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ORIGINAL ARTICLE

Inhibition of osteoclast activities by SCPC bioceramic promotes osteoblast-mediated graft resorption and osteogenic differentiation

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Abstract

Maximizing vital bone in a grafted site is dependent on a number of factors. These include resorption or turnover of the graft material, stimulation of bone formation pathway without a need for biological molecules added to the site and inhibition of cellular activities that compromise the mineralization of new bone matrix. In the present study, the dissolution profile of silica-calcium phosphate composite (SCPC) in physiological solution was measured and the data were fed to (ANN-NARX) prediction model to predict the time required for complete dissolution. The inductively coupled plasma-optical emission spectrometer ionic composition analysis of the culture medium incubated for 3 days with SCPC showed 57% decrease in Ca concentration and a significant increase in the concentration of Si (13.5 ± 1.8 μg/ml), P (249.4 ± 22 μg/ml), and Na (9.3 ± 0.52 μg/ml). In conjunction with the release of Si, P, and Na ions, the bone resorptive activity of osteoclasts was inhibited as indicated by the significant decrease in multinucleated tartrate resistant acidic phosphate stained cells and the volume of resorption pits on bone slices. In contrast, addition of SCPC to hBMSC cultured in conventional medium promoted higher Runt-related transcription factor 2 ($p < .05$), osteocalcin ($p < .01$), and bone sialo protein ($p < .01$) than that expressed by control cells grown in the absence of SCPC. The predicted dissolution time of 200 mg of porous SCPC particles in 10 ml phosphate buffered saline is 6.9 months. An important byproduct of the dissolution is inhibition of osteoclastic activity and promotion of osteoblastic differentiation and hence bone formation.

KEYWORDS

bone graft, bone marrow stem cells, osteoclast, resorption, SCPC bioactive ceramic

1 | INTRODUCTION

Bone graft materials are used in over 2 million cases per year in dental and orthopedic surgeries to aid bone regeneration.[1] Calcium phosphates and calcium sulfate ceramics were introduced as bone grafts 50 years ago, however, allografts and autografts are still the preferred choice. The primarily reason for the limited use of calcium phosphates and calcium sulfate are the inability to stimulate graft resorption and bone regeneration in a reasonable time period. Successful bone regeneration in grafted defects is partly dependent on the stimulatory effect of the graft material on osteoblast differentiation. To create more space for vital bone in the site, another valuable function is a suitable degradation rate of the graft material, corresponding to the formation of new bone. Although calcium phosphate hydroxyapatite (HA) stimulates osteoblast differentiation and function it is not bioresorbable.[2,3] This poor resorbability compromises the bioactivity

property and often leads to fibrous encapsulation rather than direct bone bonding.[4-13] Thus, the inadequate resorbability of HA due to its high chemical stability and limited solubility disabled successful bone regeneration.[14,15] In a grafted bone defect, osteoclasts attach to the surface of the graft material and create an acidic environment inside closed compartments that locally dissolves calcium phosphate locally and make resorption pits on its surface.[16] Osteoclasts cannot resorb all types of bioceramics; in particular stoichiometric HA which has a stable structure that resists dissolution. For example, data in the literature showed that tricalcium phosphate (TCP) granules (e.g., Biosorb) and bovine HA graft (e.g., Bio Oss) remained inside bone defects for 5 years postoperatively.[6,10,17] The osteoclastic dissolution of these bioceramics, does not mimic the dissolution of natural bone during remodeling for many reasons. First, in the remodeling cycle of human bone the osteoclastic resorption phases include dissolution of bone mineral and resorption of the organic phase by the action of enzymes including collagenases and cathepsin K.[18,19] The resorption phases of osteoclasts take a short time period (3–4 weeks) compared to the bone formation phase by osteoblasts which is 3–4 months [20,21]. Osteoclastic resorption of HA ceramic graft could take more than 5 years.[6,10] The lengthy osteoclastic dissolution process of the bioceramic graft and the absence of a resorption phase of organic matrix distract osteoclasts and compromise their ability to signal and recruit osteoblasts. It could be argued that osteoclastic dissolution of calcium phosphate ceramic releases calcium ions, which have a stimulatory effect on osteoblasts. However, the released calcium ions also stimulate osteoclasts differentiation.[22] The competition between osteoclasts and osteoblasts on the limited amount of calcium slows down the remodeling and maturation of the newly formed bone. This is supported by many histological analyses that demonstrated fibrous immature bone matrix in defects grafted with HA or biphasic calcium phosphate (BCP) after 3–5 years of grafting.[6,10,23] Second, osteoclastic bone resorption during remodeling releases growth factors that stimulate osteoblast differentiation and bone formation.[24] Calcium phosphate ceramics do not release any growth factors upon dissolution.

An ideal bone graft would have dissolution products that signal osteoblast differentiation and promote bone formation in the immediate postoperative period without compromising the role of osteoclasts in the subsequent remodeling process of the newly formed bone. Several studies have shown that the dissolution products of bioglass, in particular silica, stimulate osteoblasts and down regulate osteoclasts. This can explain the rapid bone formation and maturation at the interface with bioglass. However, the solubility and hence resorbability of bioglass is restricted by: (a) The rigid silicate network and (b) the formation silica rich surface layer that inhibits ionic diffusion. Previous studies from our lab reported on the stimulatory effect of silica-calcium phosphate composite (SCPC) on osteogenic gene expression by osteoblasts and stem cells.[25-27] In other histologic studies, osteoblasts have been activated in bone defects grafted with SCPC granules with complete absence of osteoclasts at the surface of the SCPC granules.[28-30] The SCPC bone graft material fulfilled two of the main requirements for osseous regenerative medicine. These included stimulations of new bone formation and complete replacement of the graft particles by functioning bone.

In the present study, we demonstrate the inhibitory effect of SCPC dissolution products on osteoclast differentiation and bone resorptive capability in vitro. Mathematical modeling of 480 hr immersion data predicted complete dissolution of 200 mg SCPC during 5,002.5 hr in physiological solution. The SCPC bone graft material induced osteogenic differentiation of hBMSC and decreased osteoclastic activity, something not seen around other graft materials. The results suggest that the resorption mechanism of silica-containing bioactive ceramic is different from that of calcium phosphate ceramics.

2 | MATERIALS AND METHOD

2.1 | Dissolution analysis and modeling

The silica-calcium phosphate composite resorbable bioactive porous granules of chemical composition (in mol% 40.7 CaO, 19.5 SiO_2, 20.3 P_2O_5, and 19.5 Na_2O) in the size range 90–710 μm (SCPC, Shefabone, Inc., Charlotte) were used for the study. SCPC granules (0.2 g, n = 5) were immersed in (10 ml) Phosphate buffered saline (PBS, pH = 7.4) in plastic bottles at 37°C for 2, 4, 8, 16, 24, 48, 72, up to 480 hr. After each time point, the solution was collected and stored in plastic centrifuge tubes at 4°C for concentration measurements. The concentrations of Ca, P, Na, and Si ions in the PBS incubated with the SCPC bioceramic samples (n = 5) were measured using the inductively coupled plasma-optical emission spectrometer (ICP-OES). A nonlinear autoregressive neural network time series prediction model with external input (NARX) was employed. All the ICP-OES ceramic dissolution data points were used for training the network, except for one set which was used to validate the predicted output. Moreover, the closeness between the predicted and the experimental dissolution data was verified.

