J Mater Sci: Mater Med (2010) 21:2175–2185
DOI 10.1007/s10856-010-4083-8

The bio-functional role of calcium in mesoporous silica xerogels on the responses of osteoblasts in vitro

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Received: 28 December 2009 / Accepted: 7 April 2010 / Published online: 22 April 2010

Abstract Mesoporous silica xerogels with various amount of calcium (0, 5, 10 and 15%, named m-SXC0, m-SXC5, m-SXC10 and m-SXC15, respectively) were synthesized by template sol–gel methods, and cell responses to m-SXCs were studied using murine pre-osteoblast MC3T3-E1 in vitro. The results showed that cell morphology was not affected by m-SXCs indicating good biocompatibility. Furthermore, cell proliferation ratio on the m-SXCs increased over time, among which m-SXC10 was highest. NO production obviously rose with the increase of Ca content in m-SXCs. ALP activity and PGE_2 level on m-SXC5 significantly improved compared with m-SXC0 while decreased with the increase of Ca content for m-SXC10 and m-SXC15. No obvious discrepancy on osteopontin mRNA expressions was observed among m-SXCs. The collagen I and osteocalcin mRNA expression on m-SXC5 were up-regulated, while decreased on m-SXC15 evidently. The phosphorylation level of ERK 1/2 for the m-SXC10 was highest after 7 days. In conclusion, calcium in m-SXCs plays an important role in osteoblast activity, which indicates mesoporous silica xerogel containing appropriate calcium could stimulate osteoblast proliferation, differentiation, gene expression via the activation of ERK 1/2 signaling pathway, and shows great prospects in bone regeneration field using as a drug controlled release filler.

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1 Introduction

An ever increasing number of people suffer every year from traumas, accidents or diseases that affect bone. Annually, more than 2.2 million bone grafting procedures (autologous bone graft and allograft) are performed worldwide to ensure adequate bone healing in many skeletal problems, such as nonunion fractures, cervical and lumbar spine fusion, joint arthrodesis, and revision arthroplasty [1–3]. In order to accelerate bone repair and reduce the sufferings of patients, bioactive materials and growth factors have been studied intensively [4–6]. In recent years, novel biocompatible silica based materials, termed silica xerogels (SX), have been developed. Low temperature processing enables them to carry biologically active agents and be useful as drug delivery systems. Recent studies have continued to report the potential use of silica xerogels, particularly as a bone substitute and drug delivering particulate carrier [7–11]. A family of modified mesoporous silica-based xerogels (m-SX) with high surface area, high pore volume, ordered pore network, silanol-containing surface [12], has received much interest on account of their advantages of larger drug loading amounts and better drug controlled-release profile [13, 14].

The composition of silica xerogels can be tuned so that they elicit bone bioactive characteristics of conventional bioactive glasses [8, 15]. Calcium phosphate-based biomaterials, such as bioglass, glass–ceramic and hydroxyapatite, have been investigated extensively for their excellent bioactivity to form stable interface with living bone [16]. It has been well recognized that the bone-bonding ability occurs from the rapid ion release and exchange to develop biological apatite layer [17] when these materials are exposed to body fluids [16, 18, 19]. From the prospective of bone metabolism, calcium acts as

a signaling molecule, affecting cell functions and gene expressions via the activation of extracellular signal-regulated kinases (ERK 1/2) signaling pathway [20].

Taking the physical and physiological roles of calcium in bone metabolism into account, alternating the chemical composition of mesoporous silica xerogels with calcium (m-SXC) seems to be meaningful to modulate bone tissue responses. As mesoporous materials have unique structural characteristics, it is reasonable to assume that mesoporous silica xerogels with different amount of calcium may possess diverse physical–chemical and biological properties [21]. The aim of this study is to synthesize mesoporous silica xerogels containing 0, 5, 10 and 15% Ca (wt%) by template sol–gel process instead of traditional methods. The functional role of Ca content in m-SXC on osteoblast proliferation, differentiation, and osteogenetic expressions were investigated and possible mechanism was speculated by analyzing specific factors production and activation of ERK 1/2 signaling.

2 Materials and methods

2.1 Mesoporous silica xerogel preparation and characterization

2.1.1 Synthesis of mesoporous silica xerogel

Tetraethoxysilane (TEOS, Sinoreagent, China), $CaCl_2$ and triethyl phosphate (TEP) were used as precursors of Si, Ca, and P oxides, respectively. Both Ca–P free and Ca–P containing gels were synthesized using one step catalyzed hydrolysis process. Sol–gel compositions obtained were as follows: 100% SiO_2 (m-SXC0), 90% SiO_2–5% CaO–5% P_2O_5 (m-SXC5), 85% SiO_2–10% CaO–5% P_2O_5 (m-SXC10) and 80% SiO_2–15% CaO–5% P_2O_5 (m-SXC15) (% in weight percent). Mesoporous silica xerogels were synthesized according to the following procedures. Cetyltrimethylammonium bromide (CTAB) was dissolved in deionized water with stirring at room temperature (RT) until the solution became clear, then TEOS was added under vigorous stirring to obtain an inhomogeneous solution ($CaCl_2$ and TEP were added when necessary in this step). Aqueous ammonia was then combined with the resulting solution in an appropriate proportion as catalyst. The synthesized sol–gel resulting from gelling, aging and drying to constant weight, were crack-free. After the reaction, the product was transferred into large surface plates for solvent evaporation at RT. The resulting solid was aged at 37°C for 3 days. The dried xerogels were heated at the rate of 1°C/h and held isothermally for 4 h at 600°C. Silica xerogel rods were ground and sieved to a grain size between 100 and 300 μm. The granules were kept refrigerated in sealed containers until in vitro testing.

