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The effect of strontium incorporation in hydroxyapatite on osteoblasts in vitro

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Abstract The purpose of this study was to test the effects of a series of strontium-substituted HA (Sr-HA) ceramics (0, 1, 5, and 10 mol% Sr substitution) on osteoblasts, thereby demonstrating whether strontium incorporation with HA would favor osteoblast metabolism. Rat primary osteoblasts were cultured with culture media containing ions released from the Sr-HA ceramics as they dissolved. MTT test, alkaline phosphatase activity, osteoblast transcription factor gene (cbfa1) expression and Alizarin Red staining were conducted at different time-points. There is no significant difference in cell proliferation between groups. However, compared with HA group, Sr-HA groups presented significant enhancement with regard to ALP activity, cbfa1 mRNA expression, and mineralization nodules. Among Sr-HA groups, 5 and 10% groups showed much better performances in ALP activity, cbfa1 mRNA expression, and mineralization nodules than 1% group, however, no significant difference was found between 5 and 10% groups. This study has demonstrated that Sr incorporation in HA ceramic enhanced osteoblastic cell differentiation and mineralization. However, further detailed studies are needed to understand the mechanistic effects of this Sr incorporation on osteoblastic cells and the optimal percentage of calcium should be substituted with strontium in HA.

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1 Introduction

The beneficial effect of strontium (Sr) at low dose in the treatment for osteoporosis was first reported over half a century ago [1]. However, the therapeutic potential was not thoroughly considered until recently with the development of strontium renelate. Strontium renelate, containing two Sr atoms, has been shown to favor bone metabolism by both increasing new bone formation and reducing bone resorption [2–6]. It stimulates the proliferation and differentiation of osteoblastic cells and inhibits the activity and differentiation of osteoclasts; thus, it enhances matrix deposition and, ultimately, encourages new bone formation [2–4, 6].

Recent studies took the advantages of the stimulatory effects of Sr on bone metabolism by incorporating it into various bone implantation biomaterials [7–10]. Calcium phosphate ceramic materials, particularly hydroxyapatite (HA) and tricalcium phosphate (TCP), own excellent biocompatibility with human hard tissues, and they have been widely used in the field of orthopaedics and dentistry. With their chemical resemblances, partial Ca^{2+} in these materials may be replaced by Sr^{2+}, which was suggested of the changes of materials dissolution behaviour and growth kinetics [11, 12]. Christoffersen et al. [11] investigated the dissolution behaviour of HA containing 1–10% Sr^{2+} in molar fraction instead of Ca^{2+} and concluded that the solubility for these apatites increases along with the content

of Sr^{2+}. Chen et al. [12] also reported that the incorporation of strontium in low doses introduce more lattice distortions into the structure of HA and lead to the increase of its solubility.

Strontium-containing hydroxyapatite (Sr-HA) bioactive bone cement is a bisphenol-A glycerolate dimethacrylate (Bis-GMA) based bone cement with 10% calcium ions in HA was substituted by strontium; a series of studies have been conducted to show its biocompatibility, osteoconductivity and bioactivity [8, 13, 14]. We previously reported that Sr-HA bioactive bone cement has the ability to stimulate new bone formation in vivo [8]. More recently, this bioactive bone cement was evaluated in a sheep hip replacement model for its effect on femoral bone remodeling, the results showed that it has positive effect to alleviate femoral bone remodeling [15]. Although in vivo evidences suggested that this Sr-substituted HA ceramic has positive effect on bone formation, a thorough understanding of its potential role in bone metabolism is required to gain mechanistic insight.

Biomaterials are subjected to numerous cellular interactions of diverse origin in vivo. Changes in the composition of calcium phosphate bioceramic can affect the cellular activity. Previous studies showed that the incorporation of Sr promoted better proliferation and attachment of osteoblastic cells on calcium phosphate bone substitutes and HA coatings in vitro [7, 9]. Recently, Gentleman et al. [16] investigated the effects of bioactive glass series, in which 0–100% of the calcium was substituted by Sr; in vitro studies showed the metabolic activity of osteoblastic cells can be enhanced with dose-dependent manner. We therefore assumed that strontium incorporation with HA would favor osteoblast metabolism. To prove this hypothesis, a series of HA, in which Sr was substituted for calcium in a mol% basis, was tested in this study for their effects on osteoblasts.

