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In vitro study in stimulating the secretion of angiogenic growth factors of strontium-doped calcium polyphosphate for bone tissue engineering

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Abstract Angiogenesis of tissue-engineered bone remains a limited factor for the engineering of larger bone tissue constructs. Attempts to stimulate angiogenesis, using recombinant protein or gene transfer of angiogenic growth factors, have been proposed; however, these approaches have been associated with some problems regarding such as complex technique, expensive prices as well as safety problems and short half-life of angiogenic growth factors. This study was performed to determine the ability of strontium-doped calcium polyphosphate (SCPP) to induce angiogenesis via researching its effect on the mRNA expressions and protein secretion of VEGF and bFGF in/from cultured osteoblasts (ROS17/2.8 cells). We cultured osteoblasts with SCPP scaffolds containing various doses of strontium as well as calcium polyphosphate (CPP) scaffold. Through the detection of MTT and SEM, we have found that SCPP could promote cell proliferation and maintain their morphology. The results of RT–PCR and ELISA indicated that, compared with those in CPP group, the mRNA expression as well as protein levels of VEGF and bFGF in/from cultured osteoblasts were dose-dependent increasing in response to increasing strontium before reaching the peak in SCPP groups, and 8% SCPP showed the optimal promoting role. Therefore, SCPP containing proper dose of strontium could be served as a potential biomaterial with stimulating angiogenesis in bone tissue engineering and bone repair.

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1 Introduction

The reconstruction of large skeletal defects is still a major orthopedic challenge. Bone tissue engineering is designed to regenerate these defects. However, the difficulty to induce rapid vascular ingrowth during new bone tissue development is a major limitation of bone tissue engineering approaches for the replacement of diseased or damaged bone tissue [1]. In bone tissue engineering, the cellular components need to be placed into a suitable environment, usually provided by a three-dimensional porous biomaterial scaffold that will promote cell viability and function. Transport of oxygen and nutrients to cells in the scaffold is initially dependent on diffusion because cells more than a few hundred micrometers away from blood vessels in the surrounding tissue are destined to die because of lack of oxygen [2]. Moreover, prompt blood supply into a tissue engineered device can improve the tissue integration, and enhance the success rate of the implant/graft similar to that achieved using an autograft [3]. Thus, enhancement of the angiogenic potential of implantable biomaterial scaffolds is receiving much attention in bone tissue engineering strategies [4].

Angiogenesis is a process tightly regulated by a variety of paracrine cytokines or peptide molecules [5]. Vascular endothelial growth factor (VEGF) is described as the most important proangiogenenic factor, which belongs to the VEGF/PDGF (platelet-derived growth factor) group of the cystine-knot super-family of hormones and extracellular

signaling molecules [6], it plays critical roles in the development and progression of the angiogenesis system both in normal physiological and pathological conditions through promoting vascular endothelial cell mitosis, permeability and survival [7–9]. Basic fibroblast growth factor (bFGF), an endogenous polypeptide growth factor, is regarded as another one of the most potent stimulators of angiogenesis; it may modulate bone formation through regulating the proliferation and differentiation of osteoblastic cells and promote angiogenesis [10, 11], at the same time, it is also considered as the inducer of VEGF in endothelial cells during the process of capillary formation [12, 13]. Cells involved with the process of bone repair all have the ability to secrete these two growth factors, such as osteoblast.

As stated previously, the angiogenesis process of biomaterials and tissue-engineered grafts after implantation is quite slow, which is not sufficient to achieve an appropriate vascularization of larger-sized grafts or highly vascularized tissues [14]. Several approaches are investigated to enhance or to accelerate the process of angiogenesis, such as partially combining angiogenic peptides [15, 16], angiogenic genes [17] and transfected endothelial cells expressing angiogenic peptides [17, 18], or integrating the growth factors bFGF and VEGF into tissue-engineering constructs [19, 20]. Nevertheless, these methods needing additional substances to modify the grafts are complex and the growth factors themselves have some shortcomings, such as expensive prices, safety problems and short half-life, all these lead to the limitation of the application of the constructs. Therefore, if the biomaterials or tissue-engineered scaffolds themselves possessed the ability to induce angiogenesis, which could stimulate the release of VEGF and bFGF from transplanted and/or host cell (such as osteoblastic cell) that have migrated into the scaffold, then the foregoing limitations would be able to overcome.

Strontium is a bone-seeking element, 99% of the strontium content accumulates in bone and accounts for 0.01% of bone weight. The role of strontium in disease therapy has attracted an increasing attention since the development of the strontium ranelate which has recently been shown to reduce the incidence of fractures in osteoporotic patients [21, 22]. Furthermore, some studies have shown that strontium presents a dual action of improving bone formation [23] as well as inhibiting bone resorption [24].

Previously, our laboratory comprehensively researched the structures, mechanical and degradable properties of calcium polyphosphate (CPP) and strontium-doped calcium polyphosphate (SCPP) with various crystal structures. Moreover, we investigated the biocompatibility of SCPP in vitro [25] and in vivo [26], found that SCPP containing low-dose strontium could stimulate osteoblasts proliferation in vitro and that SCPP scaffold could provide an ideal environment for colonization, proliferation, and differentiation of osteoblasts to form new bone in vivo, making it attractive for bone substitute application purposes. In addition, Chen et al. [27, 28] found that the degradation products of SCPP could induce the proliferation, migration and tube-like structure formation of endothelial cells in vitro; these suggested that SCPP might be of benefit to the angiogenesis.

