Bioactive strontium ions/ginsenoside Rg1–incorporated biodegradable silk fibroin-gelatin scaffold promoted challenging osteoporotic bone regeneration

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ABSTRACT

Autogenous healing of osteoporotic fractures is challenging, as the regenerative capacity of bone tissues is impaired by estrogen reduction and existing pro-inflammatory cytokines. In this study, a biofunctional ginsenoside Rg1 and strontium-containing mineral (SrHPO4, SrP)-incorporated biodegradable silk fibroin-gelatin (SG) scaffold (Rg1/SrP/SG) was developed to stimulate the osteoporotic bone repair. The incorporation of 15 wt% SrP significantly enhanced the mechanical strength, stimulated the osteogenic differentiation of mouse bone marrow mesenchymal stem cells, and suppressed the osteoclastogenesis of RAW264.7 in a concentration-related manner. The loading of Rg1 in SG and 15SrP/SG scaffolds obviously promoted the angiogenesis of human umbilical vein endothelial cells via activating the expression of vascular endothelial growth factor and basic fibroblast growth factor genes and proteins. The bioactive strontium ions (Sr2+) and Rg1 released from the scaffolds together mediated lipopolysaccharide-treated macrophages polarizing into M2 type. They downregulated the expression of inflammatory-related genes (interleukin (IL)-1β, tumor necrosis factor α, and IL-6) and stimulated the expression of genes related to anti-inflammation (Arginase and IL-10) as well as bone repair (BMP-2 and PDGF-BB) in the macrophages. The in vivo results also displayed that SrP and Rg1 significantly promoted the bone repair effect of SG scaffolds in osteoporotic critical-sized calvarial defects. Besides, the degradation rate of the scaffolds was close to the bone regeneration rate. Therefore, the simultaneous addition of SrP and Rg1 is a promising way for facilitating the osteoporotic bone repair activity of SG scaffolds via promoting the osteogenesis and angiogenesis, as well as inhibiting the osteoclastogenesis and inflammation.

1. Introduction

Along the going of age population, diseases related to musculoskeletal system were increased every year [1,2]. Among them, osteoporosis mainly happened in elderly people and postmenopausal women [3,4], increasing the risk of bone fractures and inducing mortality after fractures. It results from a disruption of the fine balance between bone formation and resorption, which is induced by estrogen reduction and
inflammatory factors increase [5,6]. Besides, the formation ability of blood vessels is impaired in osteoporotic bones [7]. These make the repairing of osteoporotic fractures challenging. In clinical, autogenous and allogeneic bones displayed good bone repairing effect [8,9]. But the donor of autogenous bone is limited, and there are risks of immunological rejection and infection using allogeneic bone. Therefore, developing a biomaterial for activating the repair potential of osteoporotic bone fractures becomes a popular trend in bone tissue engineering, as it can be made in quantity.

Silk fibroin (SF) and gelatin (GN) are two degradable natural protein-related biomaterials with good compatibility and widely used in bone tissue engineering [10–12]. The mechanical strength and degradation of them can be adjusted by altering their ratio [13]. But they lack the abilities of good osteoconductivity, angiogenesis, suppressed osteoclast activity, and inflammation required for stimulating osteoporotic bone repair [14–16]. For stimulating osteogenesis and inhibiting osteoclastogenensis, Sr2+, one of the trace elements, is widely applied [15–18]. Sr formulations have been used as the treatment for postmenopausal osteoporosis [19,20]. Our previous study showed that strontium hydrogen phosphate (SrP) clusters showed good osteogenesis when compared with calcium phosphate powders and could be used as a drug carrier [21]. Another study proved that SrP coating in Mg alloys could release Sr2+ to enhance the osteogenic differentiation of MC3T3-E1 cells via activating TLR4/PI3K/Akt signaling pathway [22]. Besides, Sr2+ doping could inhibit the inflammation [17], which would endow bone repair biomaterials with more promising functions.

Promoting the angiogenesis is also vital for the repair of bone fractures [23]. Scaffolds with interconnected pores and larger pores are beneficial for the angiogenesis. Porous SF-GN (SG) scaffolds can be manufactured by vacuum freeze drying to obtain such pores [24]. Recently, loading active ingredients of traditional Chinese medicines in porous scaffolds are widely used to promote the formation of new blood vessels [25–27]. Ginsenoside Rg1, derived from one of these medicines-ginseng, can promote the proliferation of endothelial cells [26], prevent senescence of endothelial progenitor cells [28], and stimulate the secretion of vascular endothelial growth factor (VEGF) [29]. Rg1-containing medicines were used in clinical trials of vascular-related diseases and displayed a protective role in the function of vascular endothelial cells [30,31]. Rg1 has also been proved to own multiple functions, such as immune regulation, anti-inflammatory, anti-aging, and anti-free radical effects [32–34]. Moreover, Rg1 is much cheaper than growth factors, such as VEGF. To summarize, Rg1 is promising for osteoporotic bone repair. But rare studies incorporated Rg1 in scaffolds for in vivo repair of osteoporotic bone defects.

Considering the multiple advantages of SrP, Rg1, and SG, Rg1/SrP/SF-based organic-inorganic biocomposite scaffolds for osteoporotic bone repairing are developed in this study. The influence of SrP and Rg1 on the physicochemical properties of SG has been investigated. The repair effect of Rg1/SrP/SG scaffolds has been verified by in vitro cell study and in vivo critical-sized calvarial defect model in osteoporotic rats.

