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Enhanced osteoporotic bone regeneration by strontium-substituted calcium silicate bioactive ceramics

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ABSTRACT

The regeneration capacity of the osteoporotic bones is generally lower than that of the normal bones. Current methods of bone defect treatment for osteoporosis are not always satisfactory. Recent studies have shown that the silicate based biomaterials can stimulate osteogenesis and angiogenesis due to the silicon (Si) ions released from the materials, and enhance bone regeneration *in vivo*. Other studies showed that strontium (Sr) plays a distinct role on inhibiting bone resorption. Based on the hypothesis that the combination of Si and Sr may have synergetic effects on osteoporotic bone regeneration, the porous Sr-substituted calcium silicate (SrCS) ceramic scaffolds combining the functions of Sr and Si elements were developed with the goals to promote osteoporotic bone defect repair. The effects of the ionic extract from SrCS on osteogenic differentiation of bone marrow mesenchymal stem cells derived from ovariectomized rats (rBMSCs-OVX), angiogenic differentiation of human umbilical vein endothelial cells (HUVECs) were investigated. The *in vitro* results showed that Sr and Si ions released from SrCS enhanced cell viability, alkaline phosphatase (ALP) activity, and mRNA expression levels of osteoblast-related genes of rBMSCs-OVX and expression of vascular endothelial growth factor (VEGF) without addition of extra osteogenic and angiogenic reagents. The activation in extracellular signal-related kinases (ERK) and p38 signaling pathways were observed in rBMSCs-OVX cultured in the extract of SrCS, and these effects could be blocked by ERK inhibitor PD98059, and P38 inhibitor SB203580, respectively. Furthermore, the ionic extract of SrCS stimulated HUVECs proliferation, differentiation and angiogenesis process. The *in vivo* experiments revealed that SrCS dramatically stimulated bone regeneration and angiogenesis in a critical sized OVX calvarial defect model, and the enhanced bone regeneration might be attributed to the modulation of osteogenic differentiation of endogenous mesenchymal stem cells (MSCs) and the inhibition of osteoclastogenesis, accompanying with the promotion of the angiogenic activity of endothelial cells (ECs).

1. Introduction

Osteoporosis has become one of the most universal and complex skeletal disorders for postmenopausal women, the elderly and those associated with other medical conditions or as the result of certain therapeutic interventions [1]. Osteoporosis is characterized by low bone mass and microarchitectural deterioration of the bones due to the more rapid bone resorption process than bone formation, which usually leads to bone fragility and increased risks of fractures [2,3]. Moreover, the bone defect frequently occurs in the proximal femur or distal radius after fracture [4,5], metastasis bone tumor resection, and arthroplasty revision of the knee and hip in osteoporosis patients [4]. So far, most currently available strategies used in preventing and treating osteoporosis involve agents which inhibit osteoclast activity hence bone resorption [1], and four major anti-resorptive agents including estrogen, selective estrogen

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receptor modulators (SERMs), bisphosphonates and calcitonin are widely used in clinic [1]. However, these agents cannot promote bone formation and are helpless for osteoporotic bone regeneration [6]. Recently, the inductive factors or osteoprogenitor cells were added into the grafts to improve osteogenesis in osteoporosis [1,2,4,7,8]. However, the combination of materials and growth factors or cells is usually complicated, costly and might not be suitable for mass production. Furthermore, most of the existing artificial bone grafts are largely considered to be lack of enough bioactivity to induce the differentiation of endogenous mesenchymal stem cells (MSCs) in osteoporosis patients towards osteoblasts. In addition, the inhibition of the osteoclastogenesis, and the stimulation of the new bone formation induced by the grafts play critical roles in osteoporotic bone regeneration [4,9,10]. Therefore, more effective biomaterials for regeneration of osteoporotic bone are urgently needed.

It is realized that the modulation of the *in vivo* differentiation of endogenous MSCs toward osteoblasts and inhibition of osteoclastogenesis, accompanying with the promotion of the angiogenic activity of endothelial cells (ECs) are essential for osteoporotic bone regeneration [9,11,12]. Recently, the calcium silicate ($CaSiO_3$, CS) ceramic has attracted great attentions as a potential bioactive bone graft material since it showed excellent bioactivity, osteostimulatory and biodegradable properties [13–15]. Our new investigations demonstrated that the bioactive Si ions released from CS could provide a preferential extracellular environment for directing osteogenic differentiation of BMSCs, and enhance human umbilical vein endothelial cells (HUVECs) proliferation and angiogenesis even in the absence of extra osteogenic and angiogenic reagents. *In vivo* study also showed that CS significantly promoted early bone formation comparing with the traditional calcium phosphate bioceramics [13,14]. However, it is not clear if CS can stimulate osteogenic differentiation of BMSCs derived from osteoporotic tissues, as it is known that these cells have less activity in bone regeneration [9,16].

It is well known that the strontium (Sr), as a trace element in the human body, plays a dual role in bone metabolism by stimulating bone formation and inhibiting bone resorption [17–20]. The mechanism is thought to lie in Sr ions having the ability not only to increase osteoblast-related gene expression and the alkaline phosphatase (ALP) activity of MSCs but also to inhibit the differentiation of osteoclasts by inhibiting expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in MSCs [21–23]. In addition, the Sr ions can stimulate the expression of osteoprotegerin (OPG), which blocks the interaction of RANK with its ligand, RANKL, and inhibits the differentiation and activity of osteoclasts [24,25]. Furthermore, the *in vivo* studies showed that the osteointegration could be improved through partial substitution of Ca by Sr in apatite-based ceramics and cements [26–28]. However, the low degradability of apatites limited their wider applications as bone fillers, and calcium phosphate based bioceramics are generally thought to be lack of stimulative activity for osteogenic differentiation of MSCs [29]. Moreover, our new studies showed that the Sr ions might also stimulate expression of the angiogenic factor VEGF *in vitro* [30]. In addition, the studies have shown that the Sr-substitution can apparently increase the solubility of the apatite materials [31,32].