For further research on the mechanism of the promoting effect of SCPP on angiogenesis, in this paper, we adopted SCPP containing various amount of strontium as scaffold materials for bone tissue engineering and chose VEGF and bFGF secreted by osteoblast-like ROS17/2.8 cells as the targets to investigate the ability of SCPP to induce angiogenesis at a molecular level in vitro. According to this study, it could be expected to obtain the optimal dose of strontium in SCPP in supporting osteoblastic cells growth and secretion of angiogenic growth factors from osteoblastic cells, and to provide a novel idea to solve the problem of "angiogenesis in bone repair material".

2 Materials and methods

2.1 Scaffold fabrication

Porous SCPP scaffolds containing various amounts of strontium and CPP scaffolds were prepared as previously described [25]. Briefly, strontium carbonate and calcium carbonate of various molar ratios (1, 2, 5, 8 and 10%) were added slowly into the phosphoric acid (85%) with stirring respectively. After the reaction went on at room temperature for 8 h, the solution was evaporated in vacuum. The precipitates were collected and washed by ethanol (95%) until the pH of filtrate was about 7. Then the powders were calcined at 500°C for 10 h to form SCPP and heated to 1,200°C with a heating rate of 15°C/min resulting to melt. The melt was promptly quenched on distilled water to cool down. The amorphous frits were ground and screened to yield powders in a size range of <75 μm; then the powders were mixed the stearic acid and supplemented with 3 wt% poly(vinyl alcohol), pressed at 98 MPa, and, finally, sintered to obtain porous SCPP cylinder with 10 mm diameter and 2 mm thickness. CPP scaffolds were also prepared by this method.

The crystal system of samples was tested by X-ray diffraction (XRD, Philips®, Netherlands) and the morphology of the disks was examined by scanning electron microscopy (SEM, Japan Electronics Co., Ltd.) as previously described [25].

2.2 Osteoblast culture

The immortalized osteoblastic ROS17/2.8 cell line was utilized in this study, which was purchased from West

China Hospital, Sichuan University (China). ROS17/2.8 cells are rat osteoblastic sarcoma cells and display various characteristics of osteoblast. The cells were cultured in growth medium [high Dulbecco's modified Eagle's medium (DMEM, Gibcobrl), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin (Sigma) and 100 U/ml streptomycin (Solarbio) solution] and incubated at 37°C in a humidified atmosphere (5% CO_2 in 95% air). Until confluence, cells were passaged at a 1:2 split ratio following trypsinization with 0.25% trypsin (Gibcobrl). Cells from passage 3 were used for preparation of the cell-scaffold constructs.

2.3 Osteoblast identification

The osteoblastic characters of ROS17/2.8 cells were identified by alkaline phosphatase (ALP) staining assay and mineralized nodules staining assay. For ALP staining assay, ROS17/2.8 cells were seeding on the cover slip with a density of 1×10^4 cells/cm^2. After cultured for 3 days, cells were fixed with 95% ethanol in the 4°C refrigerator for 5 min and stained by coupling azo dye method. The result was observed with inverted phase contrast microscope (Olympus Corporation, Japan). For mineralized nodules staining assay, ROS17/2.8 cells were continually cultured with induced liquid of mineralization for 20 days. Later, cells were fixed with 95% ethanol for 10 min and washed twice by phosphate buffer. Then they were stained with 1% alizarin red-Tris–HCl (pH 8.3) for 30 min before rinsed thoroughly by phosphate buffer. The consequence was observed with inverted phase contrast microscope (Olympus Corporation, Japan).

2.4 Preparation of cell-scaffold constructs

When the cells reached the stage of confluence, they were harvested by trypsinization, followed by the addition of fresh culture medium to create cell suspension. SCPP with various strontium concentrations and CPP scaffolds were gamma irradiation-sterilized for 120 min and passivated in culture medium for 12 h, prior to seeding the cells. 300 μl cell suspensions at a density of 2×10^4 cells/ml were used to seed the scaffolds kept in 24 well plates, followed by incubating the samples in a humidified environment for a period of up to 14 days. CPP was served as the controlling material. Culture medium was changed every 2 days.

2.5 Proliferation of osteoblasts seeded on scaffolds

Cell proliferation was assessed by colorimetric assay which detected the conversion of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. Briefly, one plate was taken out on the third, seventh, tenth, and fourteenth day respectively and 20 μl/well of MTT solution (5 mg/ml in phosphate buffered saline, PBS) was added, then the plate was incubated at 37°C to allow the formation of formazan crystals. After 4 h, the dimethylsulfoxide (300 ml/well) was added to all wells after aspirating the culture medium and mixed thoroughly to dissolve the dark blue crystals at room temperature. Ten minutes later, the optical density (OD) at 492 nm was measured with a Microplate Reader (Model550, Bio Rad Corp.).

2.6 Scanning electron microscopy (SEM) of cell-scaffold constructs

Cell-seeded scaffolds were pre-treated by washing with PBS for three times and immersing in PBS containing 3% glutaraldehyde (pH 7.4) for 2 h. They were then dehydrated in increasing concentrations of ethanol (30, 50, 70, 80, 90, 95 and 100%) for twice. The critical point drying of specimens was undertaken with liquid CO_2. The specimens were sputter-coated with gold and examined by SEM (Japan Electronics Co., Ltd.). The growth of cells on the scaffolds was observed on the 7th day.