2.1.2 Characterization of mesoporous silica xerogel

The morphology of m-SXC powders was analyzed using TEM (JEM2100, JEOL, Japan) working at 200 kV. N_2 adsorption–desorption isotherms were obtained using a Micromeritics porosimeter (Tristar 3000, USA) at 77 K under a continuous adsorption condition. Brunauer–Emmet–Teller (BET) and Barrett–Joyner–Halenda (BJH) analysis was used to determine the surface area, pore size and volume.

The analysis of Ca, P and Si ion release was conducted using an inductive coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer Optima 2000, USA). Granules of m-SXCs were suspended in sealed polyethylene bottles containing 20 ml of simulated body fluid (SBF) to give a sample concentration of 1% (wt/v). Immersed in SBF for durations of 1, 3, 7 days at 37°C, the sample was taken out and the concentrations of Ca, P and Si released in the media were analyzed.

2.2 Cell culture

The established MC3T3-E1 mouse newborn calvaria pre-osteoblasts (subclone 4, ATCC CRL-2593) were used to assay the osteoblast responses on m-SXCs. The MC3T3-E1 cells exhibit an osteoblastic phenotype, as evidenced by the expression of alkaline phosphatase (ALP) activity, the synthesis of extracellular matrix (ECM) components such as osteocalcin and collagen I, and their ability to mineralize in vitro. Cell culture was conducted at 37°C in a humidified 5% CO_2 atmosphere in a standard culture medium containing Eagle's Minimum Essential Medium (EMEM, GIBCO, USA) supplemented with sodium pyruvate and 10% fetal bovine serum (Sijiqing, Hangzhou, China) plus 100 U/ml penicillin and 100 μg/ml streptomycin sulfate. Prior to seeding with cells, the m-SXC granules were sterilized by dry heat under 200°C for 30 min and placed in tissue culture plates (TCP).

2.3 Cell morphology

MC3T3-E1 cells were seeded into 24-well plate preloaded with m-SXCs at 3×10^4 cells/well. After 24 h incubation, cultures were washed three times with phosphate-buffered saline (PBS) slightly, and fixed with 1% glutaraldehyde in PBS for 15 min followed by three times washes with PBS. The cultures were pictured by invert light microscope (TE2000U, Nikon, Japan).

2.4 Cell proliferation assay

MTT is a colorimetric assay that is based on measuring changes in absorbance at a specific wavelength and is widely used for measuring cytotoxicity and cell proliferation. The productions of purple formazan in the osteoblast cultures with or without m-SXCs were measured after 1, 3 and 7 days of incubation in 24-well culture plates. For this purpose, 100 µl of an MTT (MajorBiochem, Shanghai, China) solution (5 mg/ml) was added into each well (containing either just cells or pellets with adherent cells removed from the original culturing well) and the cells were incubated for 4 h. Consequently, the culture medium was aspirated and 1,000 µl/well dimethyl sulfoxide (DMSO) was added to dissolve the formazans completely for 10 min at 37°C. 100 µl of solution was transferred to 96-well ELISA plate and the absorbance was measured at 490 nm using a microplate reader (MULTISKAN MK3, Thermo Electron Corporation, USA).

2.5 Alkaline phosphatase (ALP) activity

At the end of 7 days' incubation, the culture media in 24-well plates were aspirated. 200 µl of 1% Nonidet P-40 (NP-40) was added to each well at RT for 1 h to obtain cell lysate. 50 µl of the supernatant was added to 96-well plates, to which 50 µl of 2 mg/ml *p*-nitrophenylphosphate (Sangon, Shanghai, China) substrate solution composed of 0.1 mol/l glycine, 1 mmol/l $MgCl_2 \cdot 6H_2O$ was added and incubated for 30 min at 37°C. The reaction was quenched by adding 100 µl of 0.1 N NaOH, the absorbance of ALP was quantified at the wavelength of 405 nm using a microplate reader (SPECTRAmax 384, Molecular Devices, USA). The total protein content in cell lysate was then determined using the bicinchoninic acid (BCA) method in aliquots of the same samples with Pierce protein assay kit (Pierce Biotechnology Inc., Rockford, USA), read at 565 nm and calculated according to a series of bovine serum albumin (BSA) standards. ALP levels were normalized to the total protein content at the end of experiments. All experiments were performed in quadruple.

2.6 Local factor production

2.6.1 Assessment of NO production

Osteoblast nitric oxide production was indirectly measured by cell supernatant nitrite quantification, since it is a stable metabolite of NO. Griess assay was used, according to manufacturer's protocol (Beyotime Biotech, Jiangsu, China). Cells were cultured in the presence of m-SXCs for 7 days. Culture supernatant (50 µl) was mixed with reagent Griess Reagent I (50 µl) and Griess Reagent II (50 µl). After the sample was incubated for 20 min at RT, absorbance was measured at 540 nm, and nitrite concentration was determined from a standard curve of serial dilutions of sodium nitrite dissolved in standard culture medium and normalized to protein in the cell culture supernatant.