2. Materials and methods

2.1. Material preparation

2.1.1. Extraction of SF

SF was obtained from silkworm cocoons (Sericultural Agri-Food Research Institute, China) by degumming. Briefly, 10 g of sodium carbonate (Aladdin, China) was dissolved into 2 L water and heated to boil; then 40 g cocoon silk was added and boiled for 30 min while stirred; finally, the silk fiber was washed by water for six times. The degumming step was repeated for three times, and obtained silk fiber was dried at 50°C for 24 h. Silk fiber was dissolved in lithium bromide solution (9.3 M; Aladdin, China) at 45°C for 2 h, dialyzed against water for 3 days in a dialysis bag (7 kDa; Yuanye, China) and concentrated in 10% polyethylene glycol solution (Aladdin) for 12 h at 25°C. The concentration of SF solution was determined by weighing a dish with 1 mL dialyzed solution before and after drying, then adjusted to 6 wt% and stored in a freezer.

2.1.2. Preparation of SrP clusters

SrP clusters were prepared by the chemical precipitation method as a previous study showed [21]. Briefly, 20 mM Sr(CH3COO)2 (Tianjin Keimiou Regent, China) solution was dropped into the solution containing 15 mM NH4H2PO4 (Guangzhou Regent, China) and 50 mM CO(NH2)2 (Guangzhou Regent, China). The mixed solution was stirred at 90°C and reacted for 2 h. The resulted precipitates were washed with water for five times and dried at 50°C for 24 h.

2.1.3. Preparation of porous composite scaffolds

As Fig. 1 showed, the SrP clusters were first dispersed in 1 mL of solution containing 3 wt% SF and 3 wt% GN (Yuanye) by ultrasound. The mixed SrP/SF/GN suspension was added into polystyrene cylindrical molds and placed in a −20°C fridge (Haier, China) for 12 h. The frozen samples were dehydrogenized by vacuum freeze drying instrument (Boyikang, China). Then the obtained porous samples were cross-linked by immersing in alcohol (Aladdin) for 12 h and following in 0.5% GN solution for 12 h at 25°C. The samples were washed and lyophilized again. SG scaffolds without SrP and Rg1 (Yuanye) were regarded as the blank group. SG scaffolds with the SrP/SG percentage of 5, 10, and 15 wt% were labeled as 5SrP/SG, 10SrP/SG, and 15SrP/SG scaffolds, respectively. Then 80 μg Ginsenoside Rg1 in 30% alcohol/phosphate-buffered saline (PBS) was dropped into a cross-linked dry sample and placed at 4°C for 8 h. The resulted samples were lyophilized and named as Rg1/SG or Rg1/SrP/SG scaffolds.

2.2. Physicochemical properties of the scaffolds

2.2.1. Composition analysis

Phase compositions of SrP clusters and the scaffolds were determined by X-ray diffractrometer (XRD; Bruker, GE). Data (2θ) were collected under CuKα radiation with the step size of 0.02°. The phase compositions of scaffolds and powders were analyzed by JCPDS card index. Fourier transform infrared spectroscopy (FTIR; Thermo Fisher, GER) was applied to record the structures of the prepared samples with wavenumber among 4,000–500 per cm.

2.2.2. Scanning electron microscopy observation and porosity assessment

Scanning electron microscopy (SEM; Zeiss, GER) was used to observe the morphology of SrP clusters and the scaffolds. Cross-sections of the scaffolds were spray coated with platinum for this test. Energy-disperse spectroscopy (EDS; Bruker, GER) was used to detect the element composition of SrP clusters under 20 kV energy. Pore size distribution was measured with three random images captured by SEM.

The porosity of SrP/SG groups was measured by soaking a dry scaffold (weighted as W0) in ethanol at room temperature. The scaffolds height (H) and diameter (D) were measured, and the volume (V) was calculated by Equation 1.

\[ V = \pi \times (D/2)^2 \times H \]  

(1)

The bubbles in the scaffold were removed by vacuuming for 30 min. The surface of the wet scaffold was wiped by a filter paper soaked with ethanol and then the scaffold was weighed as Ws. The porosity was calculated by the following equation:

\[ \text{Porosity} = 100\% \left( \frac{W_s - W_0}{W_0} \right) \times \rho_e \times V \]  

(2)

The label ρe is the density of ethanol at 25°C.

2.2.3. Mechanical properties

Mechanical properties of SG scaffolds with different content of SrP were measured by a dynamic mechanics testing machine (Bose, USA).
The pressing speed was 1 mm/min, and pressing distance was 3 mm. The compressive stress and modulus of the samples at 20% strain were calculated. Four samples in each group were tested.

2.2.4. Swelling behaviors

The swelling behaviors of SG and SrP/SG scaffolds (Φ5*8 mm) were tested by immersing the samples in 10 mL PBS (Gibco, USA). The samples were placed in a shaker at 37°C and 60 rpm for 1, 4, 12, and 24 h. The samples before/after immersing were weighted as W₀ and W₁, respectively. The volume of the samples before/after immersing was measured and labeled as V₀ and V₁. The absorption ratio and swelling ratio at each time point were calculated using the following equations:

Absorption ratio = $\frac{100\times(W_1 - W_0)}{W_0}$

Swelling ratio = $\frac{100\times(V_1 - V_0)}{V_0}$

2.2.5. Degradation behaviors

SrP/SG scaffolds (Φ5*8 mm) were immersed in 25 mL of PBS for 7, 14, 28, 42, and 56 days, and the solution was refreshed every 7 days and collected. The samples before immersing were weighted as W₀. At the datum time, the scaffolds were taken out, washed three times at room temperature with deionized water, dried at 37°C for 24 h, and weighted as W₂. The absorption ratio and swelling ratio at each time point were calculated using the following equation below:

Weight loss = $\frac{100\times(W_2 - W_0)}{W_0}$

The pH change of the immersing liquids was measured with a pH meter (Leici, China). The concentration of Sr²⁺ in the liquids was measured by inductively coupled plasma atomic emission spectrometer (ICP-AES; Thermo Fisher, USA).

