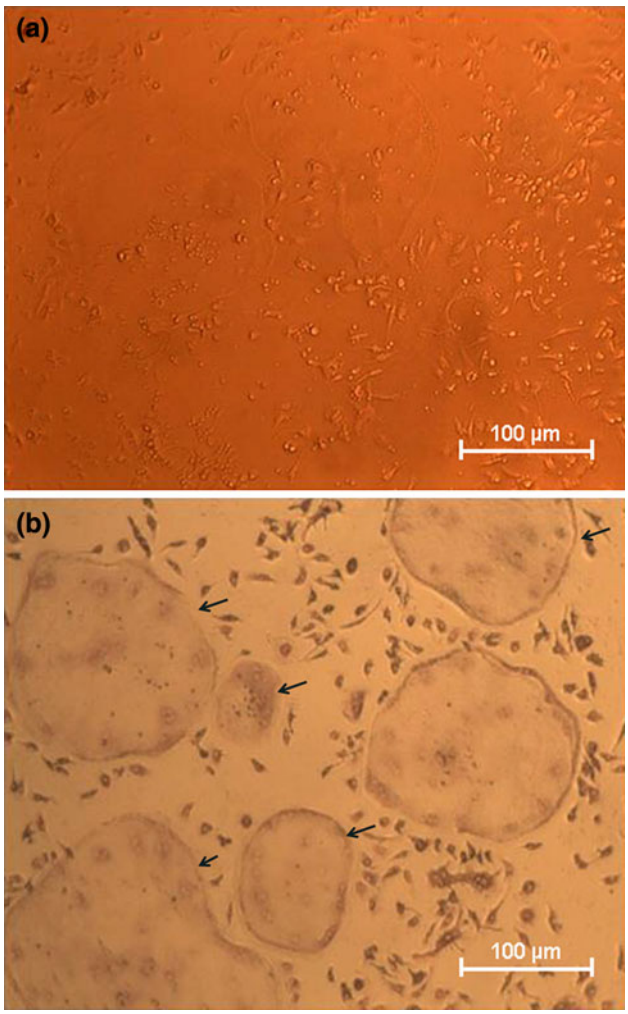

Fig. 2 Optical micrograph of **a** mouse bone marrow cells and **b** their osteoclastic differentiation following treatment with 10 ng/ml M-CSF + 1 ng/ml TGF-β1 + 50 ng/ml RANKL. Almost all cells cultured for 8 days were stained TRAP-positive and some multinucleated large cells (*arrowed*) were clearly seen

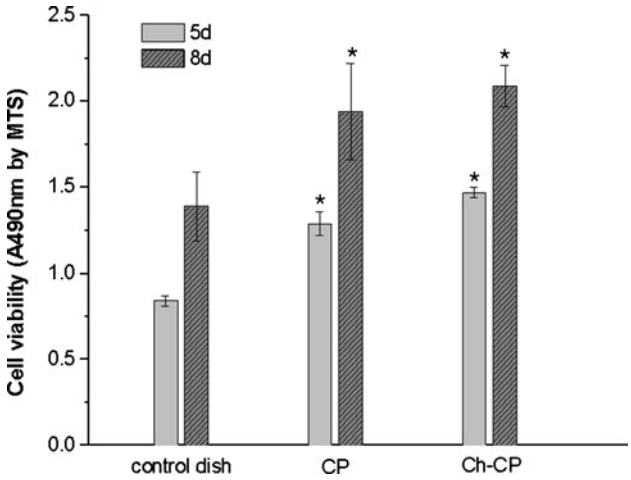

Fig. 3 The effect of cements eluants on the osteoclast-like cellular growth at day 5 and 8, as determined by an MTS assay. Cells were grown affected by the cement eluants passing a Transwell insert filter containing the cement sample. Significant differences were observed between the cement samples and the control (* $P < 0.05$, dish vs. CP or Ch-CP, Student t-test, $n = 3$)

differentiation was significantly up-regulated by the elusion products of the experimental cements.

The osteoclastic behavior was then examined when grown directly on the cements. Figure 7 shows the cell morphology grown on the CP and Ch-CP at day 8. Some extremely large cells were observed on the cements by SEM (Fig.7a), indicating osteoclast trait. In addition, confocal microscopy staining revealed that multinucleated giant cells were present around the small cells, forming an actin ring (arrows in Fig.7b). The presence of an actin ring at the cell periphery is considered to confirm the osteoclastic differentiation [18, 19]. The cell viability was also found to be significantly higher when cells were grown on both types of cements, as shown in Fig. 8. Moreover, TRAP synthesis by the osteoclast-like cells grown directly on both types of cement samples was significantly higher for up to 14 days when compared with the control ($P < 0.05$), as shown in Fig. 9. The results showed a trend similar to the indirect assay. Taken together, these findings indicate that the α-TCP based experimental cements (CP and Ch-CP) stimulated osteoclastic differentiation of mBMSCs via both direct and indirect ways.