2.6.2 Prostaglandin E 2 (PGE_2) assay

The amount of PGE_2 produced and released into the culture media by MC3T3-E1 cells was determined using a commercially available competitive enzyme immunoassay kit (R&D systems, MN, USA) after 7 days incubation. According to the manufacture's protocol, the PGE_2 concentrations were determined from a standard curve of serial dilutions of PGE_2, normalized to protein.

2.7 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were cultured in 6 cm Falcon dishes in the presence of m-SXCs for 3 and 7 days. At the end of culture, total RNAs were extracted using the mRNA purification kit (Shanghai Shenergy Bioscience & Technology Company, China) according to the manufacture's guidelines. The RNA pellet was washed with 70% ethanol treated with the RNase inhibitor diethyl pyrocarbonate (DEPC), and then solubilized in sterile DEPC/water. The concentration level of the RNA was determined from the optical density of the sample measured at 260 nm. RNA samples thus extracted were analyzed for markers of osteogenic differentiation: osteopontin, osteocalcin and collagen I. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as a housekeeping gene. Complementary first strand DNA (cDNA) was synthesized from 2 µg of total RNA using the reverse transcriptase MMLV (Promega, USA) and Oligo$(dT)_{18}$ (Takara, Japan) according to the manufacture's protocol. Template DNA was then used in gene-specific PCR for GAPDH, osteopontin, osteocalcin and collagen I (Table 1). One tenth of the cDNA products were used for PCR amplification with targeted primers designed as reported to amplify a region in GAPDH, osteopontin, osteocalcin and collagen I [22]. PCR was carried out as follows: an initial denaturation of 5 min at 94°C was followed by 32 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, followed by 10 min of final elongation at 72°C. Amplification products were analyzed through 1% agarose gel electrophoresis and stained with Goldview™ (SBS Biotech, Shanghai, China) as a succedaneum of Ethidium Bromide (EB). Photographs were taken under ultraviolet illumination system (GIS 2009, Tanon, Shanghai) and the relative expression was normalized to that of GAPDH.

Table 1 Oligonucleotide primers utilized for RT-PCR amplification

Gene	Primer	Sequence	Tm (°C)	Products (bp)
Osteocalcin	Sense	5′-TCTGACAAAGCCTTCATGTC-3′	56	198
	Anti-sense	5′-AAATAGTGATACCGTAGATG-3′		
Osteopontin	Sense	5′-ACACTTTCACTCCAATCGTC-3′	56	240
	Anti-sense	5′-TGCCCTTTCCGTTGTTGTCC-3′		
Collagen I	Sense	5′-TCTCCACTCTTCTAGTTCCT-3′	56	268
	Anti-sense	5′-TTGGGTCATTTCCACATGC-3′		
GAPDH	Sense	5′-ACTTTGTCAAGCTCATTTCC-3′	56	267
	Anti-sense	5′-TGCAGCGAACTTTATTGATG-3′		

2.8 Western blot

Cells were cultured with m-SXCs in 6-well Falcon® dishes at a density of 6×10^5/well. At the end of the treatment period indicated, media were removed immediately, and the cells were lysed by the addition of 0.5 ml of kinase extraction buffer (20 mM HEPES, 150 mM NaCl, 2 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, 10% glycerol, 1% Triton X-100, and protease inhibitor mixture, 1 tablet/10 ml of buffer). Cells were detached from dishes by scraping, and lysates were collected in 1.5 ml microcentrifuge tubes. After a 5 min centrifugation at 12,000 rpm, the supernatants were collected and stored at −80°C. The protein concentrations of the samples were determined by Bradford protein assay (Bio-Rad, USA). Activation of ERK 1/2 has been shown to occur through dual phosphorylation of threonine and tyrosine residues by upstream MAP kinase kinases. The levels of dual phosphorylation at these positions in treated samples were measured using primary antibody (Cell signaling, MA, USA) following the manufacturer's instructions. Total ERK 1/2 was served as control. Briefly, protein samples were size-fractionated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, USA) by electro blotting. The membranes were blocked in 5% non-fat dry milk for 1 h at RT followed by an overnight incubation with the primary antibody at 4°C in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). The membranes were washed three times in TBS-T and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 h at RT. Proteins were visualized by fluorography using an enhanced chemiluminescence system.

2.9 Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed with Tukey post hoc test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Characterization of mesoporous silica xerogel

Mesoporous silica xerogels with different calcium content were synthesized successfully according to our procedure. Surface morphology of m-SXCs was observed by TEM. As a representative, the surface of m-SXC5 was shown in Fig. 1a, from which a uniform mesoporous structure could be observed. N_2 sorption isotherm of m-SXC5 was shown in Fig. 1b which further demonstrated its construction. BET surface area, pore volume, and pore size of all xerogels used in this study were calculated and listed in Table 2. It was observed that the surface area and pore volume were decreased when the amount of calcium in m-SXCs was increased, whereas the pore size was maintained at $\sim$2 nm.

Figure 2 showed the changes of ion concentrations of Ca, P, and Si in SBF for m-SXCs measured by ICP for 1, 3 and 7 days. At each time point, in m-SXCs with higher amount of calcium, Ca and Si concentrations released was higher, whereas the trend of P release was opposite. As culture time increased, Ca and Si released were kept increasing, P concentration was decreased up to 7 days.

3.2 Cell morphology

Morphologies of osteoblasts cultured with m-SXCs for 24 h were observed under light microscope, as shown in Fig. 3. It revealed osteoblasts grew well, and had elongated and spindle-like needle morphologies compared to these on TCP. These results indicated that all m-SXCs had no negative effects on cell morphology, and provided good biocompatibility.