2.3. In vitro examination of cell behaviors

2.3.1. Cell culture and materials sterilization

Mouse bone marrow mesenchymal stem cells (mBMSCs; ATCC, USA), RAW264.7 (Chinese Academy of Sciences, China), and human umbilical vein endothelial cells (HUVECs; Sciencell, USA) were applied to assess the cell responses of the scaffolds. After confluence, the cells were digested by trypsin solution (Gibco) and collected. The culture medium for RAW264.7 and mBMSCs was DMEM (Gibco) with 10% fetal bovine serum (FBS; Gibco) and 1% P/S solution (Gibco). Endothelial cell medium (ECM; Sciencell) with supplements (FBS and growth factors) was used to culture HUVECs. The mBMSCs, RAW264.7, and HUVECs at 3–5 passages were used in this study. The culture medium was changed every 2 days.

SG, SrP/SG, and Rg1/SrP/SG scaffolds were prepared and sterilized under gamma-ray irradiation (cobalt source) of 5 kGy for about 4 h. Before cell seeding, the scaffolds were soaked in the basal medium for 24 h. Scaffold extracts were prepared by immersing a sample (Φ5 × 2 mm) in 1 mL basic medium and placed at 37°C for 24 h and/or 72 h. Then the supernatant was collected, supplemented with PBS and/or factors, and finally diluted by the corresponding complete culture medium with a ratio of 1:3. The concentrations of Sr, Ca, and P in DMEM extracts were measured by ICP, and the concentration of Rg1 was detected by high-performance liquid chromatography (Agilent, USA).

2.3.2. Cell adhesion, viability, and proliferation

In this study, mBMSCs and HUVECs were used to assess the cell biocompatibility of SG scaffolds. For mBMSCs, the SrP/SG scaffolds (Φ5 × 2 mm) were placed in a 96-well plate, and $5 \times 10^4$ cells were seeded on each scaffold. After 24 h, the scaffolds were moved to a 24-well plate. HUVECs were seeded in a 48-well plate with the density of
2.3.5. In vitro tube formation ability

Phosphatase kit (Sigma).

were resistant acid phosphatase (TRAP) activity of cells in SrP/SG extracts was assessed on the tube formation ability of HUVECs. ECMatrix was added to a 24-well plate and gelled at 37 °C for 15 min at 37 °C. The absorbance of the solution at 405 nm was recorded by a microplate reader (Thermo Fisher), and the relative ALP content was calculated by a standard curve. The total protein concentration of the lysate was determined by a BCA kit (Shanghai Biocolor, China). ALP activity was determined by dividing ALP absorbance by the protein content. Each group was with three replicates, and the data were displayed as mean ± standard deviation.

2.3.6. Pro-inflammation macrophage stimulation and morphogenesis phenotypes assessment

RAW264.7 cells with the density of 10^5 cells/mL were seeded on a 24-well plate and cultured in SrP/SG extracts, which consisted of 50 ng/mL receptor activator of nuclear transcription factor-κB (NF-κB) ligand (RANKL; Merck, USA), 30 ng/mL macrophage colony-stimulating factor (M-CSF; R&D), 10% FBS, and 1% P/S. After culturing for 5 days, tartrate-resistant acid phosphatase (TRAP) activity of cells in SrP/SG extracts was assessed by a TRAP Assay Kit (Beyotime). For TRAP staining, the cells were fixed by 4% paraformaldehyde for 20 min and stained by the Acid Phosphatase kit (Sigma).

2.3.7. Gene expression

The effects of the scaffolds on the gene expression of mBMSCs, HUVECs, and RAW264.7 were detected by RT-PCR. Briefly, the messenger RNA (mRNA) was first extracted by TRIzol solution (Invitrogen, USA) and reversely transcribed into complementary DNA (cDNA) by an assay (Bio-Rad, USA) following the manufacturers’ instructions. Then cDNA, gene primers (200 nM), and SYBR Green Supermix (Bio-Rad) were mixed and ran in the CFX96 system (Bio-Rad) to get the Ct value. The mRNA expression was determined by 2^(-ΔΔCt) method. For mBMSCs cultured on SrP/SG scaffolds with osteogenic induction medium after 7 days, the expression of osteogenesis-related genes, runt-related transcription factor 2 (Runx2), ALP, type I collagen (Col-I), osteocalcin (OC), and osteopontin was tested. For RAW264.7 cultured in SrP/SG extracts with RANKL and M-CSF after 5 days, the level of osteoclastogenesis-related genes, TRAP, matrix metalloproteinase (MMP9), and cathepsin K (CTK), in RAW264.7 was measured. The expression of genes related to inflammation, interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF-α), Arginase, and IL-10 and genes related to bone repair, bone morphogenetic protein-2 (BMP-2), and platelet-derived growth factor-BB (PDGF-BB) was tested in LPS-treated RAW264.7 cultured with Rg1/SrP/SG extracts for 3 days. After culturing HUVECs in Rg1/SrP/SG extracts for 3 days, the expression of angiogenesis-related genes, VEGF, endothelial nitric oxide synthase (eNOS) and basic fibroblast growth factor (bFGF) was accessed. GAPDH was regarded as the reference gene. The gene primers were synthesized from Sangon Biotech (China) and displayed in Table S1. Each group was with three replicates, and the data were displayed as mean ± standard deviation.