2.7 Angiogenic growth factors gene expression analysis

The mRNA expression of VEGF and bFGF were measured by real time fluorescence quantitative polymerase chain reaction (RT–PCR). The cell-scaffold constructs were prepared as follows: ROS17/2.8 cells were seeded on the SCPP scaffolds involved various amounts of strontium, which were cultured in high DMEM supplemented with 10% FBS ($n = 3$); 5 days later, $1,25(OH)_2VD_3$ (Sigma) was added to the medium with a final concentration of 10^{-7} mM; after 48 h, the culture was terminated. Cells cultured with CPP scaffold were served as the control in this assay.

Total RNA was extracted with Trizols (MRC, America) reagent according to the manufacturer's instruction. Briefly, lysis of the cells in Trizols was followed by centrifugation at $12,000 \times g$ and 4°C for 15 min in the presence of chloroform. The upper aqueous phase was collected and the RNA was precipitated by addition of isopropanol on the ice for 10 min, and centrifugation at $12,000 \times g$ and 4°C for 10 min. RNA pellets were rinsed with 75% ethanol before centrifugation at $12,000 \times g$ and 4°C for 5 min, then dried, resuspended in sterile water and analyzed by agarose gel electrophoresis.

Five microlitre of total RNA, following DNase treatment, was reverse transcribed into single-stranded cDNA with a RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Corp., Lithuania) using random hexaprimers. Template cDNA were then amplified in a typical 28.5 μl RT–PCR

Table 1 The primers (a) and probes (b) for real-time fluorescent quantitative RT–PCR

Gene	Primer sequences	Length (bps)
(a)		
VEGF		
Forward	5′-GTGAATGCAGACCAAAGAAAG-3′	183
Reverse	5′-GCTTGTCACATCTGCAAGT-3′	
bFGF		
Forward	5′-CCACACGTCAAACTACAGCT-3′	120
Reverse	5′-AACACACTTAGAAGCCAGCA-3′	
GAPDH		
Forward	5′-CCTCAAGATTGTCAGCAAT-3′	141
Reverse	5′-CCATCCACAGTCTTCTGAGT-3′	
TaqMan	Probe sequences	Length (bps)
(b)		
VEGF	5′-CTCGCCTTGCAACGCGAGTC-3′	183
bFGF	5′-CCGGTTCGCACACACTCCC-3′	120
GAPDH	5′-FAM-ACCACAGTCCATGCC ATCAC-TAMRA-3′	141

reaction containing 10× buffer (Mg^{2+} free), 25 mM $MgCl_2$, 25 mM dNTP, 10 μM of the respective primers, 10 μM probe, 5 U/μl Taq polymerase and ddH_2O.

Amplifications were carried out in real-time quantitative fluorescence PCR instrument (FTC2000, FUNGLYN Corp., Canada) under the following conditions: denaturation for 2 min at 94°C, followed by 20 s denaturation cycles, 20 s renaturation at 54/52°C and 30 s elongation at 60°C. Primers and TaqMan probes were designed using Primer Express™ 2.0 (Applied Biosystems) based on the sequences from the Genbank database (Table 1), and checked by Shanghai biotechnology company (China).

2.8 Measurement of VEGF and bFGF secretion

The VEGF and bFGF protein levels in the medium were determined using a double ligand enzyme-linked immunosorbent assay (ELISA). The cell-scaffolds were prepared as detailed above ($n = 3$). Supernatant liquid was collected for ELISA assay after centrifugation. ELISA assay was run according to the manufacturer's instructions (R&D Corp.). A standard curve was plotted to determine the VEGF and bFGF concentration. The values were expressed as pg/ml.

2.9 Statistical analysis

Statistical analysis was performed with SPSS13.0. Experimental data was presented as means ± standard deviation (SD). Results were analyzed by one-way ANOVA with a student's *t*-test. A p value of ≤ 0.05 was considered to be statistically significant.

3 Results

3.1 Characterization of SCPP

Various SCPP were prepared by changing the molar proportion of strontium carbonate and calcium carbonate. Figure 1 shows the XRD analysis of CPP and SCPP containing various doses of strontium (1%, 2%, 5%, 8% and 10%). The obtained CPP in this article was validated as β-CPP [$Ca(PO_3)_2$, JCPDS #77-1953] from the XRD pattern. As shown in Fig. 1, the characteristic peaks in various SCPP curves were in agreement with the curves of CPP, especially three high characteristic peaks in each SCPP curve all appeared between 20 and 30°, which indicated that the incorporation of Sr into β-CPP did not change the crystal phase of β-CPP. SEM micrographs of the CPP (Fig. 2a, b) and 8%SCPP (Fig 2c, d) scaffolds show that porous structures are obtained with three-dimensional interconnected porosity and open macropores ranging from 200 to 400 μm. Meanwhile the porosity of all the scaffolds used in this experiment was also measured, and it was about 65%. However, there was a major difference between CPP and 8% SCPP scaffolds about the surface topography (Fig. 2). Compared to CPP, 8% SCPP possessed a smoother surface and a more compact bulk. We also proved that other SCPP scaffolds (1%, 2%, 5% and 10%) possessed the same porous structures, the smooth surface and compact bulk as to 8% SCPP (data not shown).