2 Materials and methods

2.1 Sr-substituted HA synthesis

Sr-substituted HA which either contained no calcium (0%) or 1, 5 or 10% (mol%) of the calcium was substituted with Sr were synthesized by the "wet" method; such method allows mass production for small crystalline or noncrystalline HA powder as previously described [17]. In brief, H_3PO_4 was added dropwise to a basic suspension containing $Ca(OH)_2$ and $Sr(OH)_2$ in 1000 ml of distilled water while being stirred at 20 °C. The reaction mixture was stirred for 3 days, and the pH was controlled at 9.5. The slurry was filtered and the product was dried at 110 °C. An alumina ball mill was then used to pulverize the granular product into fine powder. The product under 200 mesh was calcined in a high-temperature Muffle furnace (F46240CM, Thermolyne, USA) at 800 °C for 3 h.

2.2 Dissolution ion media

0.2 g/ml of Sr-substituted HA powders were added to 10% FBS L-DMEM culture medium, incubated on a roller at 37 °C for 24 h, and then passed through a 0.2 μm filter. Samples of dissolution ion media were collected after 24 h for elemental analysis. Media were equilibrated in a 37 °C with 5% CO_2 incubator overnight before being placed on cells.

Cell culture media samples containing dissolution ions from HA were diluted by a factor of 10 with dH_2O and the elemental concentrations of Ca and Sr ions were measured with an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Ultima2, Jobin–Yvon, HORIBA, Japan).

2.3 Cell culture with dissolution ions

Rat primary osteoblasts were isolated as described previously [18]. In summary, osteoblasts were derived from postnatal day 1 rat calvariae by sequential digestions of 20, 40, and 90 min at 37 °C in 2 mg/ml collagenase A with 0.25% trypsin. Cells harvested from first and second digestions were discarded. Cells collected from the third digestion were plated at 6.4×10^3 cells/cm^2 and grown in DMEM cell culture media supplemented with 10% fetal bovine serum (FBS) containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate.

2.4 Proliferation assay

The effect of dissolution ions on osteoblasts proliferation was evaluated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma). The assay was performed at day 2, 4, and 6, respectively. The absorbance measured at a wavelength of 570 nm was plotted against with cell numbers. The cell viability was expressed as fold changes to the control (HA) group.

2.5 Alkaline phosphatase (ALP) activity

Briefly, ALP staining was carried out using an ALP kit (Sigma-Aldrich). Primary osteoblasts cultures after treatment with Sr-containing media for 3, 7, and 14 days were incubated in a mixture of naphthol AS-MX phosphate alkaline solution with fast red salt. The resulting red granular dye deposit indicates sites of ALP activity. For the quantitative determination of ALP activity, the cultured cells were incubated with p-nitrophenol phosphate as a

substrate and then washed with PBS buffer and lysed with 0.1% Triton × 100 in 10 mM Tris HCL, PH 9.0. Absorbance was determined at 405 nm and compared with p-nitrophenol standard titration curve. ALP activity was normalized to total protein measured with the Bio-Rad protein assay (BioRad, Hercules, USA).

2.6 Real-time PCR analysis

The cells were seeded in 24-well plates with an initial seeding density of 4×10^4 cells/well and cultured for 3, 7, and 14 days. Total RNA was extracted from cells cultured at each incubation time using Trizol reagent (Gibco BRL Life Technologies). To create first strand cDNA, reverse transcription was performed as described previously [18]. To determine osteoblast transcription factor gene (cbfa1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, real-time PCRs were performed as described previously using the primers shown in Table 1. The results for gene expression were normalized to the values obtained for GAPDH mRNA. Amplification was denatured at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 15 s; afterwards, the samples were annealed at 65 °C for 1 min. The temperature was then returned to 95 °C.