2.3.8. Protein expression

To further assess the regulation of the scaffolds on the protein secretion of mBMSCs, HUVECs, and RAW264.7, Western blot experiment was conducted by a procedure adapted from a previous study. Cells were under treatments, as described in gene expression detection for different times, then washed three times with PBS, and lysed using 150 μL of SDS lysis buffer (Roche, USA) containing phenylmethanesulfonfluoride (PMSF, Beyotime, China) and protease inhibitor (Beyotime) in an ice bath. The cells were further lysed by ultrasonication, and the obtained lysate was centrifuged at 12,000 rpm for 10 min at 4 °C. The protein concentration of the lysate was tested by the BCA kit (Shanghai Biocolor, China). Then 60 μg protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Beyotime) and transferred to a polyvinylidene fluoride membrane (Beyotime). After blocked with 5% skim milk (Solarbio, China) for 45 min, the membranes were incubated with TBST buffer (Beyotime) containing corresponding rabbit antibodies (mBMSCs: ALP, OC, Col-I, and Runx2; RAW264.7: CTK and MMP9; LPS-RAW264.7: Arginase, BMP-2, and PDGF-BB; HUVECs: bFGF and VEGF; Abcam, USA) overnight at 4 °C. The membranes were further stained with peroxidase-labeled secondary antibody of goat anti-rabbit immunoglobulin G (horseradish peroxidase (Abcam, USA) for 1 h at room temperature. After developed with chemiluminescent reagents (ECL-plus; Beyotime), the band images for targeted proteins in the membranes were captured using a gel imaging system (Tanon-5200; China). β-actin antibody (Abcam) was used as the control protein.

2.4. In vivo bone repair effect of Rg1/SrP/SG scaffolds implanted in critical-sized calvarial defects of osteoporotic rats

2.4.1. Surgery for animal

All experiments were conducted in a specific pathogen-free animal laboratory and approved by the Animal Research Ethics Committee of Jilin University. The ovariectomized (OVX) rats were obtained as a previous study described [35]. Briefly, the female rat was anesthetized by the intraperitoneal injection of pentobarbital (35 mg/kg). Then the ovaries were removed.
and oviduct were cut after incising the skin and muscle wall bilaterally at the abdomen. At last, the oviduct and the incision were sutured.

After the O VX surgery for 12 weeks to develop the osteoporosis, the rats were committed to the critical-sized calvarial defects (Ø5 mm) surgery. The rats were anesthetized, then the skin was incised, and the defects on both sides of the skull were made by drilling with a trephine bur. The scaffolds were filled, and the wound was closed with 4–0 sutures. The rats were divided as four groups: defects with no scaffolds (NC), with SG, 15SrP/SG, or Rg1/15SrP/SG scaffolds (n = 6). At 6 and 12 weeks, three rats in each group were sacrificed by overdose pentobarbital of 80 mg/kg, and the skulls of the rats were gained and fixed in 4% paraformaldehyde (Solarbio) solution at room temperature for 48 h.

2.4.2. Microcomputed tomography analysis
The fixed skull samples in each group were analyzed by micro-computed tomography (Micro-CT) system (Aloka co., Japan). The three-dimensional images of newly regenerated bone tissue in the calvarial defects were constructed by M imics software (Materialise, Belgium), and the bone mineral density (BMD) and bone volume fraction (BV/TV) were calculated.

2.4.3. Histology examination
The bone samples after fixed in 4% paraformaldehyde solution were decalcified in 10% EDTA (Sigma, USA) solution at room temperature for 3 weeks (refreshing solution every 3 days). The obtained samples were trimmed, dehydrated in graded ethanol (80%, 90%, 95%, and 100%), soaked in xylene, and embedded in paraffin wax (Sigma, USA). Then, the embedded samples were cut into slices with 5 μm thickness and cured at 65°C for 12 h.

The wax in slices was removed by soaking in xylene. The slices were hydrated in graded ethanol (100%, 95%, 90%, and 80%), then stained with hematoxylin and eosin dye (Solarbio) for 5 min, and then dehydrated and sealed with neutral balsam (Solarbio). The pictures of the slices were captured by a slide scanning system (3DHISTECH, HU).

2.5. Statistical analysis
All data of in vitro quantitative experiments are displayed as mean ± standard deviation. One-way analysis of variance is applied to analyze the difference between the two groups under Tukey’s test. p < 0.05 is regarded as there is a significant difference between the two groups.

3. Results
3.1. Composition, microstructure, and properties characterization
SrP clusters were successfully synthesized by chemical precipitation. XRD pattern in Fig. 2A showed that the synthesized SrP clusters were pure crystals, and the peaks matched that of strontium hydrogen phosphate (PDF#12-0368). The morphology of SrP clusters was dandelion-like, as little thin strip-like crystals were assembled together into round clusters (Fig. 2B). The diameter of SrP clusters was around 10 μm. EDS results also demonstrated that the element composition of SrP was mainly Sr, P, and O.

The SrP clusters were incorporated into SG matrix with the method displayed in Fig. 1A. SEM images of SrP/SG scaffolds in Fig. 3A showed that all scaffolds were porous with most pores around 100 μm. The pores of SG scaffold were irregular round and that of SrP/SG scaffolds were irregular rhombic. Besides, SrP clusters were dispersed uniformly in SG matrix, and the pore wall of SrP/SG scaffolds was thicker when compared with SG. The pore size distribution and porosity of SrP/SG scaffolds are displayed in Table 1. The pore size of SG was around 0–300 μm, whereas that of 5SrP/SG and 10 SrP/SG scaffolds was around 0–250 μm and that of 15SrP/SG scaffolds was around 0–200 μm. The average pore size of each group decreased from 142.7 to 95.8 with the increase of SrP content. The median pore size decreased at first and then increased while the content of SrP was more than 10 wt%. The porosity of SG, 5SrP/SG, and 10SrP/SG was close, but that of 15SrP/SG (86.91%) was significantly lower than SG (95.12%).

XRD patterns in Fig. 3B informed that all scaffolds had the peak around 20–24°, which was assigned to the β-sheet structures of SF induced by ethanol and Sr2+ (Fig. 1B and C). SG scaffolds incorporating with SrP had the peaks of SrP, and the peak intensity was increased with the increase of SrP content.