2.2 | Effect of SCPC on human osteoclasts

SCPC granules were immersed separately in α-minimum essential medium (Eagle's MEM, Sigma–Aldrich, St. Louis, MO) at various ratios (0.0, 2.5, 5.0, 10.0, and 20.0 mg/ml) at 37°C for 3 days. The SCPC-containing αMEM solutions were vortexed for 30 s and centrifuged at 1,000 rpm for 5 min. The supernatant was collected and sterilized by filtering through an 0.22 μm filter. The concentration of Si, P, Ca, and Na ions released from SCPC granules into the α-MEM after 3 days was measured using ICP-OES. Osteoclast precursor cells were isolated with Ficoll-Paque (GE Healthcare, Life Chafont, UK) from human bone marrow (BM) according to the protocol approved by the Human Subjects Committee of the University of Turku. Osteoclast precursor cells were seeded on bone slices (n = 5) at a density of 10^6 cells/cm^2 and cultured in the conditioned medium containing 10% FBS (Gibco Life Technologies), L-glutamine (Gibco Life Technologies), 100 IU/ml penicillin–streptomycin (Gibco Life Technologies), 50 ng/ml RANKL (PeproTech EC, London, UK), 25 ng/ml M-CSF (R&D Systems,

Minneapolis, MN), 5 ng/ml TGF-β (R&D Systems, Minneapolis, MN), and 1 μM dexamethasone (Sigma–Aldrich, St. Louis, MO) in a humidified atmosphere of 5% CO_2 at 37°C. Half of the medium was exchanged every 3–4 days. After 14 days, bone samples (n = 5) with attached cells were washed twice with PBS and fixed in 4% paraformaldehyde. The differentiation of osteoclast precursors into osteoclasts was evaluated by tartrate resistant acidic phosphate (TRAP, Sigma-Aldrich, St. Louis, MO) and Hoechst 33258 (Sigma–Aldrich, St. Louis, MO) staining. The number of TRAP-positive multinuclear cells per unit area was counted on each bone slice. Attached osteoclasts were removed by being scrubbed with a brush and the volume of the resorption pits on the bone slices was measured with a 3D laser microscope (Olympus, OLS4100).

2.3 | Effect of SCPC on human bone marrow stem cells

Undifferentiated immortalized human bone marrow stromal cells (CL1 subclone TERT-hBMSCs) were cultured in conventional complete medium and referred to as (Control cells). To induce differentiation, the TERT-hBMSCs (0.5×10^6 cells/ml) were grown in dulbecco's modified eagle's medium (DMEM) supplemented with 50 mg/ml L-ascorbic acid, 10 mM β-glycerophosphate, 10 nM calcitriol (1α,25-dihydroxy vitamin D3; Sigma), and 10 nM dexamethasone, referred to as (Induced cells). The induced hBMSC cells were cultured in osteogenic media in the presence and absence of SCPC granules. The ratio of the SCPC granules to tissue culture medium was 0.7 g/ml. All experiments were carried out in triplicate (n = 3). Control undifferentiated hBMSC cells were cultured in conventional complete medium (DMEM, Gibco-Invitrogen, MA) supplemented with 10% fatal bovine serum (FBS) in the presence and absence of SCPC granules. The media was exchanged every 2 days and the osteogenic gene expressed by cells was analyzed by Real-Time quantitative reverse transcription polymerase chain reaction (qRT-PCR) after 14 days in culture. The target genes were Runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osteocalcin (OSC), osteopontin (OPN), and bone sialo protein (BSP) (Table 1).

TABLE 1 Characteristics of the primers used in the study

Gene	Primer sequence (5′-3′ (forward/reverse)
RUNX2	Forward: CACCATGTCAGCAAAACTTCTT
	Reverse: ACCTTTGCTGGACTCTGCAC
ALP	Forward: GACGGACCCTCGCCAGTGCT
	Reverse: AATCGACGTGGGTGGGAGGGG
OSC	Forward: GGCAGCGAGGTAGTGAAGAG
	Reverse: CTCACACACCTCCCTCCTG
OPN	Forward: CAGTTCAGAAGAGGAGG
	Reverse: TCAGCCTCAGAGTCTTCATC
BSP	Forward: CAGTTCAGAGGGAGG
	Reverse: TCAGCCTCAGAGTCTTCATC
GAPDH	Forward: CTGGTAAAGTGGATATTGTTGCCAT
	Reverse: TGGAATCATATTGGAACATGTAAACC

2.4 | Statistical analysis

The evaluation of osteogenic genes was done with n = 3 and all other experiments were conducted with n = 5. The data were expressed as means ± SD and analyzed by using standard analysis of variance with unequal variance. Statistical significance was considered at $p < .05$.

3 | RESULTS

The ICP-OES ionic composition analysis of the tissue culture medium (TCM) incubated with SCPC showed 57% decrease in medium Ca concentration and a significant increase in the concentration of Si and P. Moreover, a slight increase in the concentration of Na ions was also noticed. The concentrations of Si, Ca, P, and Na in the TCM incubated with SCPC at a ratio 10 mg/ml for 3 days measured 13.5 ± 1.8, 29.5 ± 3.3, 249.4 ± 22, and 3,827 ± 150, respectively. The % difference in ionic composition between TCM incubated with SCPC and control TCM is shown in Figure 1.

ICP-OES data demonstrated a continuous dissolution of Si over 480 hr immersion period in PBS (Figure 2). The fluctuation of Si release is due to the dissolution-precipitation reactions that take place on the material surface and lead to deposition of a calcium phosphate surface layer. A continuous release of P was also noticed; however, since the PBS contains P, we choose to use the ionic concentrations of Si to train the ANN model to predict the dissolution of the SCPC ceramic.