2.7 Osteoblast mineralization (Alizarin red S staining)

Alizarin red S staining is a common method for visualizing nodular and calcium deposition of osteoblast cells in vitro. To evaluate the effect of strontium on the matrix mineralization of osteoblasts, Alizarin Red staining of osteoblast cultures was carried out at day 14 and 21 after the treatment of Sr-HA. Cells were fixed with formalin (10%, v/v) at room temperature for 15 min, rinsed with PBS (3 times) and stained with 40 mM Alizarin Red S (Sigma-Aldrich) with PH 4.2 for 15 min at room temperature. For the quantification assay, the stained plates were washed with distilled water to remove non-specific staining. The stained cells were then incubated for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using a spectrophotometer. The concentration of Alizarin Red staining in the samples was determined by comparing the absorbance values with those obtained from Alizarin Red standards.

Table 1 Primer sequences for PCR

Target	Primer sequences
Cbfa1	Forward 5′–3′: CCC AAC TTC CTG TGC TCC
	Reverse 5′–3′: AGT GAA ACT CTT GCC TCG TC
GAPDH	Forward 5′–3′: CAA GTT CAA CGG CAC AGT CA
	Reverse 5′–3′: CCA TTT GAT GTT AGC GGG AT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

2.8 Statistical analyses

All data values were expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) with post hoc test was used to evaluate the differences of variables among groups. A value of $P < 0.05$ was considered a significant difference. All statistical evaluations were performed with SPSS version 13.0 (SPSS, Chicago, USA).

3 Results

Table 2 showed the concentrations of released Ca and Sr ions from 4 groups. Among these groups, highest Ca concentration was found in 5% Sr-HA group, while highest Sr concentration in 10% Sr-HA group.

3.1 Proliferation assay

During day 2 until day 6, all groups had comparable increase in MTT activity (Fig. 1). After 2, 4, and 6 days in culture, there were no significant differences in the MTT

Table 2 The concentrations of released Sr and Ca ions in four groups

Group	Ca (μg/ml)	Sr (μg/ml)
10% Sr-HA	23.26	19.46
5% Sr-HA	26.12	12.75
1% Sr-HA	5.03	5.05
HA	9.12	0.00

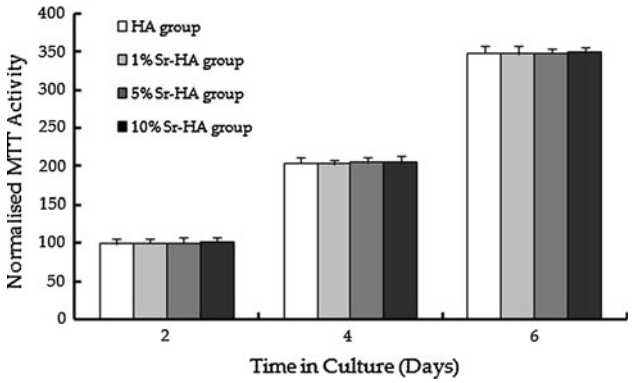

Fig. 1 Normalised MTT activity of osteoblasts treated with dissolution ions from Sr-HA groups, which was expressed as fold changes compared to HA group. Cell numbers increased with time. However, at each time-point, no statistical difference was found between groups ($P > 0.05$). Data are presented as mean ± SD ($n = 6$ per group)

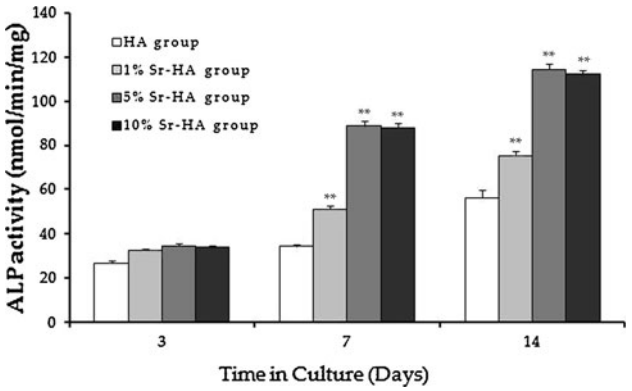

Fig. 2 ALP activity of osteoblast cells of all groups after 3, 7, and 14 days culture. There was no statistical difference between groups after 3 days of culture ($P > 0.05$). However, significantly higher ALP activity was observed in either Sr-HA group than that in HA group after 14 and 21 days of culture ($P < 0.01$). Data are presented as mean ± SD ($n = 6$ per group) (*$P < 0.05$; **$P < 0.01$)