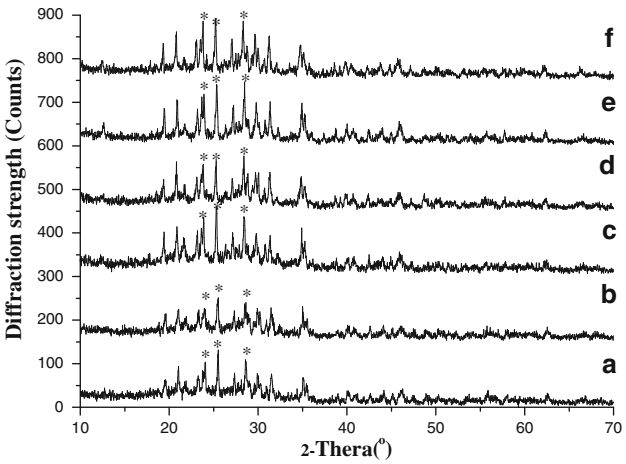

Fig. 1 XRD analysis: (*a*) CPP, (*b*) 1% SCPP, (*c*) 2% SCPP, (*d*) 5% SCPP, (*e*) 8% SCPP, (*f*) 10% SCPP. *Asterisks* (*) mean the three high characteristic peaks and the x% SCPP means x% Ca^{2+} in SCPP was replaced by Sr^{2+}

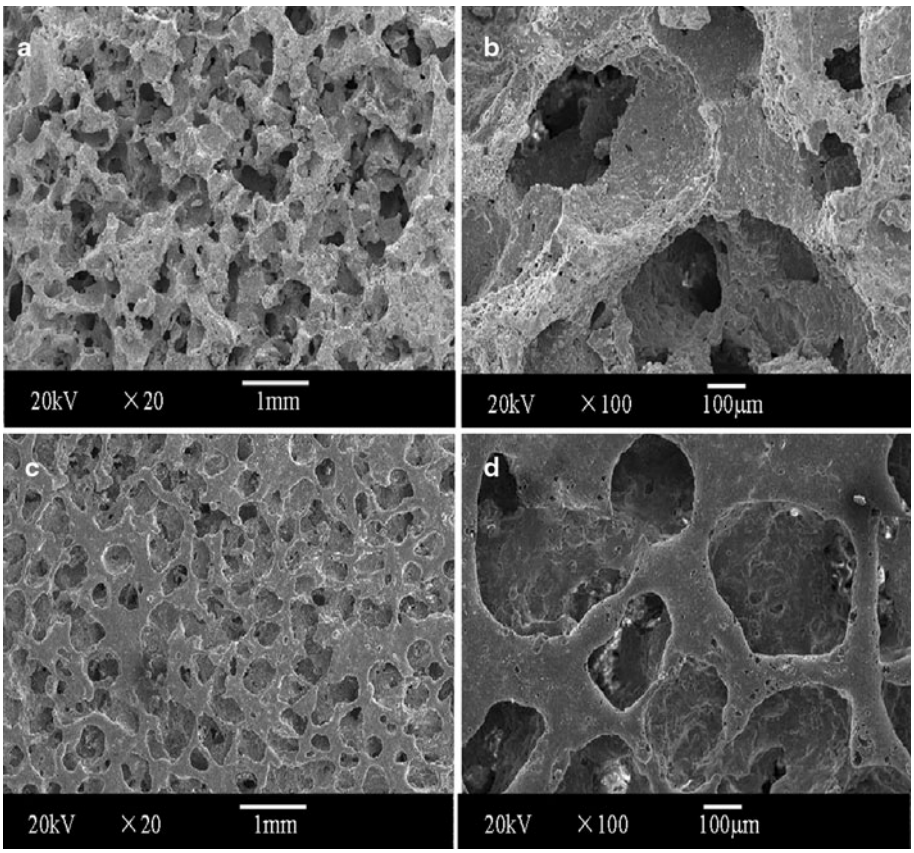

Fig. 2 SEM images of porous CPP (**a** ×20, **b** ×100) and 8% SCPP (**c** ×20, **d** ×100)

3.2 Osteoblast identification

Alkaline phosphatase (ALP) is generally considered as the character of osteoblastic cells, and the positive result of ALP staining has become an important indication to identify whether the cells possess osteoblastic property. The staining result showed that, the ALP activity department inside cytoplasm was precipitated red azo dyes and nuclei were stained aquamarine blue, the contrast was obvious and the outcome was positive (Fig. 3a).

The mineralization character in vitro could be used as another marker of osteoblasts when cells were cultured in the condition of calcification. After nodules of calcium were stained by alizarin red, color reaction occurred and Ca^{2+} formed crimson chelate complexes with alizarin red (Fig. 3b).

3.3 Effect of SCPP on proliferation of the ROS17/2.8

Proliferation of ROS17/2.8 cells on CPP and SCPP scaffolds in different periods was determined by MTT assay

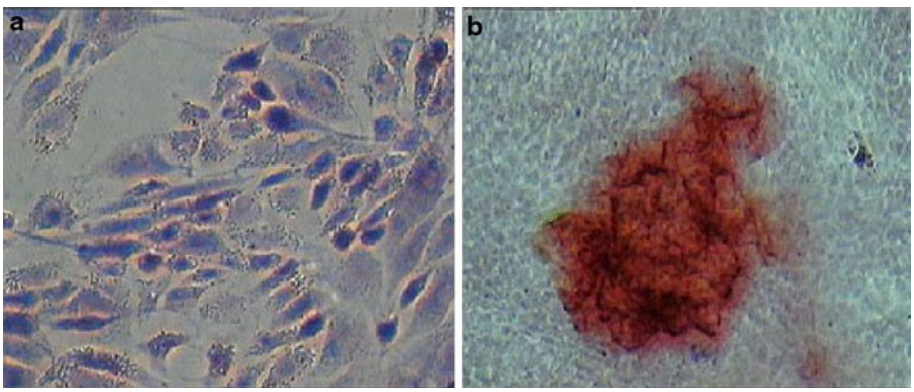

Fig. 3 ROS17/2.8 were stained by alkaline phosphatase (ALP) staining assay (**a**) and mineralized nodules staining assay (**b**)