After systematically optimizing network performance accurate prediction was obtained showing a linear sustained Si release rate

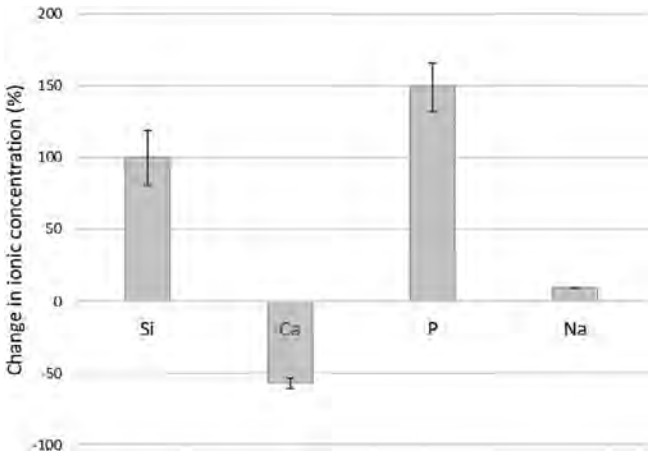


FIGURE 1 Inductively coupled plasma (ICP) analysis of the ionic concentration of tissue culture medium (TCM) incubated with SCPC at a ratio of 10 mg/mL for 3 days. A significant increase in P and Si is observed together with a slight increase in Na. On the other hand, a significant decrease in Ca ion is observed

after 48 hr. The predicted dissolution profiles were closely similar to the dissolution profiles obtained from the experimental observations with 1.195% error (Figure 3). The predicted dissolution time of 200 mg of porous SCPC particles in 10 ml PBS is 5,002.5 hrs. The ANN model could offer an effective tool to predict the dissolution profile of the SCPC ceramic in the physiological solution in vivo as well as the solution mediated resorption of SCPC bone graft. Figures 4 and 5 show the actual and predicted release profile of P and Ca ions in PBS. The fluctuation of the Ca concentration is attributed to the continuous dissolution precipitation reaction at the interface between SCPC and the physiological solution.

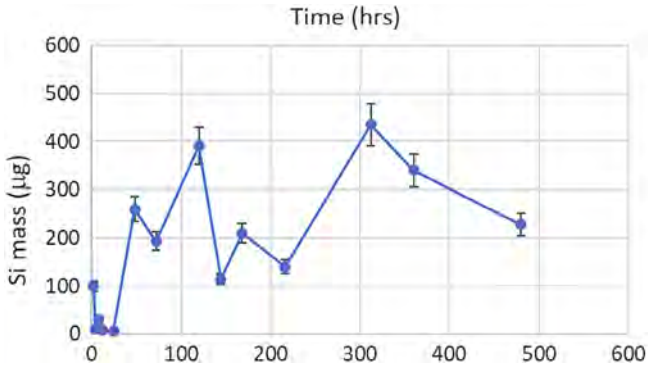


FIGURE 2 The weight (µg) of Si dissolved from silica-calcium phosphate composite (SCPC) granules into phosphate buffered saline (PBS) at each time point during 480 hr

Osteoclasts derived from human BM cultured for 14 days with the osteoclast induction factors were positively stained for TRAP on the bone slices. Multinuclear TRAP-positive cells are assumed to be mature osteoclasts. The osteoclasts contained an average of 5–20 nuclei and become larger by fusing with other multinuclear osteoclasts or with mononuclear osteoclast precursors.[31] Some small cells adhered on bone slices were TRAP-positive but not multinuclear, which are not completely differentiated into osteoclasts. Comparable number of nuclei per osteoclast was observed in all experimental groups (Figure 6). The number of multinuclear TRAP-positive cells was significantly decreased as the concentration of SCPC in the modified media increased in the order 20 µg/ml ($p < .001$) <10 µg/ml ($p < .005$) <5 µg/ml ($p < .05$) in comparison to control cells incubated in unmodified medium. Although lower number of TRAP-positive cells was observed on bone samples incubated with media modified with (2.5 µg/ml) SCPC in comparison to control, the difference was not statistically significant (Figure 7a,b).

3.1 | 3D laser microscope analysis of the resorption pits

3D laser microscope analyses of the surface of bone showed a decrease in number and size of the resorption pits created by osteoclasts in the presence of SCPC (Figure 8a). Quantification of the volume of the resorption pits using image analysis technique showed a decrease in the volume of the pits as the concentration of SCPC in the modified media increased (Figure 8b). Statistical analysis showed

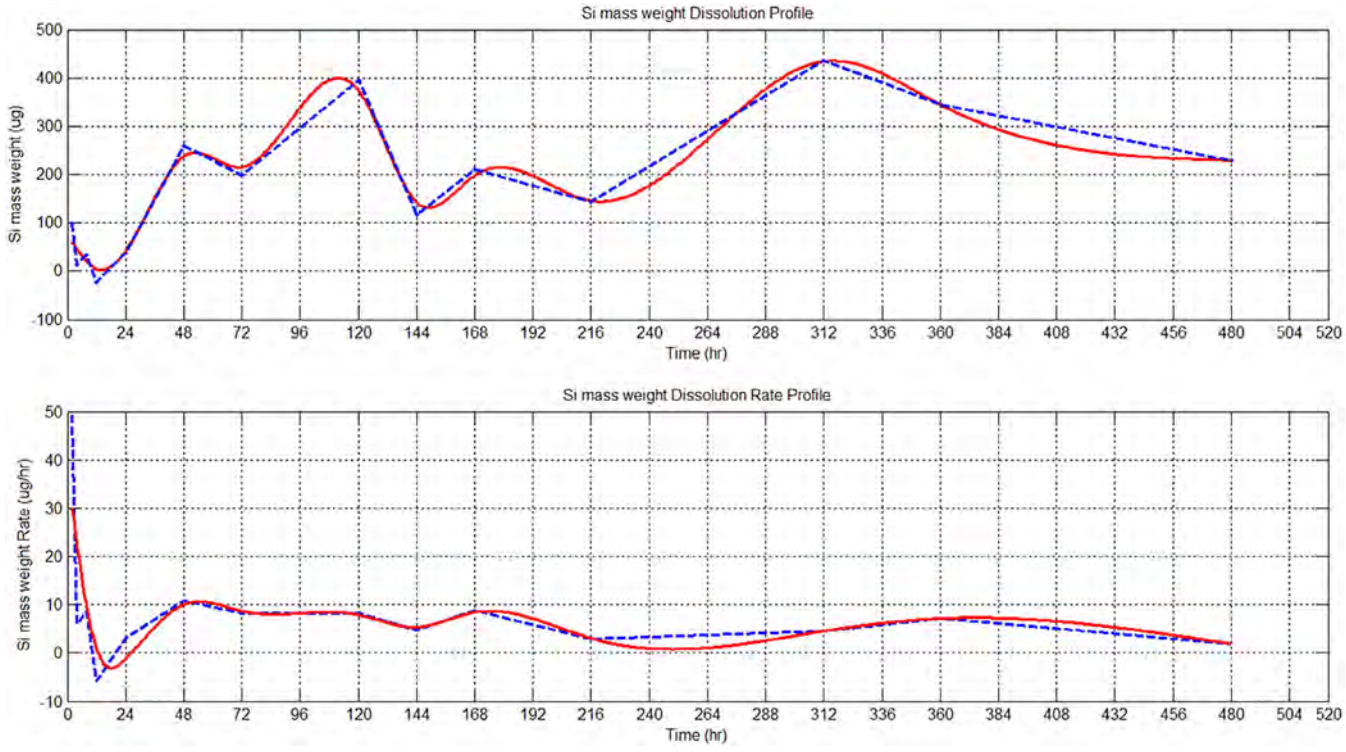


FIGURE 3 Si mass profile and dissolution rate measured experimentally "blue" and the ANN molding "red"