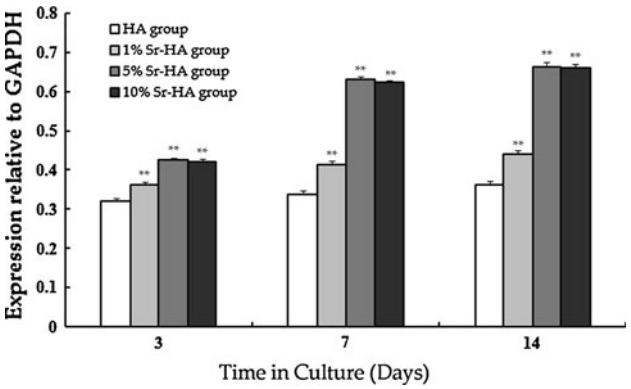

Fig. 3 Cbfa1 mRNA expression level on day 3, 7, and 14 using real-time RT–PCR. Compared with HA group, mRNA expression level was significantly higher in either Sr-HA group at each time-point ($P < 0.01$). Data are presented as mean ± SD ($n = 6$ per group) (*$P < 0.05$; **$P < 0.01$)

activity of osteoblasts treated with Sr-HA dissolution ions compared to cells treated with HA dissolution ions ($P > 0.05$), indicating that Sr incorportion in HA ceramic has little influence on osteoblastic cell proliferation.

3.2 ALP activity

The results for ALP activity are shown in Fig. 2. There was no statistical difference in ALP activity between groups after 3 days of culture ($P > 0.05$). However, significantly higher ALP activity was observed in either Sr-HA group than that in HA group after 7 and 14 days of culture ($P < 0.01$). ALP activity in 5% Sr-HA group was higher than that in 10% Sr-HA group at each time-point, but without statistical difference ($P > 0.05$).

3.3 Real-time RT–PCR gene expression levels of Cbfa1

Cbfa1 is an osteoblast-specific transcription factor which is essential for osteoblast differentiation and bone formation. Cbfa1 mRNA expression level was determined on day 3, 7, and 14 using real-time RT–PCR (Fig. 3). Compared with HA group, mRNA expression level was significantly higher in either Sr-HA group at each time-point ($P < 0.01$). Similar to ALP activity, 5% Sr-HA group has a higher mRNA expression level than 10% Sr-HA group at each time-point, but again without statistical difference ($P > 0.05$).

3.4 Mineralized nodule formation

To examine the mineralization capacity of the osteoblatic cells, mineralized nodule formation was determined by the cells cultured with the absence and presence of Sr incorporated medium for 14 and 21 days. Compared with HA group, either Sr-HA group increased osteoblast mineralization at day 14 and 21, measured by Alizarin red S staining (Figs. 4, 5). The number of mineralized nodules in primary osteoblastic cells was also counted, and the result showed considerably more nodules in either Sr-HA group than HA group at each time-point ($P < 0.01$) (Fig. 6). Similar to ALP activity and Cbfa1 gene expression, 5% Sr-HA group has more nodules than 10% Sr-HA group, though without statistical difference at each time-point ($P > 0.05$).