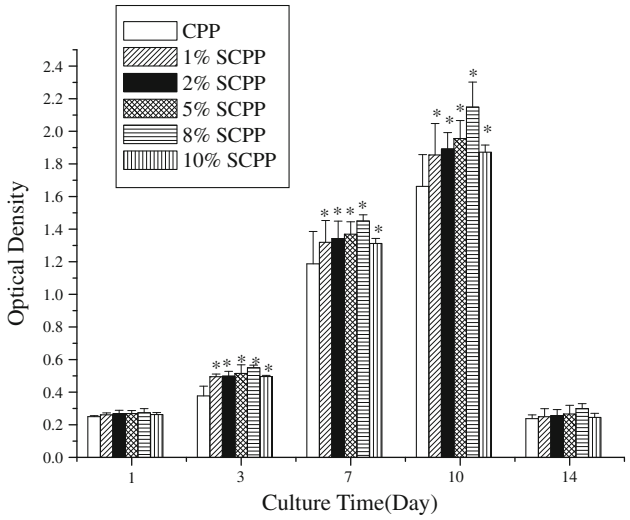

Fig. 4 Effect of CPP and various SCPP on ROS17/2.8 cells viability, the control group was without materials. (The x% SCPP means x% Ca^{2+} in SCPP was replaced by Sr^{2+}. *Asterisk* (*) means the difference attained a statistically significant increase compared to the CPP, $P < 0.05$, $n = 4$.)

(Fig. 4). Noticeably, the growth of cells on SCPP were always more superior than that on CPP over the 10 days in culture time. On the first day, there was no statistical significant difference between the ROS17/2.8 number on CPP and various SCPP. With increasing the culture duration, ROS17/2.8 cultured on all SCPP scaffolds exhibited a significantly higher proliferation rate at 3, 7, and 10 days of culture compared to those on CPP scaffold. The peaks appeared on the 10th day. Until 14th day, the number of cells on all scaffolds decreased. During the proliferation period, in encouraging osteoblast proliferation, the role of SCPP groups was more and more obvious with the increase in the doses of doped strontium; furthermore, 8% SCPP group showed the optimal effect, which suggested it was the best dose.

3.4 SEM of cell-scaffold constructs

Although SCPP could accelerate the growth of ROS17/2.8 cells, it was necessary to research whether the SCPP scaffolds could maintain the morphology of cells. SEM would be utilized to observe the morphology of cells on scaffolds. Figure 5 shows SEM-images of ROS17/2.8 cells adhered on CPP and SCPP scaffolds on the 7th day. The result was accordance with the consequence of MTT test; that is, cells adhered and grew better with their characteristic spindle-like morphology on SCPP scaffolds than those on CPP scaffold, especially the osteoblasts cultured on the