Although there have been many studies performed to evaluate the synthesis and physico-chemical properties of CPCs, very few have been conducted to evaluate the effects related with osteoclasts [18–20]. However, it is important to evaluate the effects that CPCs play on osteoclastic activity to determine the potential of developed CPCs in the bone repair process. The results of the present study revealed that two experimental cements based on α-TCP composition had the potential to stimulate osteoclastic activity. Some studies have demonstrated that CPCs play an active role in osteoblastic differentiation and mineralization, which mainly results from the transformed hydroxyapatite phase [15]. In our preliminary study, osteoblasts grown on the cements showed good viability. Furthermore, the cements evaluated herein are believed to play positive roles in the osteoblastic differentiation, based on some previous works [15]. Above all, the significantly enhanced osteoclastic viability and differentiation observed herein suggest that the developed CPCs have the potential for practical use in the regenerative process.

Based on the osteoclastic behaviors observed herein, the addition of chitosan to the α-TCP based CPC composition appeared to slightly enhance the osteoclastic activity such as the TRAP synthesis and multi-nucleated cell formation. Previous studies conducted to evaluate chitosan-added

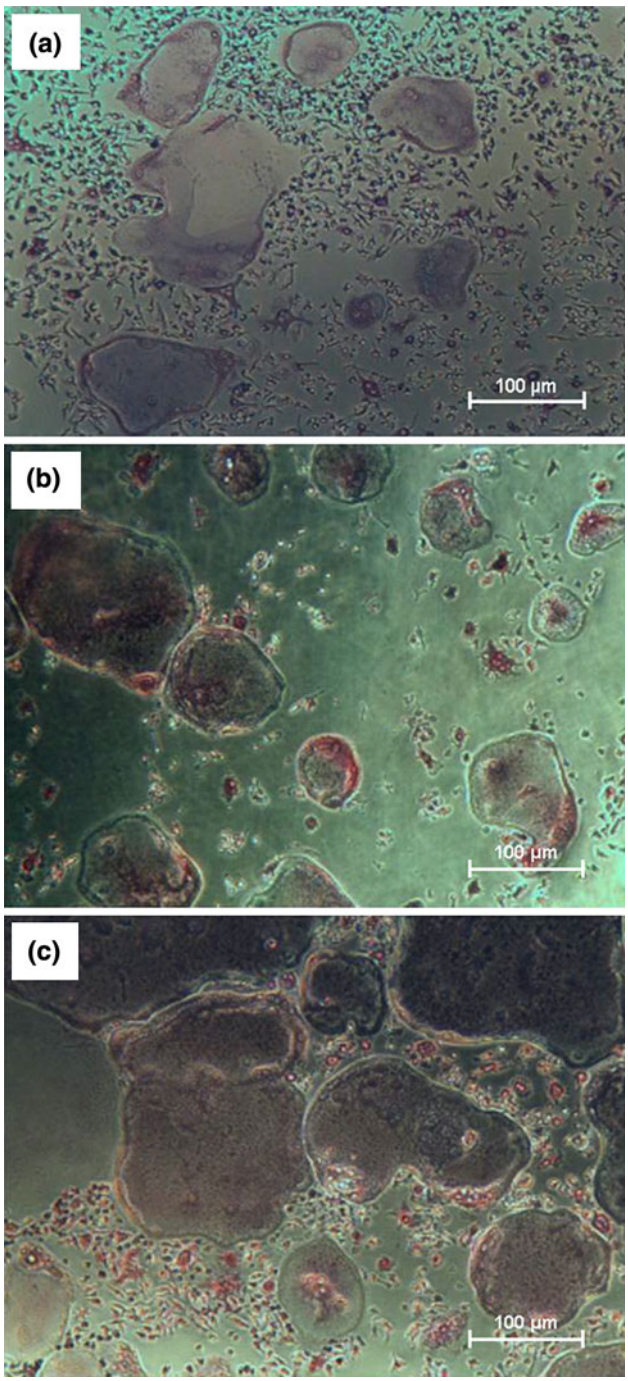

Fig. 4 Optical microscopy showing the TRAP-positive stained and multi-nucleated cells affected by the elution products of the cement samples, after culturing for 8 days: **a** control, **b** CP and **c** Ch-CP

CPCs have shown the chitosan was effective in osteoblastic differentiation and matrix synthesis [15]. In practice, chitosan has primarily been introduced into CPCs to improve their mechanical properties [13, 14], which was also observed in this study along with slight increase in setting reaction (Table 1).