FIR spectra in Fig. 3C showed that all scaffolds displayed the peaks around 3270–3290, 1620–1630, 1510–1530, and 1230–1260 per cm, which were, respectively, attributed to hydrogen bonds (-OH) and amides I, II and III. These groups were related to SF and GN. Scaffolds with SrP displayed the peaks of HPO42– around 1182, 1130, 1065, 929, and 885 per cm and the peaks of PO43– around 1007 per cm, 597 per cm, and 545 per cm. The peak around 1160–1170 per cm was related to C–O groups in genipin and that around 1060–1070 per cm was related to the covalent cross-link groups (C–N) between genipin and amino groups of lysine in SF and GN (Fig. 1C).

The compressive curve (Fig. 4A) showed that the compressive strength of 5SrP/SG and 10SrP/SG scaffolds was close to that of SG scaffold, whereas 15SrP/SG scaffold displayed distinctly higher compressive strength. Fig. 4B also showed that the static compressive modulus of 15SrP/SG scaffolds was 11.84 ± 0.96 MPa, significantly higher than that of SG scaffolds (2.06 ± 0.17 MPa; p < 0.01).

When soaking the scaffolds in PBS solution, the absorption ratio of SG scaffolds was 4.96 ± 1.32 at 1 h and 6.38 ± 1.00 at 24 h (Fig. 4C). The ratio of scaffolds was changed a little after incorporating 5 wt% SrP, then decreased when SrP concentration was 10 wt% and 15 wt%. However, the swelling ratio of the scaffolds was enhanced with the increase of SrP content (Fig. 4D). In addition, the swelling ratio of 10SrP/SG and 15SrP/SG was significantly higher than that of SG group.
Degradation behaviors of SG and SrP/SG scaffolds were plotted in Fig. 4EG. The weight loss of all scaffolds was reached approximately 8% at 7 days and reached above 23% at 56 days (Fig. 4E). The incorporation of SrP enhanced the weight loss after 28 days, and the weight loss of 15SrP/SG scaffolds was significantly higher than that of other groups at Days 42 and 56. The pH value of all scaffolds was lower than 7.4 during the immersing period and increased with the extension of time (Fig. 4F). All SrP/SG scaffolds were able to release Sr\(^{2+}\), and the concentration of Sr\(^{2+}\) was decreased with prolonging the soaking time (Fig. 4G). The concentration of Sr\(^{2+}\) was enhanced with the increased content of SrP, whereas that of 5SrP/SG, 10SrP/SG, and 15SrP/SG scaffolds was among 0.08–1.96, 0.25–2.15, and 0.84–4.81 mg/L, respectively.

### Table 1

Pore size distribution and porosity of SrP/SG scaffolds.

| Pore size (μm) | Ratio (%) | SG | 5SrP/SG | 10SrP/SG | 15SrP/SG |
|---------------|-----------|----|---------|----------|----------|
| 0–50          | 10.0      | 13.9 | 18.4     | 18.9     |
| 50–100        | 20.0      | 27.8 | 31.6     | 29.7     |
| 100–150       | 30.0      | 36.1 | 28.9     | 37.8     |
| 150–200       | 20.0      | 16.7 | 15.8     | 13.5     |
| 200–250       | 10.0      | 5.6  | 2.6      | 0.0      |
| >250          | 10.0      | 0.0  | 0.0      | 0.0      |
| Average size  (μm) | 142.7 | 114.4 | 100.7    | 95.8     |
| Median size   (μm) | 129.2 | 107.0 | 87.0     | 107.2    |
| Porosity (%) | 95.12 ± 3.73 | 95.61 ± 2.04 | 94.48 ± 0.75 | 86.91 ± 2.98* |

* Compared with SG, p < 0.05.

The effect of SrP on the osteogenic differentiation of mBMSCs was presented in Fig. 5. Live/dead cells staining images showed that most mBMSCs maintained good viability (green) on all groups, and few dead cells (red) were seen (Fig. 5A). Besides, mBMSCs were well adhered on the surface and along the pore walls of all scaffolds. The proliferation results showed there was no difference in mBMSCs number among these groups at Day 1, whereas cells on SrP/SG scaffolds were obviously more than on SG scaffolds at 7 and 14 days (p < 0.01; Fig. 5B). On Day 14, an increase of approximately 70% cell proliferation was observed between SrP/SG and SG groups.

Fig. 5C displayed that ALP activity of mBMSCs on 15SrP/SG scaffolds was significantly higher than that of SG scaffolds at 7 and 14 days (p < 0.05). The gene expression of mBMSCs cultured for 7 days was displayed in Fig. 5E. The expression of Col-I in 5SrP/SG group was obviously higher than that in SG group (p < 0.01). The expressed gene levels of ALP, Col-I, and OC of mBMSCs on 10SrP/SG scaffold were 2.3-fold, 2.4-fold, and 2.4-fold higher than on SG scaffold (p < 0.01). Cells on 15SrP/SG scaffold expressed the most ALP, Col-I, OC, and Runx2 genes, and the gene levels were 3-fold, 3.6-fold, 3.7-fold, and 3.6-fold higher than on SG scaffold (p < 0.01). The protein levels of OC, ALP, Col-I, and Runx2 of mBMSCs on the SrP/SG scaffolds were enhanced and consistent with the gene expression (Fig. 5D). The secretion of osteogenic-related proteins from mBMSCs in 15SrP/SG groups was the highest.