3.3 Effect of m-SXCs on osteoblastic proliferation

The proliferation of osteoblasts cultured with m-SXCs was assessed using MTT assay as OD values can provide an indicator of cells viability and growth on different

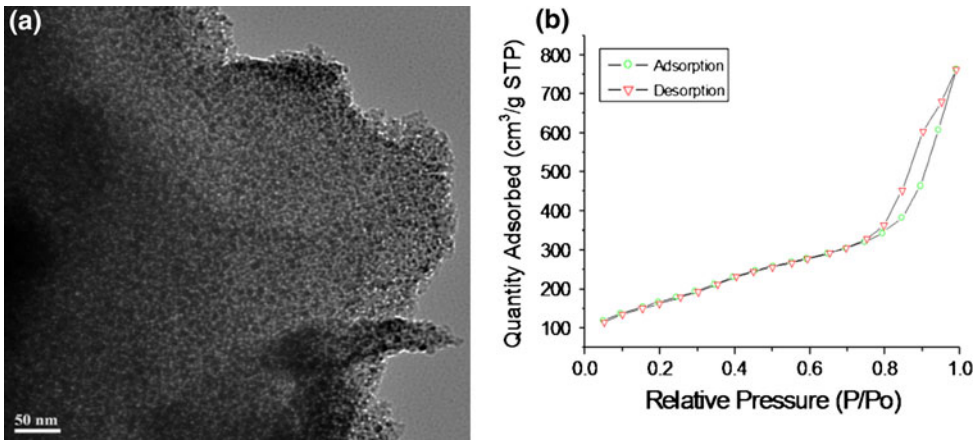

Fig. 1 Transmission electron micrographs (**a**) and N_2 sorption isotherms (**b**) of mesoporous silica xerogel containing 5 wt% Ca (m-SXC5)

Table 2 The parameters of mesoporous silica xerogels with calcium

Groups	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore size (nm)
m-SXC0	633	0.78	2.1
m-SXC5	614	1.15	2.4
m-SXC10	501	0.66	2.2
m-SXC15	405	0.46	2.0

materials. Figure 4 showed that OD values for all specimens increased with culture time, and the OD value at day 7 was significantly higher than those of at day 1 and 3; however, there was no significant difference among different m-SXCs and TCP. Furthermore, at day 7, the OD value increased with the increase of Ca content while decreased when the Ca content was 15% in m-SXC, revealing that m-SXC15 with high Ca content caused the inhibition of cell proliferation, however, it was observed that m-SXC10 facilitated osteoblast viability when compared to that on TCP.

3.4 Effects of m-SXCs on osteoblastic differentiation

Cell differentiation was assessed in terms of ALP activity of MC3T3-E1 cells at the end of 7 days of culture, as shown in Fig. 5. ALP activity on m-SXC5 was higher than that of m-SXC0, suggesting that little Ca content in m-SXC improved the ALP expression levels. However, ALP

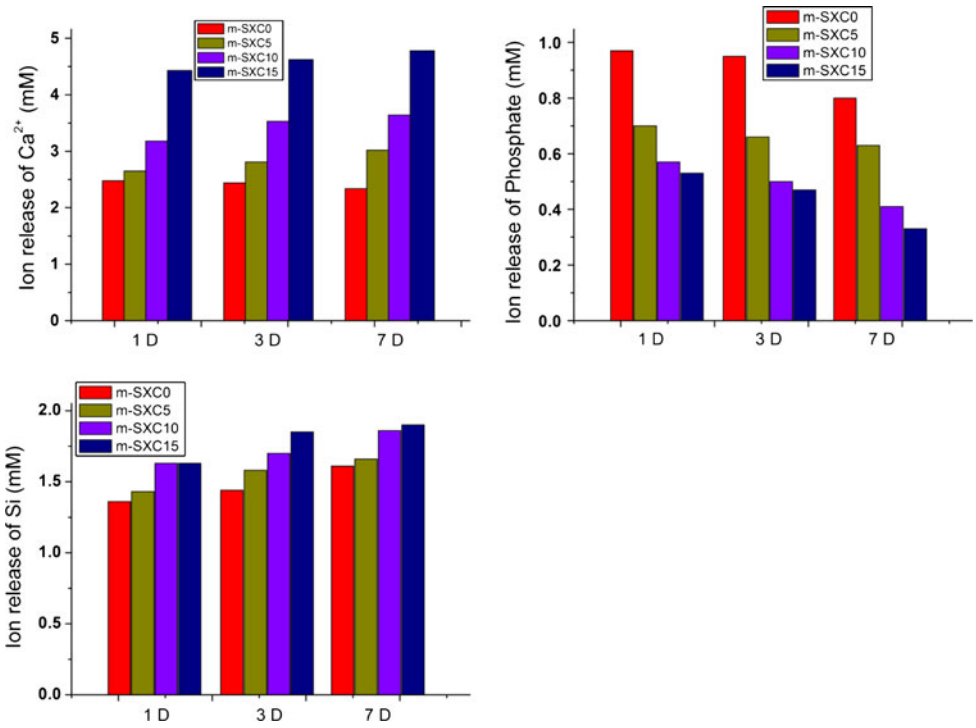

Fig. 2 Changes of ion concentrations of Ca, P and Si after m-SXCs immersed in SBF for 1, 3 and 7 days. (The basal ion concentration of Ca, P and Si in SBF is 2.5, 1.0 and 0 mM, respectively.) Results shown are mean values from three parallel experiments