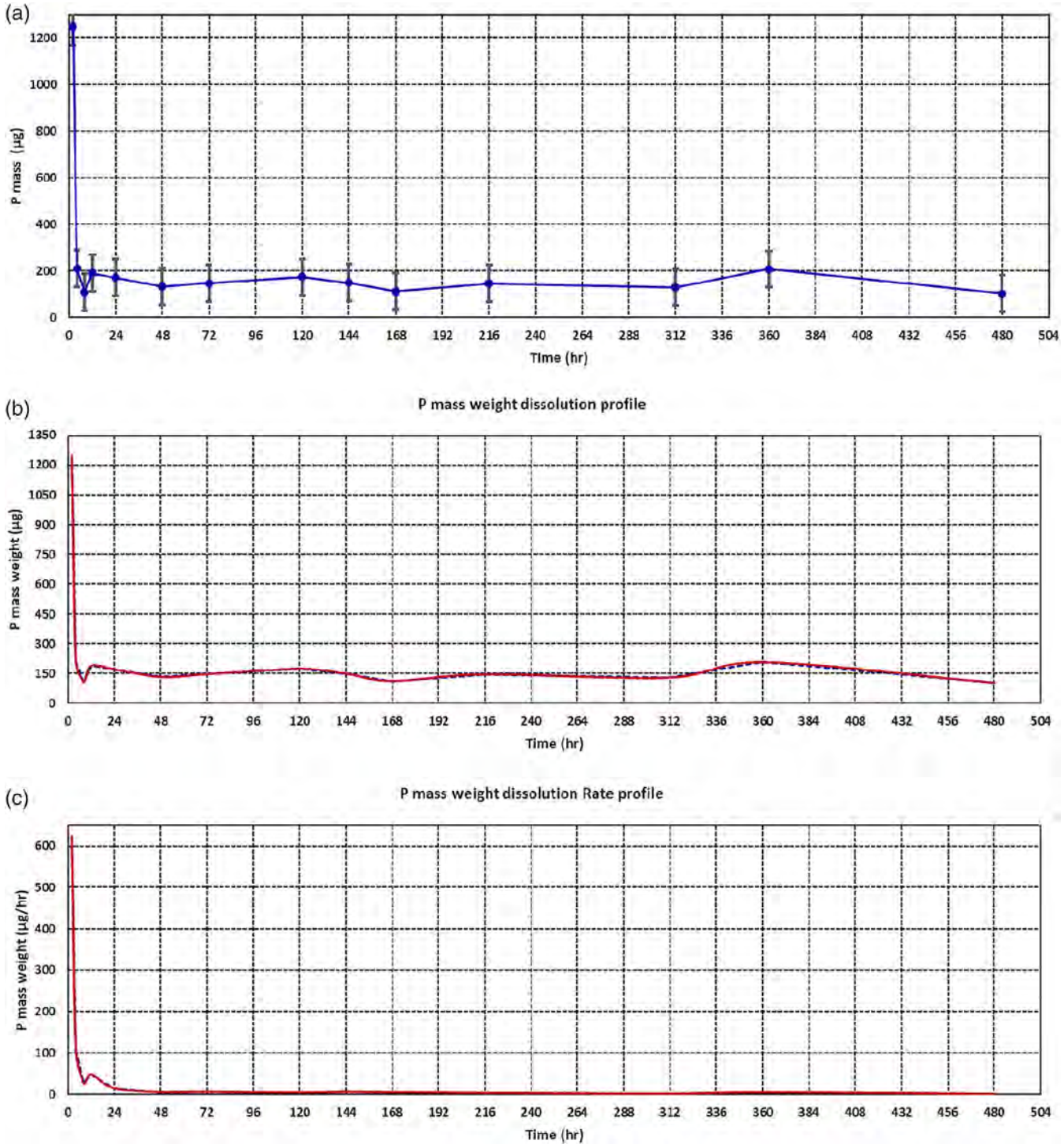


FIGURE 4 (a) The weight (µg) of P dissolved from silica-calcium phosphate composite (SCPC) granules into phosphate buffered saline (PBS) at each time point during 480 hr. (b) The experimental (dotted blue curve) and the predicted (solid red curve) weight (µg) of dissolved P. (c) The P ion dissolution rate profiles measured experimentally (dotted blue) and the predicted dissolution profiles (solid red)

that although the volume of resorption pits on bone samples incubated in media modified with 2.5 µg/ml SCPC was lower than that observed on substrates incubated in control unmodified media, the difference was not statistically significant. On the other hand, a statistically significant ($p < .05$) lower volume of the resorption pits was observed on the bone substrates incubated with media modified with higher (5, 10, and 20 µg/ml) SCPC concentrations (Figure 8b). Although lower volume of resorption pits is observed as the medium concentration of SCPC increased from 5 to 20 µg/ml, statistical analysis showed that the difference is not significant.