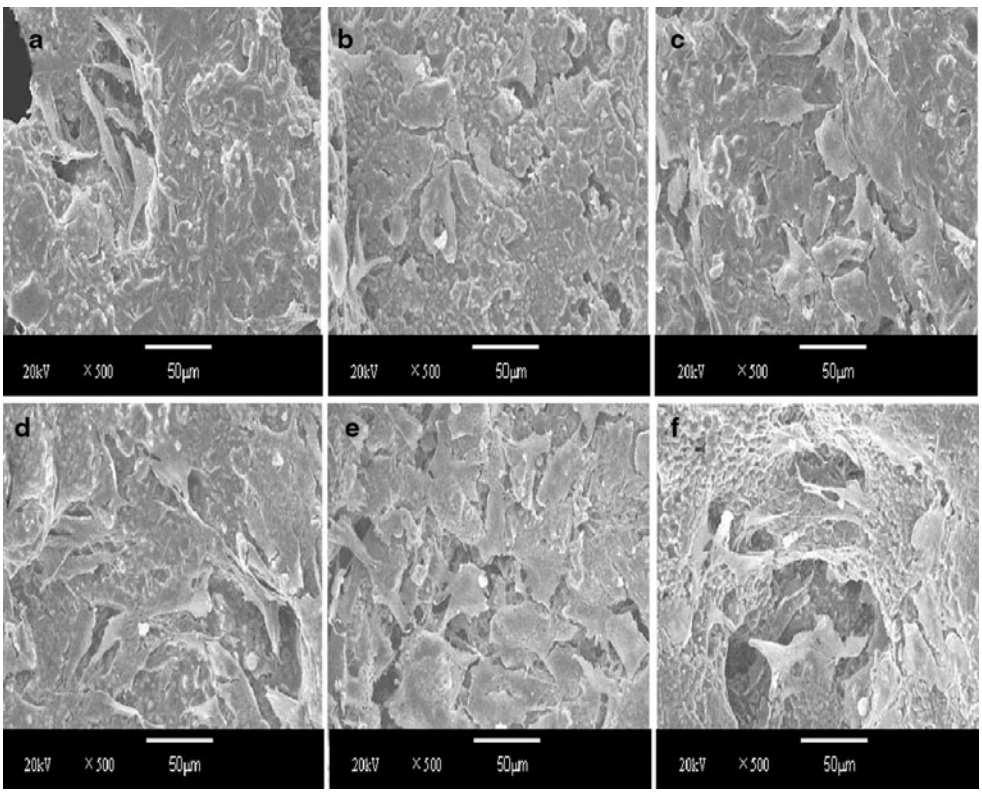

Fig. 5 SEM images of the osteoblasts grown on porous CPP and SCPP scaffolds on the 7th day. (**a** CPP, **b** 1% SCPP, **c** 2% SCPP, **d** 5% SCPP, **e** 8% SCPP, **f** 10% SCPP; ×500; the x% SCPP means x% Ca^{2+} in SCPP was replaced by Sr^{2+}.)

surface of 8% SCPP scaffold appeared to be well spread and nearly reached the continuous cell layer. Also, the result of SEM suggested that 8% SCPP exhibited the optimal role in promoting cell growth and maintaining their morphology.

3.5 Effects of SCPP on mRNA expression of VEGF and bFGF

In this paper, comparative Ct method was chosen to calculate mRNA expression, which simultaneously determined the target gene and some endogenous housekeeping gene (beta-actin), then utilized beta-actin as a reference standard to convert the relative numerical relationship of the initial number of template in each sample [29]. Its calculate formula was as follows: relative expression of the target gene (X fold of control) $= 2^{-\Delta\Delta Ct}$. As shown in Table 2, with the increasing in strontium, the mRNA expression of VEGF and bFGF appeared an upward trend before reaching the peak in 8% SCPP group, subsequently, it declined. Moreover, compared to that in the CPP group, the mRNA expression of VEGF and bFGF in the 8% SCPP group were approximately 4.01-fold and 8.01-fold increase, respectively. And all SCPP groups exhibited a significantly higher mRNA expression of VEGF and bFGF relative to control ($P < 0.05$). Moreover, as shown in the Fig. 6, we normalized relative expression of the target gene to its total cell numbers, which was expressed as $2^{-\Delta\Delta Ct}$/OD (on the 7th day), the result was similar to Table 2.

3.6 Measurement of VEGF and bFGF protein secretion

ELISA assay was broadly applied to detect little amount of cytokine due to its biological catalysis and specificity. The limits of sensitivity of the VEGF and bFGF measured by ELISA were >25 and >2 pg/ml, respectively. In SCPP groups, the amounts of VEGF were all higher than that in CPP group ($P < 0.05$) and the levels were dose-dependant increasing in response to the increasing strontium before reaching the peak; 8% SCPP group was also the optimal one (Fig. 7a). The phenomenon similar to VEGF secretion was also observed in bFGF secretion, excepting that no statistically significant differences were found between 10% SCPP group and the control group (Fig. 7b).

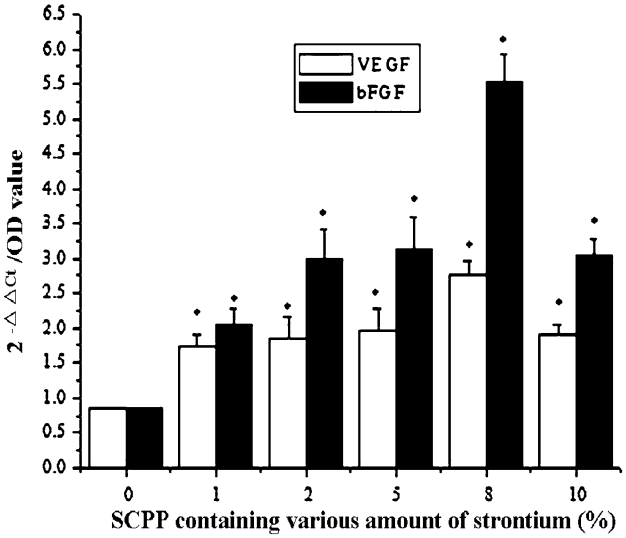

Fig. 6 mRNA expressions of VEGF and bFGF in per unit cells after 7 days of culture: $2^{-\Delta\Delta Ct}$ value/OD value. (The x% SCPP means x% Ca^{2+} in SCPP was replaced by Sr^{2+}. *Asterisk* (*) means the difference attained a statistically significant compared to CPP, $P < 0.05$, $n = 3$.)

4 Discussion

Angiogenesis or vascularization is essential not only for tissue engineered bone or orthopedic implant but also for the regulation of bone remodeling and fracture repair in the

Table 2 mRNA expressions of VEGF and bFGF in osteoblasts cultured with scaffolds after 7 days of culture: Ct values mean the values of the fluorescence signal strength detected by real-time quantitative PCR instrument; $2^{-\Delta\Delta Ct}$ values mean the varying multiples of the gene expression in experimental group compared with that in the control group