### 3.2. In vitro osteogenesis and osteoclastogenesis of SrP/SG scaffolds

Scaffold extracts were prepared for RAW264.7 culture, and the concentration of Sr\(^{2+}\) was increased with the content of SrP (Table 2). Sr\(^{2+}\) concentration was increased as the content of SrP increased, which was among 0.45–0.65 mM. When RAW264.7 cells were cultured with...
scaffold extracts containing RANKL, some cells differentiated into osteoclasts and secreted TRAP (indicated in Fig. 6A and B). Fig. 6A showed that TRAP expression of RAW264.7 in all SrP/SG extracts at Day 5 was remarkably lower than that in SG extracts \( p < 0.01 \). A decrease of approximately 40% TRAP activity was observed between SrP/SG and SG extracts. Less differentiated osteoclast-like cells were also observed in SrP/SG extracts when compared with SG extract (Fig. 6B).

Osteoclastogenesis-related genes of RAW264.7 in scaffold extracts were displayed in Fig. 6C–E. Cells in all SrP/SG extracts expressed less TRAP, MMP9, and CTK than in SG extract. The expression of TRAP and CTK was decreased linearly with the increased content of SrP. The gene level of MMP9 of RAW264.7 in 10SrP/SG extract was 0.01-fold lower than that in SG extract \( p < 0.01 \) and that of TRAP, MMP9, and CTK of cells in 15SrP/SG extract were also 0.5-fold, 0.48-fold, and 0.16-fold lower than in SG extract \( p < 0.01 \). The protein levels of MMP9 and CTK of RAW264.7 in SrP/SG extracts were also inhibited (Fig. 6F), and the secretion of osteoclastic-related proteins in 15SrP/SG groups was the lowest.

3.3. In vitro angiogenesis of Rg1/SrP/SG scaffolds

For the stimulation of angiogenesis, SG and 15SrP/SG scaffolds were selected based on osteogenesis and osteoclastogenesis assessments, immersed in Rg1 solution overnight, and lyophilized (Fig. 1A). Rg1 was absorbed by SrP and SG matrix through Van der Waals’ force, such as hydrogen bonds (Fig. 1D). Table 3 showed that Rg1 with a concentration of approximately 47.85 \( \mu g/mL \) at Day 1 and 16.12 \( \mu g/mL \) at Day 3 was released from the obtained scaffolds, and the incorporation of SrP decreased the release rate of Rg1 (42.27 \( \mu g/mL \) at Day 1 and 13.12 \( \mu g/mL \) at Day 3).
The proliferation of HUVECs in all scaffold extracts was good, and Rg1/15SrP/SG and Rg1/SG extracts significantly enhanced the cell number compared with SG extract (Fig. 7A). More viable cells (green) were observed in 15SrP/SG, Rg1/15SrP/SG, and Rg1/SG extracts than in SG extract, and fewer dead cells (red) were found in all groups (Fig. 7F).

For angiogenesis-related gene expression, 15SrP enhanced the expression of VEGF, bFGF, and eNOS genes, but there was no significant difference when compared with other groups (Fig. 7B–D). However, the expression levels of VEGF and bFGF genes of HUVECs in Rg1/15SrP/SG extract were 7.4-fold and 2.4-fold higher than in SG extract ($p < 0.01$) and that in Rg1/
SG extract was 5.2-fold and 1.8-fold higher than in SG extract \( (p < 0.01) \). Besides, the expression of VEGF and bFGF in Rg1/15SrP/SG group was obviously higher than in 15SrP/SG group \( (p < 0.05) \). The expression of eNOs displayed no difference among the groups. The protein levels of bFGF and VEGF of HUVECs were promoted by loaded SrP and Rg1, which were consistent with the gene expression \( (\text{Fig. 7E}) \). Moreover, the angiogenic proteins secreted by HUVECs in Rg1/15SrP/SG groups were the highest. In vitro tube formation results showed that most HUVECs in extracts contain Rg1 linked into stripes and formed junctions \( (\text{nodes}) \) at 4 h when cells in SG and 15SrP/SG extracts displayed fewer stripes and junctions \( (\text{Fig. 7G}) \). At 8 h, more vessel nodes and loops were observed in Rg1/15SrP/SG and Rg1/SG extracts when compared with SG and 15SrP/SG extracts. Quantitative calculated data \( (\text{Fig. 7H}) \) also proved that the numbers of vessel nodes and loops as well as tube length in all groups were increased with time. Besides, the numbers of vessel nodes and loops in Rg1/15SrP/SG and Rg1/SG group were significantly higher than that in 15SrP/SG and SG group at 8 h. The tube length showed the same tendency at 8 h.

### 3.4. Inflammatory regulation of Rg1/SrP/SG scaffolds

Under LPS stimulation for 8 h, RAW264.7 cells had more pseudo-podia, indicating it had a tendency to polarize from M0 macrophages to M1 phenotype (pro-inflammatory state; \( \text{Fig. S1} \)). After treating with SG, 15SrP/SG, Rg1/15SrP/SG, and Rg1/SG extracts for 3 days, the flow cytometry results displayed that SrP and Rg1 retarded the expression of
M1 marker CD86 and promoted the expression of M2 marker CD206. The expression of CD86 in Rg1/15SrP/SG group was decreased from 42.2% (SG group) to 38.5% and that of CD206 was increased from 46.3% to 52.0% (Fig. 8A). Moreover, Rg1/15SrP/SG extract remarkably downregulated the expression of pro-inflammatory genes (TNF-α, IL-1β, and IL-6) and obviously promoted the expression of anti-inflammatory genes (IL-10 and Arginase) as well as bone repair–related genes (BMP-2 and PDGF-BB) in comparison with SG group (p < 0.05; Fig. 8B). 15SrP/SG and Rg1/SG showed a similar effect on the gene expression of RAW264.7 in Rg1/15SrP/SG extract, and both displayed a significant difference in the expression of BMP-2 gene. The protein secretion of Arginase, BMP-2, and PDGF-BB of RAW264.7 in scaffold extracts with Sr2⁺ and Rg1 was enhanced and consistent with the gene expression (Fig. 8C). Among them, LPS-treated RAW264.7 cells in Rg1/15SrP/SG extract secreted the most proteins related to anti-inflammation (Arginase) and bone repair (BMP-2 and PDGF-BB).