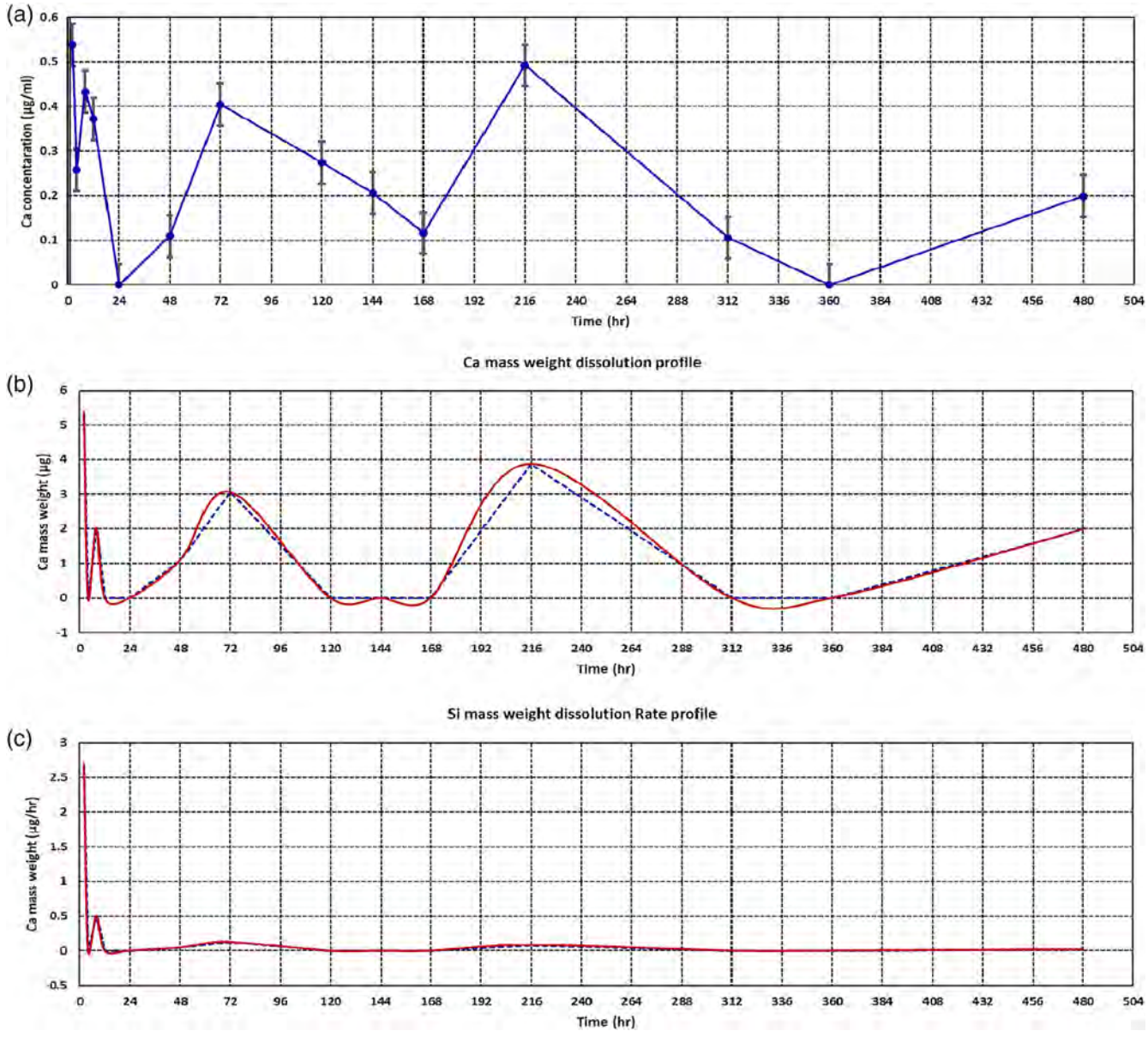


FIGURE 5 (a). The weight (µg) of Ca dissolved from silica-calcium phosphate composite (SCPC) granules into phosphate buffered saline (PBS) at each time point during 480 hr. (b) The experimental (dotted blue curve) and the predicted (solid red curve) weight (µg) of dissolved Ca ions. (c) The Ca ion dissolution rate profiles measured experimentally (dotted blue) and the predicted dissolution profiles (solid red)

3.2 | Effect of SCPC on human osteoblasts

Control hBMSC showed minimal expression of osteogenic genes. Addition of SCPC to control cells cultured in conventional medium promoted higher RUNX 2 ($p < .05$), OSC ($p < .01$), and BSP ($p < .01$) than that expressed by control cells grown in the absence of SCPC (Figure 9). The upregulation of the osteogenic gene expression is indicative of the osteoinduction property of SCPC. Induced hBMSC cultured in osteogenic medium expressed twice as much ALP genes compared to control cells grown in conventional medium. However, addition of SCPC to the induced cells promoted significantly higher OSC ($p < .001$), OP ($p < .01$), and BSP ($p < .01$) than that expressed by control induced cells cultured in differentiating medium without SCPC (Figure 9). The % increase in osteogenic genes were 700, 270, and 351% for OSC, OPN, and BSP, respectively. Previous work from our lab showed that the expression of osteogenic genes by cells incubated with SCPC was associated with the synthesis of the corresponding bone proteins.[27]

4 | DISCUSSION

Results of the present study showed a significant increase in Si, P, and Na ions in physiological solution incubated with SCPC granules.

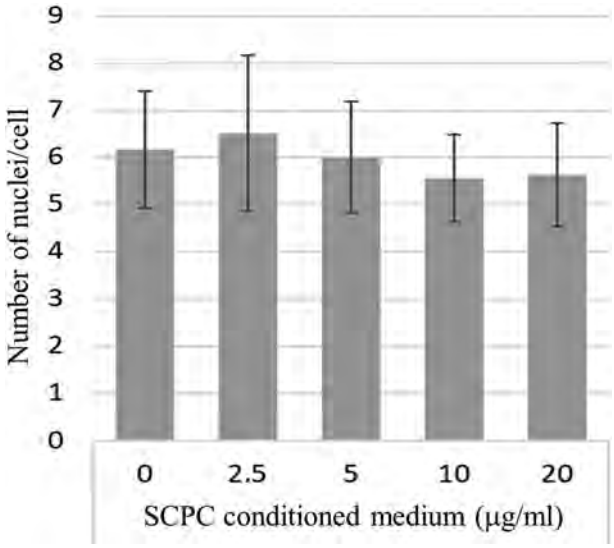


FIGURE 6 Average number of nuclei per osteoclasts incubated with medium modified by different concentration of silica-calcium phosphate composite (SCPC)

Mathematical modeling using Si dissolution data over 480 hr predicted complete dissolution of 200 mg of SCPC in 5,002.5 hr (6.9 months). In conjunction with the release of SCPC dissolution products, a dose dependent decrease in osteoclast differentiation and function was observed. The inhibitory effect of dissolution products on osteoclast differentiation and function undermined the role of these cells in the resorption of the SCPC bone graft. Indeed, histology analyses in several animal and clinical studies reported absence of osteoclasts at the interface with remnant SCPC granules.[28,30,32] Incubation of undifferentiated hBMSC with SCPC induced osteogenic gene expression in the absence of any medium differentiation stimuli, such as beta glycerophosphate, ascorbic acid, or dexamethasone. These results suggest that the resorption of SCPC bone graft is controlled by the dissolution mechanism and is mediated by osteoblasts not osteoclasts. The Si, P, and Na ions released from SCPC into the physiological solution play important role in stimulating osteoblast activities and downregulating osteoclast function. The osteoblast-mediated resorption of the dissolution products of SCPC explains the vascularization and rapid maturation of the newly formed bone in grafted defects reported in the literature.[25,28,33]