4 Discussion

During the past few years, Sr-HA bioactive bone cement, in which 10% calcium ions in HA were substituted with Sr, has been extensively evaluated in the applications of vertebroplasty and hip replacement [8, 10, 13–15, 17]. These in vivo studies confirmed that this strontium-substituted HA biomaterial has positive effect on bone formation under both weight-bearing and non-weight-bearing conditions. To further understand the potential role of Sr incorportion in HA in bone metabolism, a series of strontium-substituted HA bioceramics, in which 0, 1, 5, and 10% of the calcium was substituted with strontium, were tested and compared in this study for their effects on osteoblasts in vitro. The results showed that Sr incorportion in HA ceramic enhanced osteoblastic cell differentiation and mineralization, which coincided with our previous in vivo studies. On the other hand, our study showed that Sr incorportion in HA ceramic did not enhance cell proliferation. It seems that the stimulatory effect of Sr incorporation was most likely associated with the enhancement of cellular differentiation, but not with an increase of cell number.

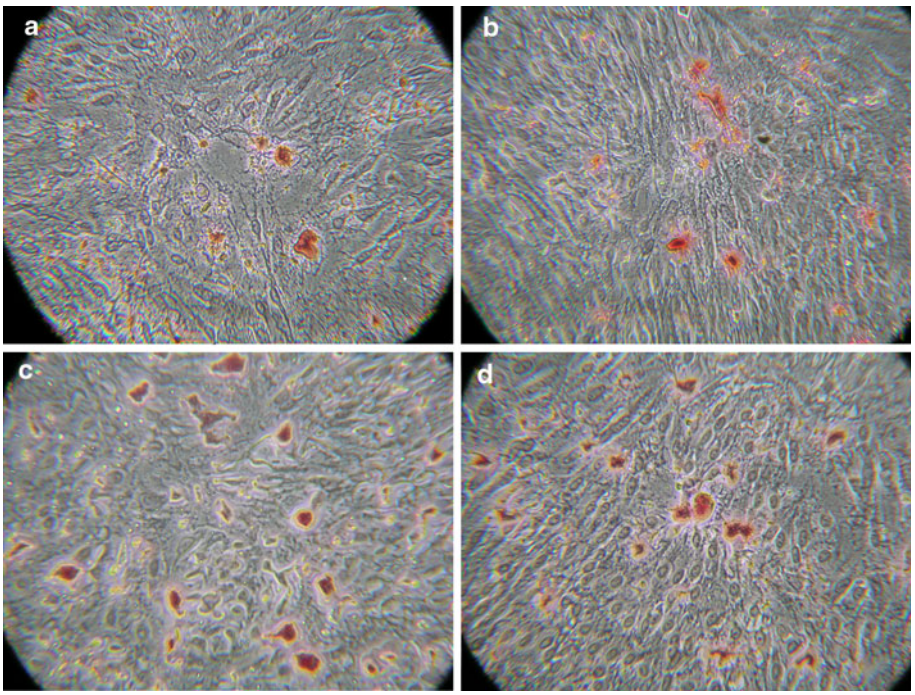

Fig. 4 Effect of Sr incorporation in HA on mineralization. Cells were subjected to Alizarin red S staining at day 14. (**a** HA group, **b** 1% Sr-HA group, **c** 5% Sr-HA group, **d** 10% Sr-HA group)