	CPP	1% SCPP	2% SCPP	5% SCPP	8% SCPP	10%SCPP
Ct						
VEGF	20.7 ± 0.1	21.9 ± 0.1	27.5 ± 0.2	21.6 ± 0.2	22.5 ± 0.1	22.0 ± 0.1
bFGF	27.8 ± 0.3	28.8 ± 0.2	33.8 ± 0.2	28.0 ± 0.2	28.6 ± 0.1	28.2 ± 0.1
GAPDH	14.7 ± 0.1	17.1 ± 0.2	22.7 ± 0.5	17.0 ± 0.1	18.5 ± 0.5	17.1 ± 0.3
$2^{-\Delta\Delta Ct}$						
VEGF	1	2.3 ± 0.2*▲	2.5 ± 0.4*▲	2.7 ± 0.4*▲	4.0 ± 0.3*	2.5 ± 0.2*▲
bFGF	1	2.7 ± 0.3*▲	4.0 ± 0.6*▲	4.3 ± 0.6*▲	8.0 ± 0.6*	4.0 ± 0.3*▲

The x% SCPP means x% Ca^{2+} in SCPP was replaced by Sr^{2+}

Asterisk (*) means the difference attained a statistically significant compared to CPP

Triangular form (▲) means the difference attained a statistically significant compared to 8% SCPP

$P < 0.05$, $n = 3$

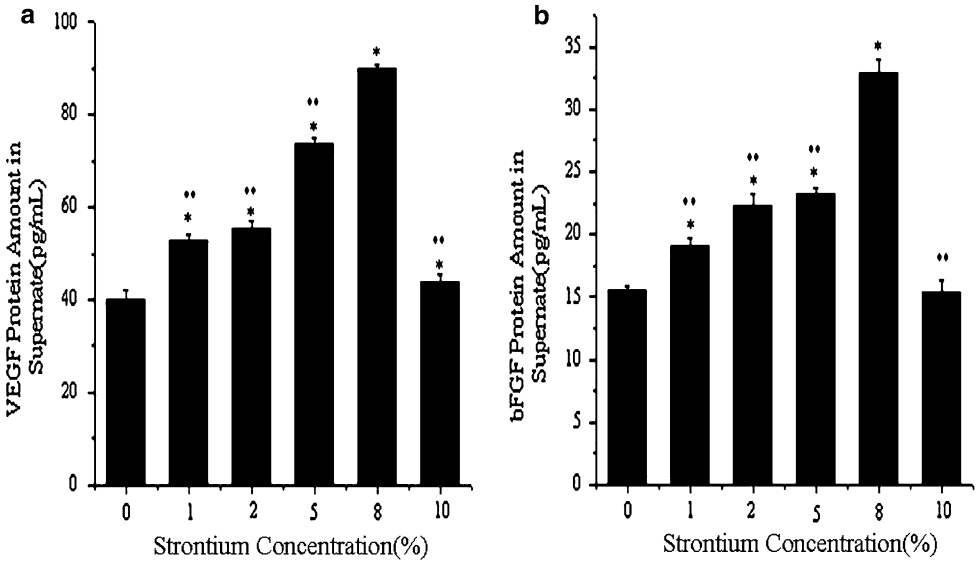

Fig. 7 Effect of CPP and various SCPP on VEGF (**a**) and bFGF (**b**) protein production from osteoblasts cultured with scaffolds. (The x% strontium means x% Ca^{2+} in SCPP was replaced by Sr^{2+}. The culture time was 7 days. *One asterisk* (*) means the difference attained a statistically significant compared to CPP; *double asterisks* (**) means the difference attained a statistically significant compared to 8% SCPP; $P < 0.05$, $n = 3$.)

long term. In this paper, we researched the role of SCPP in inducing angiogenesis via measuring the release of VEGF and bFGF from ROS17/2.8 cells cultured with SCPP scaffolds, which is a relatively new field with only few efforts to investigate the ability of SCPP to influence the behavior of osteoblastic cells. Meanwhile, CPP was chosen as the controlling material due to its similar chemical elements to bones, the good biocompatibility and degradability in biological environments.

In the present study, the results of XRD (Fig. 1) and SEM (Fig. 2) demonstrated that the crystal system of the prepared materials were monoclinic and the developed CPP and SCPP scaffolds possessed interconnected porous network, large pore size (200–400 μm) and an overall porosity of 65%. These properties are essential for enhancing bone ingrowth into scaffolds and all developed materials could be used in subsequent experiments. The identified experiments proved that ROS17/2.8 possessed the characters of osteoblasts (Fig. 3); these were in line with expectations and the cells could be also used in subsequent experiments.

From the MTT assay, no cytotoxic responses were observed when ROS17/2.8 cells cultured with SCPP scaffolds, which indicated the good biocompatibility of SCPP. The reasons of cell proliferation rate dropped overall on the 14th day were due to the contact inhibition or density inhibition which resulted from the limitation of cellular growth space. In addition, with increasing the culture duration, the accumulation of metabolic waste in the culture medium is increasing because a large numbers of cells consume nutrition, and then the medium will be rapidly acidulated even if it is changed frequently, which result in the death of the cells. The results of MTT and SEM assays all illuminated that SCPP containing low-dose of strontium was beneficial to the growth of osteoblasts, and 8% SCPP was the best one. The reasons for this may be related to two reasons as follows. Firstly, the incorporation of strontium into CPP changed the surface topography of SCPP and made SCPP possessed a smoother surface as well as a more compact bulk, which resulted in better adherence and growth of ROS17/2.8 cells. Secondly, some studies had shown that strontium could promote osteoblast proliferation through activating calcium-sensing receptor (CaSR) and inositol trisphosphate (IP3) as well as mitogen-activated protein kinase (MAPK) [30, 31]. 1–10%SCPP released strontium into the medium at concentrations <5 μg/ml (which is harmless to nodule formation and bone mineralization in vitro) [32] for 14 days (data not shown). The released strontium can diffuse into the osteoblasts cultured with SCPP scaffolds, and stimulate cells proliferation via the manner mentioned above.