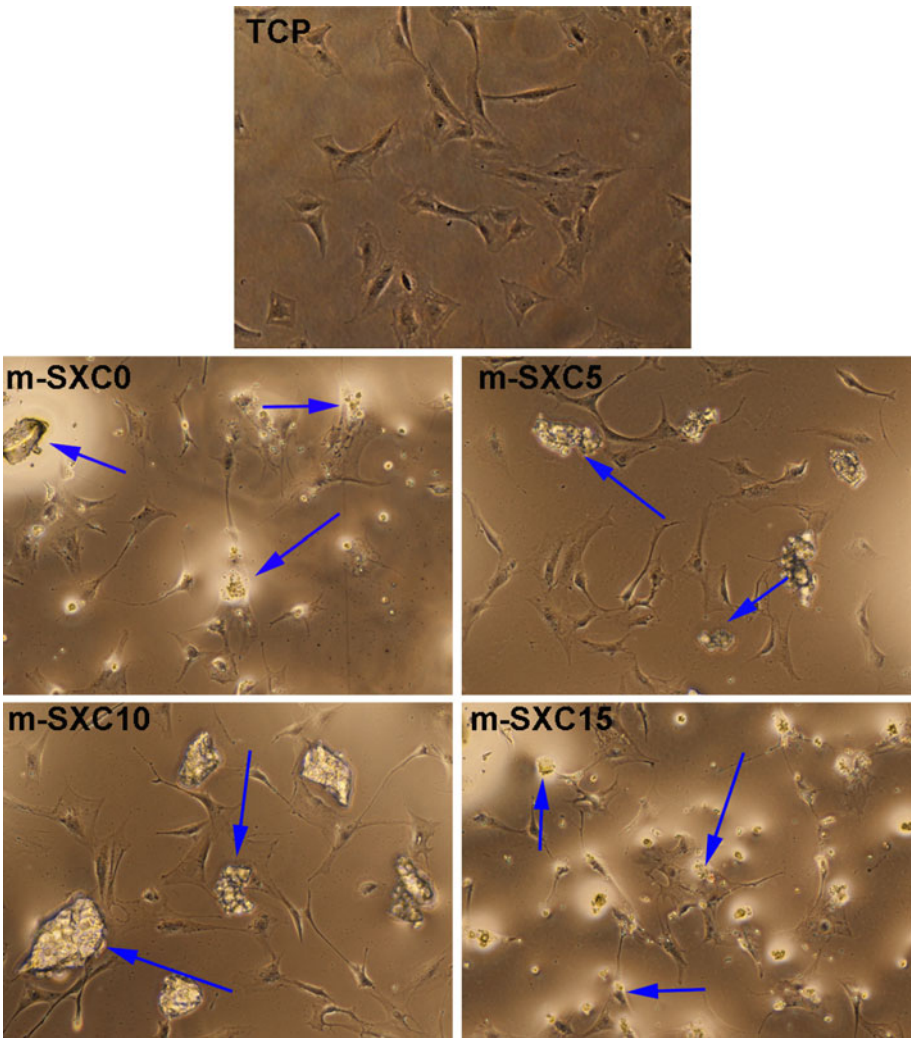

Fig. 3 Light micrographs of MC3T3-E1 osteoblasts cultured with m-SXCs for 24 h with m-SXC0, m-SXC5, m-SXC10, m-SXC15 and tissue culture plastic (TCP). The *arrows* indicate silica particles (magnification: ×10)

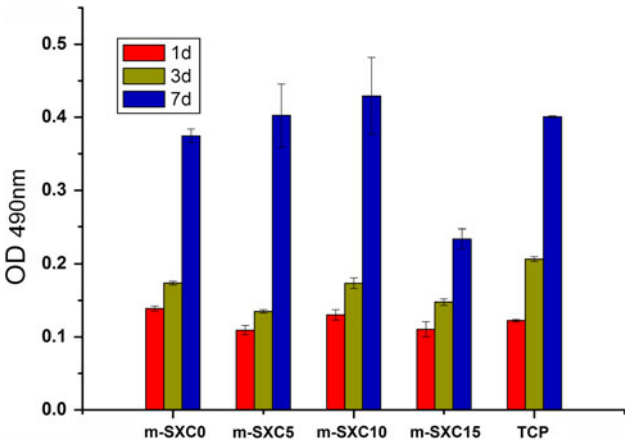

Fig. 4 Effect of calcium content in m-SXCs on cell proliferation. MC3T3-E1 osteoblast cells cultured with m-SXC0, m-SXC5, m-SXC10, m-SXC15 and tissue culture plastic (TCP) for 1, 3 and 7 days. Results are the mean ± standard deviation ($n = 4$)

activity significantly decreased when the Ca content was more than 5% in m-SXC, indicating that a little Ca in m-SXC could stimulate cell differentiation, m-SXC with high Ca content would be detrimental to cell differentiation.

3.5 Effect of m-SXCs on NO production

Nitric oxide (NO) acts as an important mediator of cytokine affecting osteoblast and osteoclast activity in vitro [23]. The effects of different m-SXCs on NO production levels of cultured MC3T3-E1 cells were shown in Fig. 6. There was no significant difference of NO production in osteoblasts between m-SXC0 and m-SXC5. However, the amount of NO production was obviously increased with higher Ca content in m-SXC.