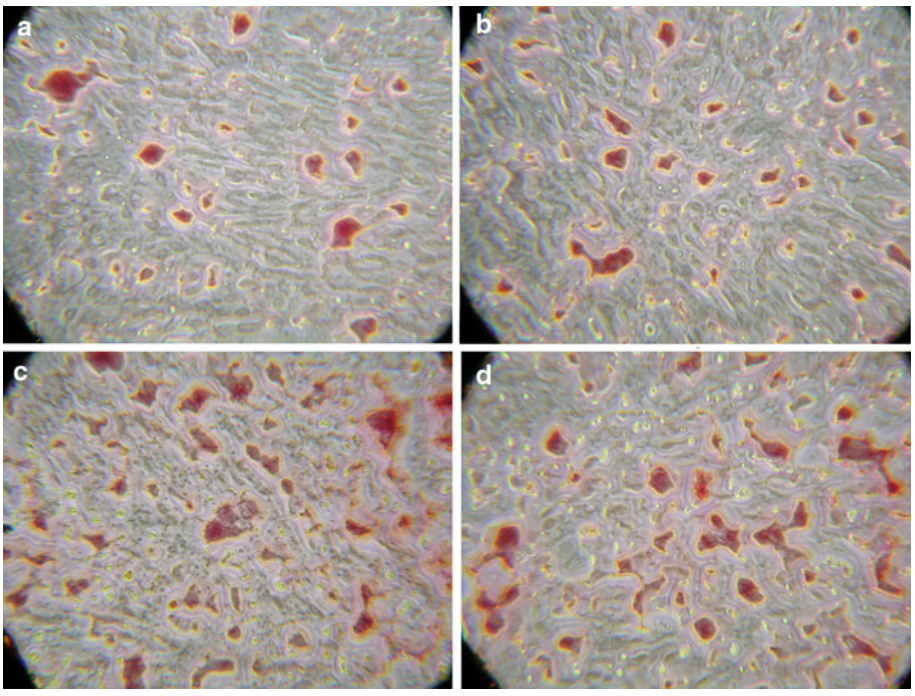

Fig. 5 Effect of Sr incorporation in HA on mineralization. Cells were subjected to Alizarin red S staining at day 21. (**a** HA group, **b** 1% Sr-HA group, **c** 5% Sr-HA group, **d** 10% Sr-HA group)

In this study, Sr incorportion in HA significantly increased the ALP activity and the expression of Cbfa1 of osteoblast cells. These results agree with previous in vitro studies regarding the stimulatory effect of Sr incorportion into various materials [7, 9, 16, 19]. Sr incorporation into calcium polyphosphate scaffolds stimulated ALP activity of ROS17/2.8 cells [9]. A thin-film of Sr-substituted HA coatings produced by pulsed laser deposition significantly increased the ALP activity and osteocalcin production of osteoblast-like MG-63 cells on materials' surfaces compared to HA coatings without any Sr content [7]. Sr-containing Ti–6Al–4 V surface produced by hydrothermal treatment enhanced ALP activity and osteoblast gene expression of MG63 cells compared with untreated Ti–6Al–4 V surface [16]. In addition, Gentleman et al. found that Sr incorporation into a series of bioactive glasses also

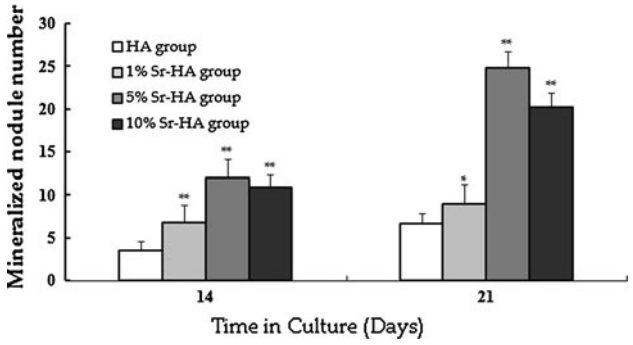

Fig. 6 Compared with HA group, considerably more mineralized nodules in primary osteoblastic cells was found in either Sr-HA group ($P < 0.01$). Data are presented as mean ± SD ($n = 6$ per group). ($^*P < 0.05$; $^{**}P < 0.01$)

enhanced the metabolic activity of osteoblasts in a dose-dependent manner [19].

Cbfa1 mRNA expression level was determined in cultures with and without strontium ions in this study, and the results showed that, along with ALP activity, Cbfa1 gene expression was significantly higher in Sr-HA groups compared with HA group, indicating that Sr may promote differentiation of osteoblast cells through activating the downstream Cbfa1. Detailed signaling pathway and mechanism may need further investigation. What is more, Sr incorportion in HA ceramic was found to enhance osteoblastic cell mineralization. In addition to the stimulatory effect of released strontium ions on osteoblastic cell mineralization, another reason may be the increasing solubility of HA ceramic after Sr incorportion, which could lead to apatite formation [11, 12]. On the other hand, among three Sr-HA groups, the highest strontium concentration was found in 10% Sr-HA group, however, no significant differences in ALP activity, Cbfa1 gene expression and the formation of mineralized nodules of the osteoblastic cells were found between 5% Sr-HA group and 10% Sr-HA group, suggesting that the stimulatory effect of strontium incorportion on cellular differentiation and mineralization does not always enhance with the increase of strontium concentration. Recently, Zhang et al. [20] compared the effect of strontium-substitution (10, 40, 100%) in HA on osteoblasts, and found that 10% Sr-HA group has the best performance. Sila-Asna et al. [6] ever suggested the optimal concentration of strontium ranelate was between 0.2107 and 21.07 μg/ml, whereas the high concentration up to 210.7 μg/ml have delayed effect on osteoblastic differentiation. It was therefore assumed that there might exist a negative feedback mechanism for the regulation of strontium on osteoblastic differentiation when the concentration of strontium amounts to a certain level. Whether this is the case or not deserves further investigation.