The enhanced dissolution of SCPC is attributed to (a) the modified crystalline structure of cristobalite (α-SiO_2) and β-rehnanite (β-$NaCaPO_4$) as indicated by the X-ray diffraction analysis.[33] The crystalline phases in SCPC are in the form of solid solution due to silicate-phosphate ionic substitution induced by thermal treatment during ceramic preparation. The $NaCaPO_4$ solid solution phase serves as a delicate binder for the (SiO_2)ss grains and offers a preferential dissolution sites. The silica substitution in the $NaCaPO_4$ regulates its dissolution. The P substitution in the silicate network introduces stresses and strain within the SiO_2 crystals and enhances dissolution. Incorporation of other alkali ions such as Ca and Na within the silicate network modifies the silicate structure and enhance dissolution. (b) the high density of grain boundaries due to the nano size of the SCPC crystalline phases, and (c) the high percentage of nanopores that maximizes the surface area in contact with physiological solution.[34] The accumulation of the ionic dissolution products and the negative surface charge of SCPC trigger precipitation of a poorly crystalline carbonate apatite surface layer similar to the mineral phase of bone. These structure features of SCPC have been correlated to the significantly higher controlled dissolution rate of Si, rapid bone regeneration and graft resorption compared to bioglass in vivo.[33] Several other clinical and animal studies have demonstrated near complete resorption of SCPC in bone defects and that the resorption is associated with bone regeneration and maturation[28-30,32,35-39] in the time frame ideal for dental implant placement. The SCPC resorption and bone regeneration increased as the silica content in SCPC increased from 29.2 to 63.7 mol%.[34] The present study demonstrates decreased osteoclastic activities as the concentration of dissolved silica from SCPC increased. Therefore, the difference in dissolution rate from different SCPC formulations controls osteoblasts and osteoclasts activities. The resorption mechanism of an HA bone graft is mediated by osteoclasts which produce acid and lytic enzymes locally in closed compartments in an attempt to dissolve the graft material.[40] The high chemical stability of HA ceramic resists dissolution regardless whether it is from bovine bone or synthetic and therefore poses a challenge for the replacement of HA by new bone. Reports in the literature showed that HA ceramic is not resorbable.[2] The incorporation of carbonate ions in an apatite crystal structure has an inductive effect on osteoclast differentiation and resorption in vitro and in vivo.[41,42] Although carbonated apatite showed better dissolution than HA in physiological solution, osteoblastic differentiation, assessed by qRT-PCR of RUNX2 and COLIA2, and pre-osteoclastic proliferation and differentiation were not significantly different on carbonate HA or HA ceramics.[43] In addition to the limited resorbability, a common observation in defects grafted with HA or BCP ceramics is the limited bone maturation and frequent formation of fibrous rather than mineralized tissue. Increasing the dissolution rate of calcium from calcium phosphate ceramics, such as TCP or from calcium sulfate, is challenged by the adverse effect of high Ca concentration which is associated with inflammatory response that lead to fibrous encapsulation of the graft material.[44]

Results of the present study indicate that dissolution products of SCPC promote osteogenic differentiation of stem cells and inhibit osteoclast differentiation and mineral dissolution capability. The inhibitory effect on osteoclast activities correlates well with the dissolution of Si, P, and Na released from the SCPC into the physiological solution. This agrees with data in the literature, which reported that dissolved silica down regulates osteoclasts, upregulate osteoblasts and increased the density of bone [45-48]. Previous studies demonstrated a strong stimulatory effect of Si ions on osteogenesis of bone marrow mesenchymal stem cells derived from ovariectomized rats (rBMSCs-OVX) as indicated by the expression of high ALP activity and promotion of osteogenic gene expression of collage Type I and osteocalcin.[48] The osteogenic gene expression was correlated to the promotion of new bone formation and mineralization in mandibular

defects grafted with silicate bioceramic but not for control group treated with β-TCP.[48] The promoted cell activity have been correlated to the effect of the silanol (Si—OH) groups on increasing cell membrane fluidity.[49] The enhanced membrane fluidity facilitates conformation flexibility of membrane proteins and lipids thus enhancing enzymatic reaction.[50] The diffusion of SiO_3^{2-} ions intracellularly through the cell membrane channels appeared to enable the stabilization and translocation of β-catenin into the nucleus.[51] The WNT/β-catenin signaling pathway plays important role in cell proliferation and differentiation.[52,53] In addition to β-catenin, silicon ions upregulated AXIN2, SHH, and PTCH1 gene expression. The activation of WNT and SHH-related genes of BMSCs led to upregulation of bone related gene expression including OCN, OPN and ALP of BMSCs as well as the production of the corresponding proteins.[50,54] Moreover, the dissolved silicon ions enhance the apoptosis of injured cells and eliminate their death signaling effect on healthy cells.[49]

The incorporation of Si into the newly formed bone matrix increases its resilience and lowers osteoclast activity.[55] Analysis of the chemical nature of Si in glycosaminoglyeans pointed at the role of silicate ions as a cross linker of polysaccharide molecules present in connective tissue through ester compounds R—(O—Si—O)$_n$—R.[56] The biological cross-linking and stabilization of collagen Type I and glycosaminoglycans are thought to take place by involvement of silicate ions in the radical-dependent prolyl-hydroxylase pathway during tissue formation.[56,57] It is also possible that activation of osteoblasts by bioactive ceramic ions upregulates osteoprototegerin production which in turn inhibits the RANK–RANKL signaling pathway that leads to osteoclast development and differentiation.[58,59] Indeed, results from our lab showed that osteoblasts attached to the SCPC surface released significantly lower amount of inflammatory and osteoclastogenic cytokines, IL-6 ($p < .01$) and RANKL ($p < .05$) compared to cells attached on Ti-6Al-4V.[27] Moreover, data in the