3.6 Effect of m-SXCs on PGE_2 production

PGE_2 level of MC3T3-E1 cells was also affected by different m-SXCs (Fig. 7). It was found that the PGE_2 levels on m-SXC5 was significantly higher than that of m-SXC0 ($P < 0.05$), suggesting that Ca in m-SXC obviously

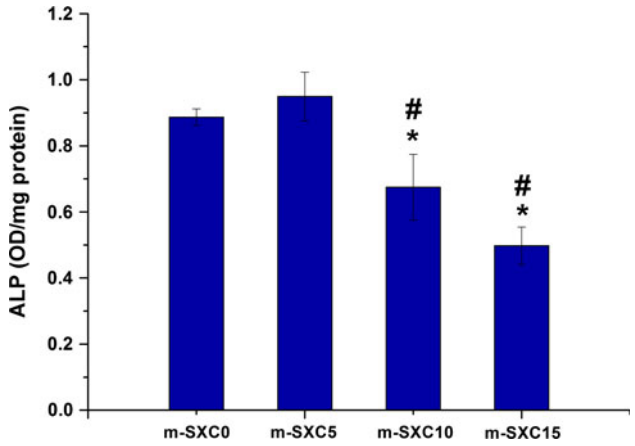

Fig. 5 Effect of calcium content in m-SXCs on alkaline phosphatase activity of cultured MC3T3-E1 cells for 7 days. Data are mean ± standard deviation ($n = 4$). * $P < 0.05$, m-SXC10 and m-SXC15 versus m-SXC0; # $P < 0.05$, m-SXC10 and m-SXC15 versus m-SXC5

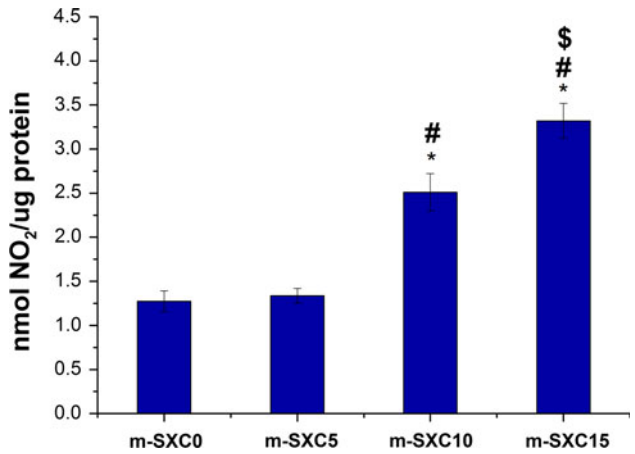

Fig. 6 Effect of calcium content in m-SXCs on nitric oxide levels of cultured MC3T3-E1 cells for 7 days. Data are mean ± standard deviation ($n = 4$). * $P < 0.01$, m-SXC10 and m-SXC15 versus m-SXC0; # $P < 0.01$, m-SXC10 and m-SXC15 versus m-SXC5; $ $P < 0.05$, m-SXC15 versus m-SXC10

improved the PGE_2 level. However, PGE_2 level significantly decreased when the Ca content was more than 5% in m-SXC, indicating that only a little Ca in m-SXC could stimulate PGE_2 expression positively, m-SXC with high Ca content would inhibit it.

3.7 Effect of m-SXCs on osteogenic expressions

Osteogenic expressions were regulated by different m-SXCs. As shown in Fig. 8, after 3 and 7 days culture of MC3T3-E1 cells, there was no obviously effect of Ca content on the all m-SXCs samples for the osteopontin mRNA expressions as determined by RT-PCR. However, the collagen I mRNA expression on m-SXC5 was highest

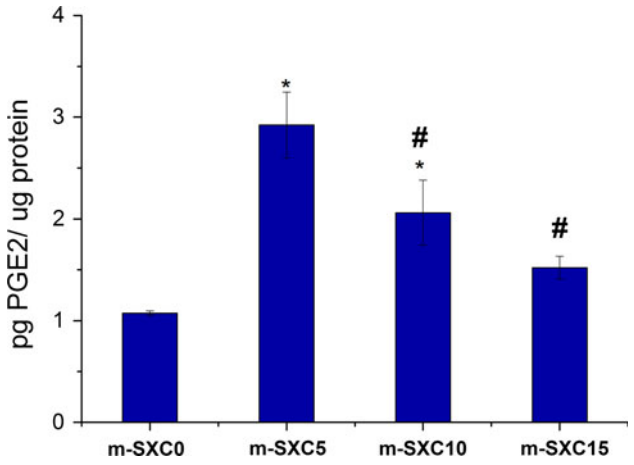

Fig. 7 Effect of calcium content in m-SXCs on PGE_2 level of MC3T3-E1 cells for 7 days. Data are mean ± standard deviation ($n = 4$). * $P < 0.05$, m-SXC5 and m-SXC10 versus m-SXC0; # $P < 0.05$, m-SXC10 and m-SXC15 versus m-SXC5

among all m-SXCs at both day 3 and 7. For osteocalcin, at day 3, the expression level in m-SXC5 was highest. After 7 days of culture, the gel density in m-SXC10 was increased and comparable to that in m-SXC5, the expression in m-SXC15 was negligible. These results indicated that a little Ca in m-SXC could enhance some osteogenic expressions, while m-SXC with higher Ca content would down-regulate osteoblastic gene expression.

