



Study of bone repair mediated by recombination BMP-2/ recombination CXC chemokine Ligand-13-loaded hollow hydroxyapatite microspheres/ chitosan composite

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ARTICLE INFO

Keywords:

Bone graft
Recombination BMP-2
Recombination CXC chemokine ligand-13
Hollow HA microspheres/CS composite
Bone regeneration
Bone repair

ABSTRACT

Aims: The present study aimed to investigate the mechanism of bone repair mediated by recombination BMP-2 (rhBMP-2)/recombination CXC chemokine ligand-13 (rhCXCL13)-loaded hollow hydroxyapatite (HA) microspheres/chitosan (CS) composite.

Materials and methods: Firstly, the biological activity of rhBMP-2 and rhCXCL13 released from the complex was investigated. Secondly, the effect of rhBMP-2 sustained release solution on ALP activity and rhCXCL13 sustained release solution on cell migration of rat bone marrow mesenchyme stem cells was tested. Thirdly, osteoblasts differentiation test, X-ray scoring and three-point bending test were performed. Finally, the mRNAs expression of osteogenic marker genes and the protein expression of Runx2 was tested by reverse transcription-polymerase chain reaction (RT-PCR) and western blotting (WB), respectively.

Key findings: RhBMP-2 could significantly promote the proliferation and differentiation, and RhCXCL13 could promote the migration of rat bone marrow MSCs. Detection of ALP activity and calcium salt deposition showed that rhBMP-2 and rhCXCL13 could significantly improve the biological activity and promote cell differentiation ability. X-ray scoring of radius and flexural strength test showed that rhBMP-2 and rhCXCL13 could promote bone healing and improve the bending resistance of bone tissue. The in vitro molecular experiments including RT-PCR and WB further demonstrated the roles of rhBMP-2 and rhCXCL13 in bone formation and bone repair. **Significance:** Our results indicated that the hollow HA microspheres/CS composite could be effective as a delivery vehicle for rhBMP-2 and rhCXCL13 in bone regeneration and bone repair. In this process, rhBMP-2 may promote bone regeneration by regulating bone marrow MSCs cells recruited by rhCXCL13.

1. Introduction

Bone defect and nonunion caused by trauma, tumor resection and deformity correction are common problems in orthopaedics. Clinically, treatment of bone defect and nonunion mainly depends on autologous or allogeneic bone transplantation. However, limited sources of autologous and new trauma may cause some complications (such as infection and pain in the donor bone area), which restricted the wide clinical application of autologous bone transplantation [1–4]. Allogeneic bone transplantation is prone to immune rejection, infection and even biological infectious diseases including hepatitis b and AIDS, which greatly

affect the use of allogeneic bone transplantation. In addition, there are many uncertainties and long-term clinical safety problems of gene and stem cell therapy in the treatment of bone defects [5]. Therefore, the application of bone growth factor to promote bone regeneration may be a more realistic and feasible method to solve the treatment problem of bone defect [4,6].

Bone morphogenetic proteins (BMPs) are important growth factors in the process of osteogenesis, bone induction and bone repair. Among which, bone morphogenetic protein 2 (BMP-2) is the most commonly used bone growth factor in tissue engineering [7]. Recombinant human bone morphogenetic protein 2 (rhBMP-2) is synthesized by

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<https://doi.org/10.1016/j.lfs.2019.116743>

Received 28 June 2019; Received in revised form 24 July 2019; Accepted 7 August 2019

Available online 10 August 2019

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recombinant DNA and molecular biology technology, which can be used in clinical practice to effectively promote repair of the bone defect and bone nonunion, fracture healing and vertebral fusion [8,9]. However, rhBMP-2 must have an appropriate vector to maintain its local function and promote bone regeneration [10]. It is noted that synthetic bone graft substitute is a good substitute choice for conventional natural graft [11,12]. Moreover, a number of biomaterials and synthetic bone substitutes are used as scaffolds, such as hydroxyapatite (HA) [13]. It has been demonstrated that HA has several significant advantages over other carriers for BMP-2 delivery of large bone defects regeneration [14–18]. In addition, chitosan (CS) also has unique characteristics for drug delivery platforms. It is noted that CS can be compounded with HA to form a good mechanical delivery scaffolds. It is reported that [19,20] rhBMP-2-loaded hollow hydroxyapatite (HA) microspheres/chitosan (CS) composite could slow release rhBMP-2, which provided three-dimensional porous scaffolds for bone growth.