Although Sr incorportion in various materials favors osteoblastic cell behaviors, the optimal concentration of Sr remains controversial. With regard to SR, a number of in vitro studies explored the optimal concentration of Sr, and the results suggested that relatively high concentrations of Sr (17.5 ppm or above) are required to enhance osteoblastic cell proliferation and differentiation [2, 3, 21, 22]. On the other hand, however, Park et al. [19] found that Sr ions released from Ti64/Sr surface were 103–135 ppb, which enhanced differentiation of osteoblastic cells. In the present study, Sr ion with the concentration of 5.05 ppm could stimulate the differentiation and mineralization of osteoblastic cells. It seems that relatively lower concentration of Sr is needed to stimulate the metabolic effect of osteoblast cells in Sr-substituted material than SR. Unlike SR, several dissolution ions dissolved from a Sr-substituted material may co-act with Sr ion. For instance, ions, such as calcium, phosphorus, silicon, and strontium, were found to be dissolved from Sr-substituted bioactive glasses [16]. Our previous in vivo study demonstrated that ions including calcium, phosphorus, Na, strontium, and oxygen, were detected at the interface between bone and bone cement after Sr-containing HA bioactive bone cement was implanted [23]. It is now known that a number of divalent and trivalent cations, including Ca^{2+} and Sr^{2+}, are agonists and modulators of the calcium-sensing receptor (CaR) [24, 25], which has been shown to be expressed at all stages of osteoblast development, including proliferation, differentiation and matrix mineralization [26, 27]. However, whether the presence of other dissolution ions can co-act with strontium needs further investigations to stimulate the metabolism of osteoblastic cells.

Our previous studies demonstrated that Sr-HA bioactive bone cement stimulates bone growth under both weight-bearing and non-weight-bearing conditions, in which, 10% calcium ions in HA were substituted by Sr. However, it remained unclear as to the optimal percentage of calcium should be substituted with strontium in HA. In this study, a series of Sr-substituted HA bioceramics was tested for their effects on osteoblasts in vitro, and the results suggested that 10% Sr-HA ceramic was not superior to 5% Sr-HA ceramics in ALP activity, Cbfa1 gene expression and mineralization nodule formation. The reason of this phenomenon is unknown, and further investigation is required to determine the relation of Sr concentration with metabolic activity of osteoblast cells. Furthermore, Chen et al. [28] even compared the mechanical properties of a series of Sr-HA ceramics, and found that 5% Sr-HA has the best mechanical properties among these ceramics. However, more studies, especially in vivo studies, should be conducted to evaluate these Sr-HA ceramics for their biological and biomechanical properties.

5 Conclusion

In this study, the effects of a series of strontium (Sr)-substituted HA ceramics (0, 1, 5, and 10 mol% Sr substitution) were tested on osteoblasts, and the results suggestd that HA ceramics with 5 and 10 mol% Sr substitution may enhance osteoblastic cell differentiation and mineralization. It was assumed that Sr may promote differentiation of osteoblast cells through activating the downstream Cbfa1, and there might be a negative feedback mechanism for the regulation of strontium on osteoblastic differentiation. However, further detailed studies are needed to understand the mechanistic effects of this Sr incorporation on osteoblastic cells and the optimal percentage of calcium should be substituted with strontium in HA.

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