3.5. In vivo repair effect of Rg1/SrP/SG scaffolds

Micro-CT results showed that the calvarial bone defects of OVX rats treated by Rg1/SrP/SG scaffolds were exhibited the best bone regeneration effect, and most areas of the defects were filled with the newly generated bone at 12 weeks (Fig. 9A). The NC and SG groups showed limited bone formation, and SrP/SG groups displayed more newly formed bone when compared with NC or SG group. Quantitative analysis further demonstrated that 15SrP/SG and Rg1/15SrP/SG scaffolds at 6 and 12 weeks had higher BMD and total BV/TV ratio than NC and SG group (Fig. 9B and C). The BV/TV ratio of 15SrP/SG group at Weeks 6 and 12 was 2.6-fold and 2.3-fold higher than that of NC group and that of Rg1/15SrP/SG group were 2.6-fold and 4.1-fold higher than NC group (p < 0.01). The BMD of 15SrP/SG and Rg1/15SrP/SG groups at Week 6 was, respectively, 0.51 and 0.53 and significantly higher than that in NC group (0.33; p < 0.01). At Week 12, the BMD of 15SrP/SG and Rg1/
15SrP/SG groups was, respectively, increased to 0.59 and 0.65, whereas that of NC group was 0.38. Besides, the BV/TV ratio and BMD of Rg1/15SrP/SG scaffolds at Week 12 were the highest.

New bone tissue formation stimulated by the scaffolds was characterized by H&E staining and Masson’s trichrome staining (Fig. 10). After implantation of 6 weeks, the NC group displayed a little of new bone formation along the margins and center of the defects. All scaffolds were integrated with the margins. Moreover, 15SrP/SG and Rg1/15SrP/SG scaffolds presented more generated new bone with the external in-growth pattern than NC and SG groups. After 12 weeks, most scaffolds were degraded, and the amount of generated bone was the highest in Rg1/15SrP/SG scaffolds. The defect with SG scaffolds was filled with fibrous tissues, and little bone tissues were formed at the margins of the defect. Besides, all scaffolds were degraded gradually during the implantation, and the bone tissue regenerated rate of Rg1/15SrP/SG group was more matched the degraded rate when compared with other groups.

4. Discussion

To promote the regeneration of osteoporotic bone defects, numerous researchers and clinical investigators were committed to develop an ideal scaffold with good osteogenesis, angiogenesis, suppressed osteoclastogenesis, and inflammation. In this study, a novel Rg1/SrP-loaded inorganic–organic porous scaffolds with proper degradation behaviors and mechanical strength was developed, and the combination of Rg1 and SrP
promoted the critical-sized calvarial defect repairing of osteoporotic rats.

SF and GN are proved to be promising natural biomaterials in tissue engineering and widely applied in bone repair, as they are biocompatible, degradable, and can mimic the organic components of bone tissue [36]. The mechanical strength and degradation rate of the SF/GN composite scaffold could be adjusted by their ratio [13]. As GN was degraded too fast and SF with β-sheet structure was degraded slowly, SF/GN with a ratio of 1:1 was chosen in this study. The obtained SG scaffolds contained SF with β-sheet structure induced by ethanol treatment and Sr2⁺, whereas covalent bonds between SF and GN cross-linked by genipin (Figs. 1 and 3). It resulted in the high compressive modulus of SG scaffolds (approximately 2.06 MPa) and a proper weight loss (23–38%) at 56 days (Fig. 4E). The incorporated SrP clusters were dispersed randomly in SG matrix to mimic the inorganic composition of bones, which enhanced the mechanical properties of SG scaffolds. Pore size and porosity of scaffolds were generally negatively correlated with strength [37,38]. In our study, the pore size of scaffolds displayed a decreased tendency with the increased content of SrP (Table 1), but the porosity and strength of 5SrP/SG and 10 SrP/SG was close to that of SG. However, the porosity of 15SrP/SG was significantly lower than that of SG, resulting in remarkably stimulated compressive strength. It is because the samples of 15SrP/SG shrunk a little after cross-linking and freeze drying. Previous studies showed that cation ions (Ca²⁺) could aid the formation of β-pleated sheet structure in SF and enhanced the strength of SF-based scaffold [39]. In our study, the released Sr²⁺ also strengthened SG scaffolds in a concentration-dependent manner. Moreover, 15SrP/SG samples swelled to the size close to samples in other groups after soaking in PBS, resulting in higher swelling ratio (Fig. 4D). In addition, the water absorption of SG scaffolds was decreased a little by 15 wt% SrP. When soaking in PBS, scaffolds with 15 wt% SrP were degraded almost 38% at

Fig. 9. Micro-CT analysis. The superficial 3D-reconstructed images of new bone formation (A), morphometric analysis of BMD (B), and BV/TV ratio (C) for each group at 6 and 12 weeks postoperation. The diameter of the brown circles was 5 mm *p < 0.05, **p < 0.01 (n = 3).
CTK (Fig. 6), which was essential for osteoporotic bone repair [42]. The differentiation of RAW264.7 cells by downregulating TRAP, MMP9, and further, SrP incorporation in SG scaffolds suppressed the osteoclast concentration of Sr²⁺ among 0.45–0.65 mM (Table 2), which was among the positive concentration range of Sr²⁺ for stimulating osteogenesis and inhibiting osteoclastogenesis, 0.01–1 mM [43]. Besides, scaffold pores with a diameter >50 μm can promote the growth of blood vessels [44], whereas that greater than 100 μm can promote the growth of bone tissues [45,46]. The SrP/SG scaffolds in this study possessed pores most with the diameter of around 100 μm (Table 1). Therefore, the incorporation of SrP in SG scaffolds is a potential method to promote osteoporotic bone repair.