Table 2 and Fig. 6 respectively revealed the effects of CPP and 1–10%SCPP on mRNA expression and protein secretion of VEGF and bFGF released from osteoblasts cultured with scaffolds for 7 days. The reasons for detecting VEGF and bFGF at 7 days of culture time are that at this point of time, the osteoblasts seeded on scaffolds were just confluent and lying in differentiation period. In these experiments, the aim of adding $1,25(OH)_2VD_3$ was to stimulate osteoblasts differentiation [33], so that the expression of VEGF and bFGF in osteoblasts and medium could be amplified in the same benchmark and measured by RT–PCR and ELISA assays. The mRNA expression of VEGF and bFGF in osteoblasts were enhanced in SCPP groups versus in the control (CPP group). Specifically, to VEGF mRNA expression, all SCPP groups existed

significant difference with the control ($P < 0.05$) and 8% SCPP group was the best one. On the other hand, with reference to bFGF mRNA expression, all groups existed marked increasing compared to the control group ($P < 0.05$), 8% SCPP also showed the most apparent promoting effect. Meanwhile, compared to the control, strontium doped CPP groups all stimulated protein secretion of VEGF as well as bFGF from cultured osteoblasts and all these possessed the significant differences ($P < 0.05$) excepting bFGF in 10% SCPP group. Whether the protein secretion or mRNA expression of VEGF and bFGF, the trends were dose-dependent increasing earlier and then falling in response to the increasing strontium, and 8% SCPP was always the optimal one. However, the results between ELISA and RT–PCR presented some discrepancies, the reason was possible due to the loss of growth factors' activity in a certain period of time after secretion for the short half-life of growth factors, and then they failed to be detected by ELISA assay. Despite this, both the results of ELISA and RT–PCR indicated that the stimulating effects of our materials on the expression of VEGF and bFGF were consentaneous; especially the stimulating effect of 8% SCPP was the optimal. Furthermore, our laboratory also proved that SCPP containing low doses of strontium could promote the expression of VEGF and bFGF in endothelial cells, and 8% SCPP showed the optimal effect (data not shown). To further demonstrate the potential impact of SCPP scaffolds for tissue engineering with view to a better angiogenesis, the tests of the angiogenic potential of SCPP scaffolds in osteoblast-endothelial cell co-culture systems will be carried out in our follow-up work.

The mechanism by which SCPP causes up-regulated expression of VEGF and bFGF remains uncertain and warrants further investigation. We inferred that these results occurred because of the following aspects.

First, SCPP could promote osteoblasts proliferation versus CPP [28, 30, 31], then there were more cells to secrete the cytokines on SCPP scaffolds than that on CPP scaffold; therefore, the amounts of VEGF and bFGF in culture medium could be enhanced in SCPP groups. Second, surface chemistry, structure and porosity of biomaterials are known to affect cell attachment, growth and cell-specific functions such as the expression of cytokine and growth factor production [34, 35]; the incorporation of strontium into CPP changed the surface topography and made SCPP possessed a smoother surface as well as a more compact bulk, which might up-regulate the expression of VEGF and bFGF. However, we could conclude from Fig. 5 that SCPP prompted the expression of VEGF and bFGF in this experiment not only through increasing in number of osteoblasts but also by other ways. In this context, third, some scholars confirmed that strontium could promote osteogenic differentiation [36], while VEGF and bFGF were highly expressed by osteoblasts in their differentiation period [37, 38]; therefore, accelerating the osteogenic differentiation might be another way to regulate cytokines expression by strontium. In addition to these, strontium is known as "bone seeker", and 98% of the total body Sr content can be found in the skeleton. Doping strontium into CPP could make material more biomimetic; therefore, culturing cells with materials could create a micro-environment that was similar to the circumstance of normal bone, which not only promoted cell proliferation, but also increased the expression of cytokines. The result shown in Fig. 5 validated the reasons mentioned above to a certain extent.

As regards why did 8% SCPP show the optimal effect, we inferred that it might be because there was an optimum dose of strontium about the effect of strontium on cell proliferation and the expression of factors in vitro; while the Sr^{2+} release of 8% SCPP was just to close to the dose.

In this experiment, the porosity and connectivity of scaffolds as well as the culture conditions were kept at the same condition under strict control to avoid the action of other influence factors such as hypoxia which affect the secretion of VEGF and bFGF from cultured cells. Thus, we could ensure that the factors affecting the ability of these scaffolds to stimulate the secretion of VEGF and bFGF are focused on the formulations and surface topography of scaffolds. However, further studies on induction of stimulation for hypoxia-inducible factor are mandatory because VEGF gene up-regulation is triggered by hypoxia-inducible factor.

5 Conclusion

Our findings demonstrated that SCPP containing low-dose of strontium could promote the expression of VEGF and bFGF, including the expressions of mRNA in osteoblasts cultured with SCPP scaffolds and the amount of protein secretion in the culture medium. The rising trend was dose-dependent with the increasing of strontium during the cell proliferation period, and 8% was the optimal proportion. These findings strongly suggested that SCPP containing proper dose of strontium could be used as a potential material with stimulating angiogenesis, which provided a novel thought for resolving the problem of angiogenesis in bone tissue engineering.

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