3.8 Effect of m-SXCs on the activation of ERK 1/2 signaling pathway

Figure 9 presented the effect of calcium content in m-SXCs on ERK 1/2 signaling of MC3T3-E1 cells for 6 h, 3 and 7 days. No obvious differences in band intensities among all m-SXCs were observed for the activation of ERK 1 and ERK 2, using a phospho-antibody that recognized the dual phosphorylated proteins at 6 h. The activation of ERK 1/2 increased on all m-SXCs except m-SXC15, and the m-SXC5 was the highest in all samples at 3 days. The phosphorylation level of ERK 1/2 on m-SXC10 was the highest than other samples, and band intensity on m-SXC0 was very low that almost could not be detectable at 7 day.

4 Discussions

It has been demonstrated that cellular responses depend not only on physical status (such as surface morphology of biomaterials) but also on the material's chemical composition [24]. As various chemical compositions play a crucial role in determining cell responses, they affect the quantity of ions released from a material and the

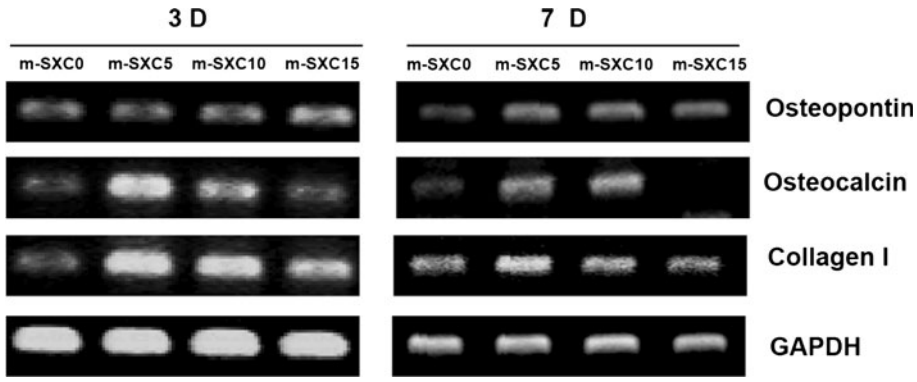

Fig. 8 Effect of calcium content in m-SXCs on osteogenetic expressions of MC3T3-E1 cells for 3 and 7 days. Representative agarose gels from three independent samples are shown

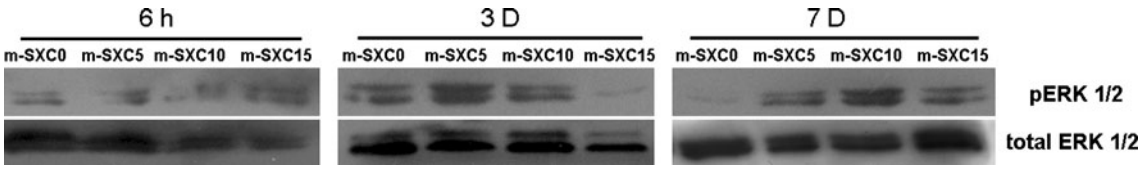

Fig. 9 Effect of calcium content in m-SXCs on phosphorylation of ERK 1/2 of MC3T3-E1 cells for 6 h, 3 days, and 7 days. The expression level of total ERK 1/2 was served as control. Data shown are representative from two independent experiments

consequent cell-material interaction [25]. This study confirms the importance role of calcium in mesoporous silica based materials resulting to marked changes in osteoblast proliferation, differentiation, local factor production and even intercellular signaling pathway.

Using CTAB as template, mesoporous silica xerogels were fabricated with different amount of calcium. However, with the addition of calcium amount in m-SXCs, the surface area of m-SXCs was decreased which might be explained that the incorporating calcium destroyed silica framework (Table 2). During the process of interactions between materials and osteoblasts, ionic dissolution productions from materials play a crucial role. Some authors believed that silicon ions form silanol groups which are deemed to act as nucleation sites during apatite deposition. Calcium and phosphorous ions lead to the supersaturation of SBF solution around the hybrid membrane and accelerate the formation of bioactive apatite-like layer [26]. In the present study, the ICP results revealed that Si and Ca ions could be released from the m-SXC into SBF, and that m-SXC with Ca resulted in a more rapid increase of Ca and Si ion concentrations, providing a higher basic ion concentration in the SBF solution, which might be helpful to osteoblasts responses. The decrease of P release over time might be correlated with the apatite layer formation. Some viewpoints showed that ionic dissolution products containing Ca and Si from materials can stimulate osteoblast proliferation and gene expression [19, 27, 28]. We observed that osteoblast viability and proliferation increased in m-SXCs except m-SXC15. This is in agreement with previous findings that show a positive correlation between alkalinization and osteoblast or chondroblast proliferation. It was also demonstrated that osteoblasts present voltage-activated calcium channels in their membrane, and that alkalinization increases channel sensitivity, enhancing cellular calcium entry, and excessive Ca is harmful to osteoblast function [29].

M-SXCs have the capability to promote early differentiation of osteoblasts as indicated by the expression of ALP, NO and PGE_2, respectively. ALP activity is routinely used as an early marker of osteoblast differentiation, bone turnover and remodeling in vitro [30]. The ALP activity of osteoblasts on m-SXC5 was higher than that on m-SXC0, suggesting that Ca in m-SXC improved the ALP expressive levels, which was supported by previous study that ALP activity and mineralization of MC3T3-E1 pre-osteoblast cells are under the influence of extracellular Ca^{2+} [31]. Some recent perspectives believed that high extracellular Ca has stimulatory effect on ALP activity partly via a transcription factor, SMAD3 as well as TGF family [32]. The expression of TGF-1 in ROS17/2.8 osteoblastic cells was up-regulated by Ca^{2+} on the surface of hydroxycarbonate (HCA) layer of bioactive apatite- and wollastonite-containing glass ceramics [33].