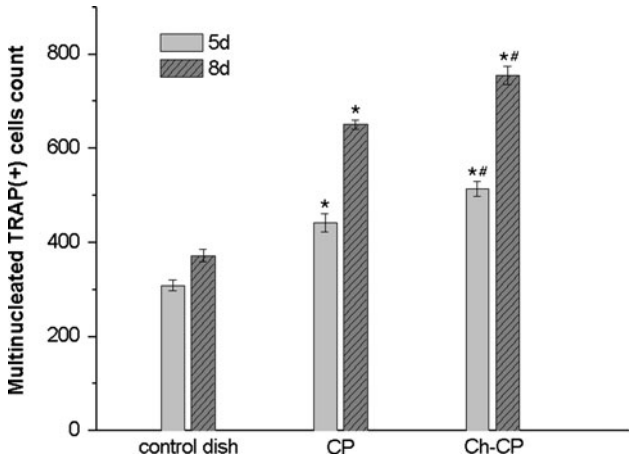

Fig. 5 Count of multi-nucleated TRAP-positive cells affected by the elution products of the cement samples in comparison with the control dish. Cells with more than 3 nuclei were counted as the multi-nucleated cells. A significantly higher number of cells were observed in the CP and Ch-CP treatments than in the control. (* $P < 0.05$, control vs. CP or Ch-CP; # $P < 0.05$, CP vs. Ch-CP, Student t-test, $n = 3$)

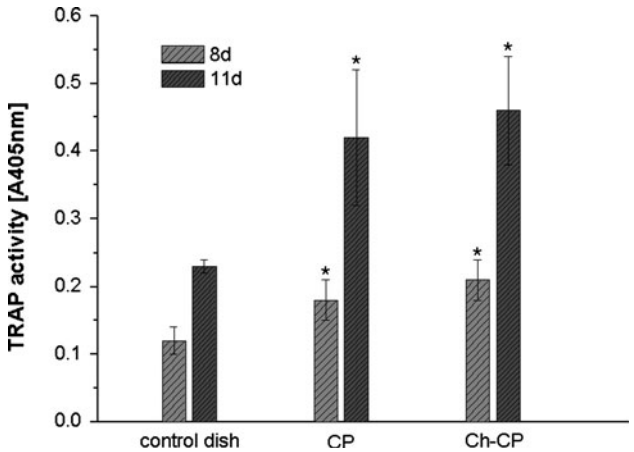

Fig. 6 TRAP synthesis by the osteoclast-like cells in response to the elution products of the cements at day 8 and 11. TRAP synthesis differed significantly between cement treatments and the control (* $P < 0.05$, control vs. CP or Ch-CP, Student t-test, $n = 3$)

Together with some recent studies that have evaluated the role of chitosan in the stimulation of osteoblastic differentiation [15], the enhanced osteoclastic activity observed herein suggests that chitosan-CPC would be useful in bone regeneration. However, the osteoclasts were cultured alone in this study; therefore, future studies should be conducted under co-culturing condition with osteoblasts to understand the cellular resorption behavior of the experimental CPCs. Moreover, the evaluation of the osteoclastic response to dentin slices and comparison with experimental groups need to be conducted to establish the desorption potential of the cements relative to bone.