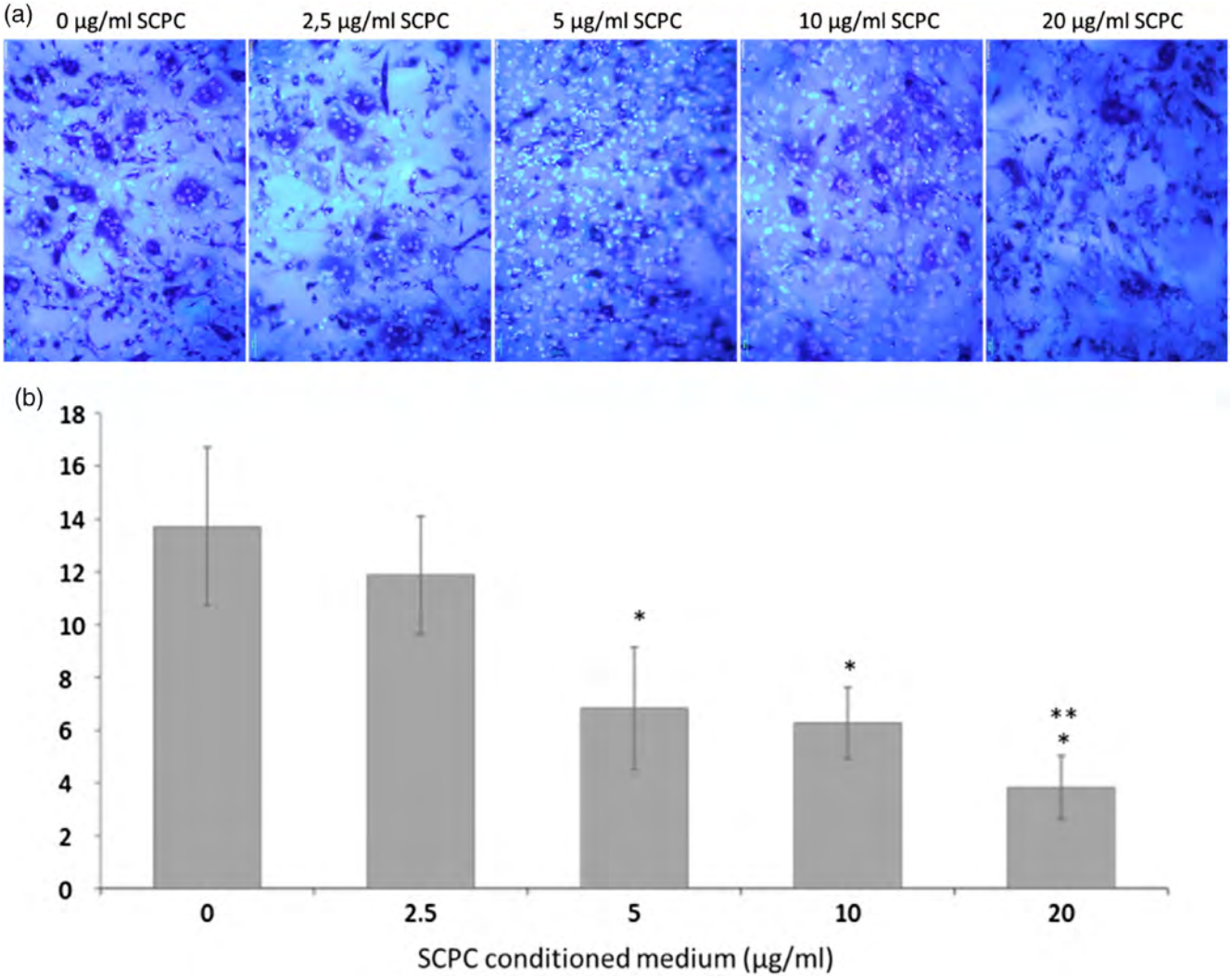


FIGURE 7 (a) Tartrate resistant acidic phosphate (TRAP)-staining showing multinuclear osteoclasts on the surface of bone incubated in medium modified with silica-calcium phosphate composite (SCPC) bioactive ceramic. The number of osteoclasts decreased as the concentration of the SCPC in the medium increased. (b) Quantitative analysis of the number of TRAP positive multinucleated osteoclasts attached to the surface of bone slices ($n = 5$) in the presence of various concentrations of SCPC particles. * $p < .05$, ** $p < .005$, *** $p < .001$

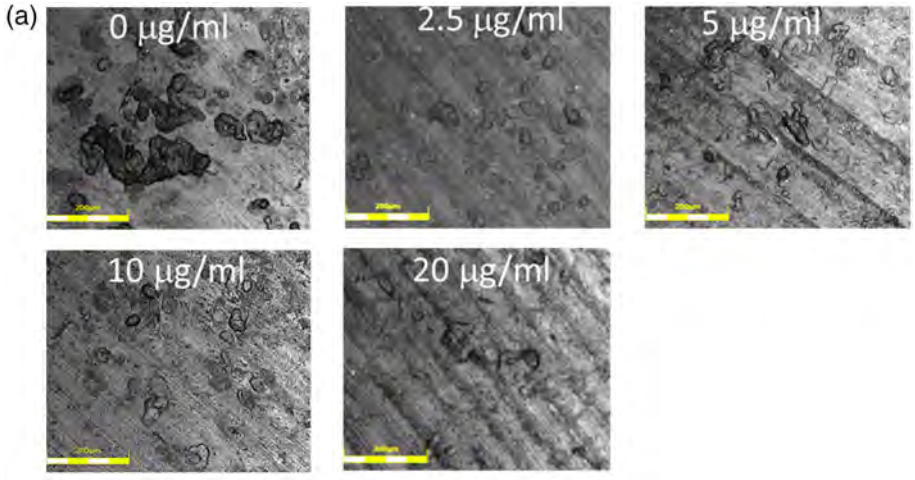
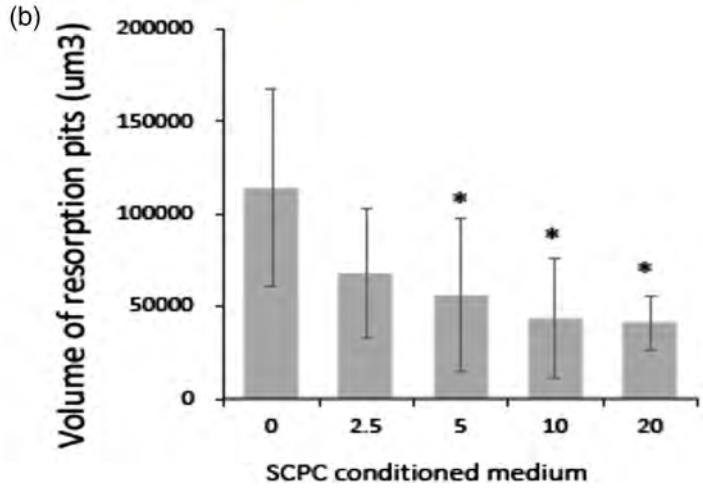


FIGURE 8 (a) SEM analyses of the pits created by osteoclasts attached on bone slices in the presence of various concentrations of the silica-calcium phosphate composite (SCPC) dissolution products. (b) Quantitative analyses of the volume of the pits created by osteoclasts attached on bone slices (n = 5) in the presence of various concentrations of the SCPC dissolution products. A statistically significant decrease in the pit volume is observed for samples incubated with media modified with SCPC in the concentration range 5–20 µg/ml (* p < .05) compared to samples incubated in control unmodified medium



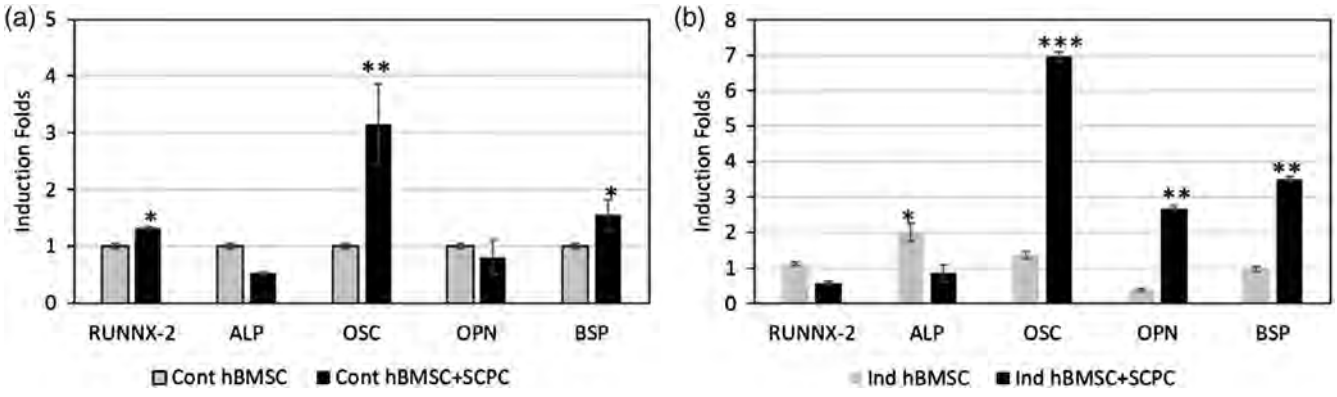


FIGURE 9 Osteogenic gene expression analysis in control and induced hBMSCs cultured in conventional and differentiating media (n = 3), respectively in the presence and absence of silica-calcium phosphate composite (SCPC) particles: (Cont hBMSC) control cells grown in conventional medium, (Cont hBMSC + SCPC) cells grown in conventional medium with SCPC, (Ind hBMSC) induced cells grown in differentiating medium, and (Ind hBMSC + SCPC) cells grown in differentiating medium in the presence of SCPC. *p < .05; **p < .01; ***p < .001

literature showed that RANKL-induced formation of osteoclasts and the expression of osteoclastic markers, including TRAP, DC-STAMP, V-ATPase a3, and NFATc1, were significantly suppressed by Sr and Si-containing bioceramics. It is suggested that dissolved Si ions released from bioceramics may rebalance the ratio of OPG/RANKL of BMSCs under osteoporotic condition at the early stage as well as repress RANKL-induced osteo-clastogenesis at late stage.[48]