It is likely that the cytokines expression induced by m-SXCs contributed to osteoblast differentiation locally. Nitric oxide is a gaseous molecule as a result of L-arginine metabolism catalyzed by nitric oxide synthases (NOS) and is known to play a crucial role during bone remodeling [23, 34, 35], which is demonstrated Ca^{2+} sensitive [23]. In this study, NO production obviously increased with the increase of Ca content in m-SXCs. NO produced in

response to pro-inflammatory cytokines participates in the modulation of ALP activity via the activation of COX pathway, which indicated that the levels of NO production in m-SXC with low calcium content were suitable for osteoblast functions. Prostaglandin E 2 (PGE_2) is one of the prostanoids produced as a result of initial action of cyclooxygenases (COX-1 and COX-2) on arachidonic acid. Indeed, PGE_2 is known as a potent mediator for bone modeling, since it has ability to activate osteoclasts and osteoblast differentiation. The PGE_2 levels results was consistent with previous ALP tested results, which was supported by viewpoint that osteoblast differentiation was positively correlated with PGE_2 concentration [36].

Osteogenic expressions are strictly regulated by different materials surfaces, which have unique expression profiles that lead to either cell differentiation and osteogenesis or cell growth [22]. Gene expression of osteopontin, osteocalcin and collagen I was also influenced by different m-SXCs. Collagen I is the most abundant protein synthesized by the active osteoblasts and its expression represents the beginning of the osteoblasts differentiation. Osteocalcin and osteopontin is another important osteoblast-related marker produced by mature osteoblasts during mineralization and is found in fully developed mineralized matrix [37]. Calcium is a well established regulator of transcriptional change in gene expression that affects the transcription of numerous phenotypic genes and transcription factors. Our results indicated that no obviously effect of Ca content on all m-SXC for the osteopontin mRNA expressions was found after 3 and 7 days of culture. But the collagen I and osteocalcin mRNA expressions on m-SXC5 were much higher than that of m-SXC0, indicating up-regulated osteoblastic differentiation. It was reported that higher collagen production, mineralization was closely related to calcium deposition and collagen I mRNA was further stimulated by alkalinization [38]. Therefore, it is explainable that probably high extracellular Ca^{2+} levels have initiated these effects on collagen synthesis and osteocalcin expressions, which finally resulted to stimulated osteoblastic differentiation, such as ALP and PGE_2 excretions.

In osteoblasts, modulation of mitogen-activated protein kinase (MAPK) signaling pathways has been implicated in the regulation of cell proliferation and differentiation as well as in other cellular responses triggered by extracellular stimuli [39]. Extracellular signal-regulated kinases (ERKs), one of four different subgroups within MAPK family, are not only essential for the growth and differentiation of osteoblasts but also are important for osteoblast adhesion, spreading, migration, and integrin expression [40]. Studies have also shown that intercellular Ca^{2+} is important to ERK 1/2 activation in osteoblasts [41]. The obvious influences for m-SXCs on ERK 1/2 activation up to 7 days might result to the discrepancy in osteoblast proliferation and differentiation. Differences in complicated ions release system, protein adsorption ability of m-SXCs containing varied calcium contents and the capability to form apatite layer on the surface of materials may regulate focal adhesion formation and integrin signaling, and conformation of cell attachment proteins like fibronectin [42, 43], finally result in different extent in activation of ERK 1/2 signaling pathways.

Taking these factors into account, it seems that osteoblasts tend to differentiate in m-SXCs with low calcium content, proliferate in m-SXC with high calcium contents, however, excessive calcium is harmful to the cellular functions. On the meanwhile, it is also demonstrated that modulation on the chemical compositions of mesoporous silica based materials could result to different responses of osteoblast. Therefore, the data obtained in this paper would provide some guidance to the chemical design of mesoporous materials used in bone regeneration.

5 Conclusions

Osteoblasts have different responses on mesoporous silica xerogels containing different amount of calcium. Among them, m-SXC10 could promote cell proliferation, whereas m-SXC5 showed significant enhancement on osteoblast differentiation, including ALP activity, PGE_2 as well as osteogenic (osteocalcin and collagen I) expressions. The promotion on osteoblast proliferation and differentiation of m-SXC10 and m-SXC5 might result from the activation levels of ERK 1/2 signaling pathway. The present experimental results demonstrated that the calcium content in m-SXC had some obvious effects on the cell behavior and showed critical roles of chemical compositions on enhancing bioactivity of biomaterials. Our basal data also provide some guidance on the design of inorganic-based materials. In conclusion, the m-SXC containing appropriate calcium had good biocompatibility, and could promote osteoblast proliferation, differentiation, gene expression and activation of ERK 1/2 signaling pathway, indicating it could be used as potential bioactive controlled-release materials in bone tissue engineering.

Acknowledgements The authors are indebted to the financial support from the National Natural Science Foundation of China (No. 30670568, 50732002), Program of Shanghai Subject Chief Scientist (No. 07XD14008), and Shanghai Nanotechnology Special Foundation (No. 0852nm02700), Major Program of National Natural Science Foundation of China (No. 50732002), Program for Changjiang Scholars and Innovative Research Team in University.

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