Therefore, we hypothesize that the combination of Si and Sr in bioceramics may enhance osteoporotic bone regeneration by synergistic effects to stimulate the osteogenic differentiation of mesenchymal stem cells (MSCs) and angiogenesis of umbilical vein endothelial cells (UVECs) on one side, and to inhibit osteoclastogenesis on the other side. The ovariectomized rat (OVX rat) model is ratified by the Food and Drug Administration (FDA) as the primary model system to evaluate the prevention and treatment of postmenopausal osteoporosis [33,34]. In addition, the OVX rat exhibits most of the characteristics of human post-menopausal osteoporosis. With the fast generation time, rats are often a starting point for preclinical evaluation model used for the prevention and treatment of osteoporosis, followed by verification in large animal models, before undertaking clinical trials in human patients. Moreover, comparing with other animal models, the OVX rat model also possesses other advantages including low-cost, easy to house and to be recognized by general public in clinical research field [34]. In this study, the Sr-substituted calcium silicate (Sr–$CaSiO_3$, SrCS) ceramic scaffolds were fabricated, and the stimulative effects and related mechanisms of SrCS on the growth and differentiation of bone mesenchymal stem cells derived from OVX rats (rBMSCs-OVX), as well as the effects on angiogenic differentiation of human umbilical vein endothelial cells (HUVECs) were evaluated. Finally, the OVX rat calvarial defect model was used to investigate the regulatory effect of SrCS on the *in vivo* bone formation ability.

2. Materials and methods

2.1. Fabrication and characterization of macroporous bioceramic scaffolds

The calcium silicate (CS) powders and strontium-substituted CS powders (SrCS) with 10 mol% of Ca replaced by Sr were synthesized by chemical precipitation method as previously described [21]. The analytical grade reagents with purity ≥99.0% (Shanghai Chemical Co., Ltd., China) without further purification were used in this study. The $Ca(NO_3)_2$ solution or the mixed solution of $Ca(NO_3)_2$ and $Sr(NO_3)_2$ was firstly prepared by the expected substitution degree, and then dropwisely added into Na_2SiO_3 solution at room temperature under rigorously stirring with (Sr + Ca)/Si molar ratio of 1.0. After complete addition, the white precipitates were further stirred for 24 h, and then the precipitates were filtered and washed by deionized water and anhydrous ethanol three times, respectively, then dried at 60 °C for 24 h, and finally calcined at 800 °C for 2 h to obtain CS and SrCS powders.

The obtained CS and SrCS powders were sieved to obtain the powders with particulate size less than <200 mesh, and then mechanically mixed respectively with 300–450 μm polyethylene glycol (PEG) particulates at a weight ratio of 40:60. The mixture was dry pressed at a uniaxial pressure of 6 MPa in a stainless steel die to obtain green disks with a diameter of 6 mm and a thickness of 4 mm. Then the green disks were heat treated at 400 °C for 2 h at a firing rate of 2 °C/min to drive off the porogens, and then sintered at 1090 °C for 2 h. After sintering, the samples were cooled to room temperature in the furnace [13].

The phase of samples was characterized by X-ray diffraction (XRD, Geigerflex, Rigaku Co., Japan) with mono-chromated CuKα radiation. The open porosity of the fabricated CS and SrCS ceramic scaffolds was determined by the Archimedian method using distilled water as the determination medium [35]. The cross-section of the porous structures was observed by scanning electron microscopy (SEM: JSM-6700F, JEOL, Japan). The Ca and Sr content of the fabricated SrCS scaffold was quantified by X-ray fluorescence spectroscopy (XRF: PW-2404, Philips, Netherlands). Five samples from each group were tested to obtain an average value of the porosity.

2.2. Preparation of ceramics extracts

The extracts of macroporous CS and SrCS ceramic scaffolds were prepared according to the International Organization for Standardization (ISO 10993-12). Briefly, a stock solution of 200 mg/mL was first prepared by adding the granules with size of 300–450 μm grinded from each ceramic scaffold into DMEM culture medium. After incubation at 37 °C for 24 h, then the mixture was centrifuged and the supernatant was collected. Subsequently, the extract was sterilized by filtration through 0.2 μm filter membranes and stocked for further experiments. To investigate the effect of the proper ion concentration on rBMSCs-OVX, a serial diluted extracts with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 concentrations were prepared by diluting the stock solution with serum-free DMEM. The concentrations of Ca, Sr and Si in the extracts were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES: 710-ES, Varian, USA).

2.3. Isolation and culture of rBMSCs-OVX

All experimental protocols concerning animals were approved by the Animal Care and Experiment Committee of the 9th People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Briefly, six-month-old female Fisher 344 were raised in individual cages in an animal room that was maintained at 22 °C with a 12 h light and 12 h dark cycle and given an ovariectomy through two dorsal incisions [36]. After culture for three months, the OVX rats were sacrificed by an overdose of pentobarbital sodium. Bilateral femurs were harvested under aseptic conditions and all soft tissues were removed. Metaphyses from both ends of femur were cut off and the marrow was flushed out with 10 mL Dulbecco's modified Eagle's