Improving the angiogenesis of bone repair materials is not only beneficial to tissue growth but also can transport nutrients and metabolic substances to bone repair cells [47]. In addition to increasing the pore size, the ability to stimulate endothelial cells surrounding the defect to form new blood vessels is also essential. Ginsenoside Rg1, one of the most abundant monomers in ginseng, was demonstrated to have the role to facilitate the formation of new blood vessels. Leung et al. [48] found that 120 μg/mL Rg1 promoted VEGF secretion in HUVECs through HIF-1α pathway. Our experiments also demonstrated that SrP clusters could delay the release of Rg1, as they had dandelion-like structure formed by nanorods, which could help the capture of Rg1 (Fig. 1 and Table 3). Rg1 released from the 15SrP/SG and SG scaffolds was with concentration among 13–50 μg/mL and promoted the tube formation ability and gene expression (VEGF and bFGF) of HUVECs (Fig. 7). Besides, it was also proved that 4 μg/mL of Rg1 could help the osteogenic differentiation of stem cells by upregulating BMP-2 [49], and 1–100 μg/mL of Rg1 suppressed the formation of osteoclastic cells by downregulating TRAP, CTK, and MMP9 [50]. These together made Rg1 a promising angiogenic therapeutic for osteoporotic bone repair.

As previous studies have proved that the inflammatory factors secreted by immune cells (especially macrophages) could exacerbate the osteoporosis [5,6]. Macrophages are the main inflammatory regulating cells inherent in bone tissues [51,52]. Therefore, mediating the macrophages polarized from M1 (pro-inflammation) to M2 (anti-inflammation) type could suppress the inflammation and accelerate the bone regeneration [53,54]. In our study, the incorporation of Rg1 in SG scaffolds retarded the formation of M1-type macrophages, whereas SrP stimulated the formation of M2-type macrophages (Fig. 8). It has been proved that Sr-doped hydroxyapatite could suppress the inflammation [17], and Sr-doped bioglass could release Sr²⁺ to stimulate the formation of new bone and vessels by modulating the macrophages polarized to M2 type [41,54]. Therefore, Sr²⁺ released from SrP/SG scaffolds could create a microenvironment favorable for bone regeneration. The role of ginseng monomers (such as Rb1, Rg1, and Rg3) on the immune regulation and inflammation inhibition was widely proved in different organs of animals and humans [32,55]. But rare studies revealed these effects of Rg1 on bone regeneration. In this study, the combination of Rg1 and SrP in the scaffolds not only inhibited the expression of pro-inflammatory genes (TNF-α and IL-1β) and stimulated the expression of anti-inflammatory genes (Arginase and IL-10) but also upregulated the expression of angiogenic (PDGF-BB) and osteogenic (BMP-2) genes and proteins.

Fig. 10. Histological staining of the osteoporotic bone defects after implantation of different scaffolds for 6 and 12 weeks. Images of H&E staining (A) and Masson’s trichrome staining (B). The images in the second and fourth row of HE and Masson staining were the zoomed pictures.
The previously mentioned results all indicated that the addition of Rg1 and SrP could improve the bone regeneration efficiency of SG scaffolds in osteoporotic critical-sized calvarial defects. In vivo animal experiments showed Rg1/15SrP/SG accelerated the bone formation and almost degraded at Week 12 (Figs. 9 and 10). The repair mechanism of it is shown in Figure S2. After SrP and Rg1 were introduced into SG matrix, the obtained scaffold was implanted into the osteoporotic critical-sized calvarial defects. Immediately, in flowing blood with bone repair cells was absorbed by Rg1/SrP/SG, whereas Sr2+ and Rg1 are slowly released from the scaffold into the defects during its degradation. Sr2+ has the dominating effect on directly stimulating osteogenesis and suppressing osteoclastogenesis, whereas Rg1 exerts an essential role in angiogenesis. They together mediate macrophages polarized into M2 type, which is important for inflammation suppression and bone repair [51,54,56]. In summary, the results in the present study indicate that Rg1/SrP-loaded SG scaffolds promote osteoprotic bone repair, which shed light on the development of next-generation tissue-engineered scaffolds for osteoporotic bone repair.

5. Conclusion

In summary, the Rg1/SrP-loaded SG scaffolds for osteoporotic bone repair were successfully fabricated. Incorporating SrP clusters into SG scaffolds significantly increased the mechanical properties of the constructs and delayed the release of Rg1. Notably, SrP in the scaffolds could create microenvironments preferable for stimulating the osteogenesis and suppressing the osteoclast differentiation in vitro. Moreover, Sr2+ and Rg1 released from the scaffolds significantly promoted in vitro angiogenesis and suppressed the inflammation by regulating LPS-treated macrophages into M2 type. Rg1/15SrP/SG scaffolds with a proper degradation rate remarkably enhanced the regeneration of in vivo osteoprotic critical-sized calvarial defects. These results suggest that SG scaffolds incorporating with Rg1 and SrP could serve as a promising bone substitute for the treatment of critical-sized bone defects in osteoporotic patients.

Authors’ contributions

T.W. contributed to methodology, investigation, writing the original article, data curation, and funding acquisition. W.L. contributed to methodology, investigation, validation, and writing the original article. S.H. contributed to methodology and validation. S.H. contributed to investigation and formal analysis. F.H. reviewed and edited the article. H.W. contributed to visualization. X.Z. contributed to funding acquisition. Z.L. contributed to investigation, reviewing and editing the article, and funding acquisition. Z.L. contributed to funding acquisition. Z.L. contributed to investigation, project administration, reviewing and editing the article, and funding acquisition. Y.C. contributed to conceptualization, reviewing and editing the article, funding acquisition, investigation, and supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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