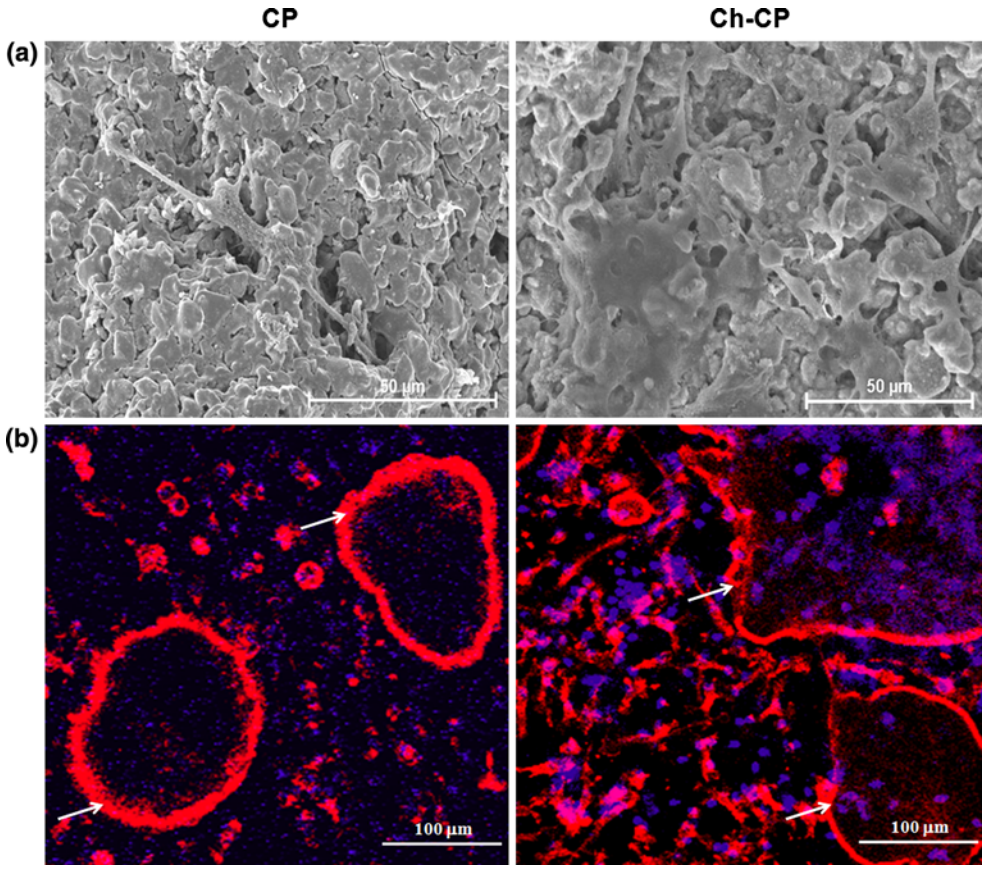

Fig. 7 **a** SEM and **b** CLSM stained images of the cells grown on CP and Ch-CP at 8 days. Multi-nucleated large cells were revealed around the small cells indicated by the *arrows*. Multiple nuclei were stained *blue* within the *red*-colored border of the osteoclast membrane (Color figure online)

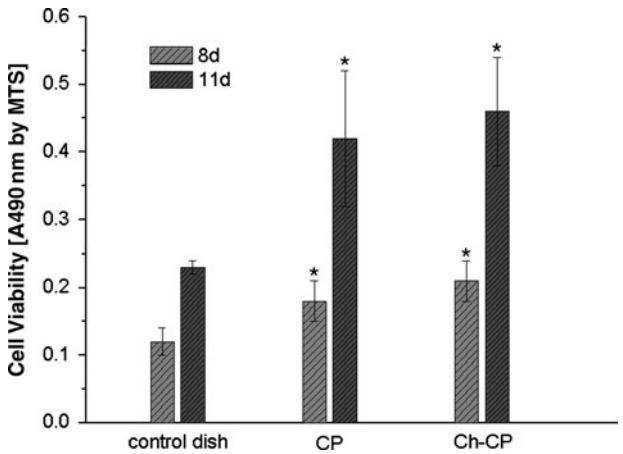

Fig. 8 MTS assay of cells grown directly on the cement samples at day 5 and 8. A significant difference was observed between the cements and control (* $P < 0.05$, control vs. CP or Ch-CP, Student t-test, $n = 3$)

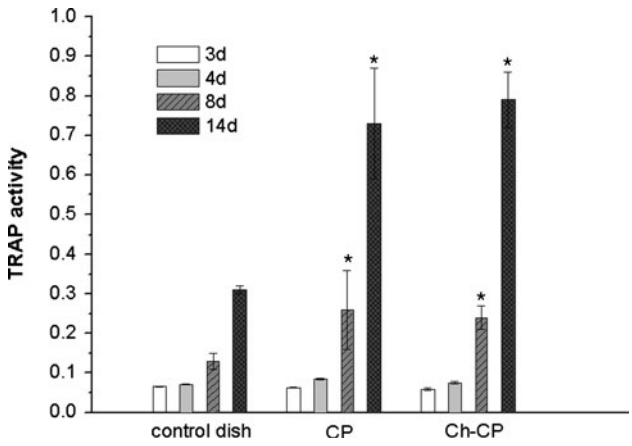

Fig. 9 TRAP synthesis by the osteoclast-like cells grown directly on the cement samples for up to 14 days. The synthesis by the cells differed significantly between the cements and control, particularly after 8 days (* $P < 0.05$, control vs. CP or Ch-CP, Student t-test, $n = 3$)

4 Conclusions

The effects of two types of α-TCP based experimental cements (chitosan added or not) on osteoclastic differentiation of bone marrow derived cells were investigated. TRAP-positive and multi-nucleated cells were well-developed under appropriate differentiation conditions. In addition, the cement substrates and elution products led to significant stimulation of the osteoclastic cell growth and

differentiation activity. Moreover, the addition of chitosan to the cement slightly increased the osteoclastic development. Together with other properties such as setting time, mechanical properties and osteoblastic viability, the osteoclastic stimulatory role of the CPCs observed herein indicates that they would be useful as a degradable matrix for bone regeneration.

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