The P ions released from SCPC synergies the inhibitory effect of Si on osteoclast activity and bone resorption. Data in the literature demonstrated the adverse effect of high phosphorus ion

concentration on generation of new osteoclasts and resorptive activity of mature osteoclasts.[60,61] On the molecular level, high P concentration upregulated OPG leading to blocking the stimulatory effect of RANKL on osteoclast differentiation.[14,62] Contrarily, the increase of P concentration demonstrated a stimulatory effect on osteoblasts proliferation.[63] Moreover, data from our previous work showed that the controlled release of P from SCPC obviated the need for exogenous phosphate supplementation to stimulate osteoblast differentiation in vitro.[64] The decreased production of the inflammatory and osteoclastogenic cytokines, IL-6, and RANKL leads to minimal immune pro-resorptive functions of bone cells and facilitates bone formation and maturation.

A significant decrease in the volume of resorption pits was observed in the presence of SCPC dissolution products. Data in the literature demonstrated that human and animal osteoclasts are almost inactive at pH 7.4 and that bone resorption increases steeply as pH is reduced, reaching a plateau at about pH 6.8.[65-68] Therefore, the significant decrease in pit volume could be attributed in part to the effect of the SCPC on the pH that creates unfavorable environment for osteoclasts activity. Previous studies have shown that the SCPC surface is negatively charged in physiological solution with a zeta potential equal to (– 40.36 ± 1.76 mV).[69] The negatively charged surface attracts H^+ and Ca^{2+} ions from the solution onto the surface of the SCPC. The ICP-OES analysis (Figure 1) confirmed the significant decrease in the Ca^{2+} ions in the medium incubated with SCPC and also showed an increase in Na^+ ion concentration (Figure 1). The release of Na^+ ions and the withdrawal of H^+ and Ca^{2+} raises the pH of the surrounding physiological solution and create hostile environment for osteoclast formation and activity. It is also possible that the release of Na^+ ions form SCPC and the withdrawal of Ca^{2+} ions from the surrounding solution by SCPC signal osteoclasts to shut down the resorption activities. In addition, data in the literature reported greater release of Na^+ than Ca^{2+} ions during osteoclastic resorption of bone.[70-72] Therefore, it is also possible that the release of Na^+ from SCPC gives a negative feedback signal to osteoclasts to shutdown activities. Taken all together, the enhanced dissolution of Si, P, and Na ions from the SCPC prohibits osteoclast from participating in the graft resorption process and makes room for osteoblasts to manipulate ceramic surface and dissolution products to produce new vascularized bone.[25] This mechanism explains the rapid bone regeneration and maturation of the newly formed bone in defects grafted with SCPC. As the SCPC granules are resorbed, the remodeling cycle of the newly formed bone is resumed normally.

Maturation of the newly formed bone is achieved through a bone remodeling cycle that naturally takes place in sequenced phases. The initial phase of remodeling is the activation of osteoclasts followed by dissolution of the mineral phase and resorption of the organic matrix. Following the resorption phase the osteoclasts signal the recruitment of osteoblasts among other mechanistic steps to start the bone formation phase. The coordinated balance between chemical dissolution and biodegradation by osteoclasts as well as the osteogenesis by osteoblasts stimulate bone regeneration and ensure no net change in bone mass or quality at the end of each remodeling cycle. In the presence of HA graft, the osteoclasts are exhausted in prolonged HA dissolution and phagocytosis which compromise their ability to signal and promote osteoblast activities. The continuation of the osteoclastic dissolution activities of the grafted mineral also does not result in the release of any growth factors as the case of natural bone remodeling. Therefore, the involvement of osteoclasts in prolonged resorption activity of the HA ceramic compromises their ability to participate in the normal remodeling phase. This delays the transition from the resorption phase to the osteoblast signaling phase. Another important factor that could reverse the mineralization of the new formed bone matrix is the acidic pH created by active osteoclasts at the interface with HA ceramics. In contrast, bone maturation in SCPC-grafted defects is attributed to the upregulation of osteoblasts and down regulation of osteoclasts inside the site. The increased activity of osteoblasts is enhanced by calcium uptake from the surface of SCPC in the initial phase of dissolution. Rather than releasing Ca ions, the initial phase of SCPC dissolution includes Ca uptake from the tissue fluid onto the surface of the material. The attraction of positively charged calcium ions to the negatively charged surface of SCPC exposes stem cells inside the defect to high Ca transport intracellularly which prompt osteoblast differentiation and production of calcified nodules.[73,74] After 6–9 days immersion in PBS the Ca released from SCPC in PBS fluctuates between a maximum of 1.226 ± 0.528 μg/ml and zero due to the dynamic dissolution precipitation reaction.[25,75] This explains the rapid bone maturation in defects grafted with SCPC compared to those grafted by HA ceramic. The slow HA dissolution and the limited availability of calcium ions needed to promote osteoblasts are two main reasons for the slow bone formation and poor maturation in defects grafted with HA. Studies have showed limited bone formation associated with the presence of bovine bone mineral particles (Bio-Oss) even after 5 years of grafting in human.[6-12] Therefore, the involvement of osteoclasts on prolonged resorption activity of the HA ceramic compromises their ability to participate in the normal remodeling phase of the newly formed bone and delay the transition from the resorption phase to the osteoblast signaling phase.

5 | CONCLUSION

The results of the present study point at the role of osteoblasts rather than osteoclasts in the resorption of SCPC bone graft material. The controlled dissolution of SCPC provided a natural inhibitor of osteoclast functions and a stimulus for osteoblast differentiation. The osteoblast-mediated resorption of SCPC is considered a major difference in comparison to the osteoclast mediated resorption of HA ceramic. The SCPC dissolution increased the concentration of Si, P, and Na ions in the physiological solution. The dissolution products of SCPC induced osteogenic differentiation of hBMSC. The upregulation of the osteoblasts is enhanced by the calcium uptake by the surface of SCPC in the initial phase of dissolution.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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