However, as lacking of local seed cells and presenting conversion obstacle of seed cells, it is difficult for rhBMP-2 to play an effective role in large bone defects. How to recruit and supplement sufficient number and vitality of seed cells to participate in the bone repair has become one of the important subjects of bone tissue engineering. It is worth mentioning that the directional migration of stem cells mediated by chemokines is particularly important [21]. CXC chemokine ligand-13 (CXCL13), an important chemokine for functional maintenance of osteoblasts, is produced by osteoblasts and bone marrow mesenchyme cells (MSCs) [22,23]. The binding between CXCL13 and its receptor CXC chemokine receptor-5 (CXCR5) is crucial to recruiting of MSCs for bone repair [24]. It has been demonstrated that CXCL13 promotes the effect of MSCs on tendon-bone healing in rat experiment [25]. Significantly, hollow HA microsphere is also an effective controlled release vector of rhCXCL13, which has a good chemotaxis effect on MSCs. In view of this, we proposed a hypothesis: could CXCL13 be added to the rhBMP-2-loaded hollow HA microspheres/CS composite to break through the problem of large bone defects repair? In this study, we prepared rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite, and explored the mechanism of bone repair mediated by the complex in mice osteoblast progenitor (MC3T3-E1) cells and rabbit radial bone defect model.

2. Materials and methods

2.1. Preparation of CS temperature sensitive hydrogel

0.2 g CS was dissolved in 9 mL (0.1 M) hydrochloric acid solution. Under aseptic conditions, the mixture was magnetically stirred for 12 h to dissolve and filtered to obtain transparent CS solution. 1 M NaHCO₃ solution was used to adjust the pH (> 5) of the CS solution. The CS temperature-sensitive hydrogels were obtained by dissolving 560 mg β-sodium glycerophosphate (GP) in 1 mL de-ionized water, which was completely dissolved by ultrasound for 10 s, and slowly dropping GP solution into CS solution under agitation.

Preparation of rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites.

Firstly, the hollow HA microspheres were firstly sterilized in anhydrous ethanol and dried under 100 °C for further use. Then, rhBMP-2 and rhCXCL13 were adsorbed to the hollow HA microspheres under the role of pressure permeation. Detailed procedure was as follows: [1] 10 μg rhBMP-2 and 5 μg rhCXCL13 was respectively dissolved in 50 μL PBS (final concentration: 0.2 mg/mL and 0.1 mg/mL, respectively) and stored at 0–4 °C; [2] The mixture of 10 μL rhBMP-2, 10 μL rhCXCL13 and 20 mg hollow HA microspheres was put in 1 mL centrifuge tube. The centrifuge tube was vacuuming at 0–4 °C so that rhBMP-2 and rhCXCL13 could diffuse into the internal hollow HA microspheres; [3] 0.05 g rhBMP-2/rhCXCL13-loaded hollow HA microspheres was added to 10 mL of CS temperature-sensitive hydrogels. In order to obtain the stable composite hydrogel at room temperature, the mixture was stirred

so that no precipitate existed. The hydrogel of rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites were injected to the cylindrical model (h = 15 mm, Φ = 10 mm) and stored for 24 h at 38 °C constant temperature moisturizer for completely gelation.

2.2. Measurement of rhBMP-2 and rhCXCL13 release in vitro

In order to measure the release of rhBMP-2 and rhCXCL13, 100 mg of rhBMP-2/rhCXCL13-loaded HA microspheres/CS composites was added to 5 mL of PBS (pH = 7.04), and incubated with slowly shaking (135 r/min) at 37 °C. At 2, 6, 12, 24 h and 2, 3 days, and at each additional 1d interval until 35 days, 100 μL of supernatant sample was removed for testing (by ELISA Kit), and replaced with equal volume of PBS. Three duplicate samples were set at one time point. The release percentage of rhBMP-2 and rhCXCL13 was calculated followed by the drawing of the release curve.

2.3. Effect of rhBMP-2 on ALP activity of rat bone marrow MSCs cells

Rat bone marrow MSCs were subcultured in vitro for the study. Cells (1×10^4 /mL) were cultured with DMEM medium containing 10% FBS and added into 96-well plates (100 μL/well). After 24 h of cell growth, 100 μL DMEM medium containing 1% FBS was replaced with the previous medium. The cells were divided into A group (containing rhBMP-2 sustained-release solution released from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites) and B group (blank control). Three wells were set in each group. The medium was discarded after culture for 1, 2, 4 and 7 days. 50 μL 0.2% Triton X-100 was then added. The ALP activity procedure was tested according to the detection kit. The absorbance value was tested at 450 nm.

2.4. Effect of rhCXCL13 on the migration of rat bone marrow MSCs cells

RhCXCL13 released from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites was collected. In order to evaluate the biological activity of rhCXCL13, Boyden chamber method was used to test the migration of bone marrow MSCs. The whole bone marrow culture method was used to collect the third-generation rat bone marrow MSCs with a good growth state. The cells were inoculated in turn on the upper layer of Boyden cells with Matrigel gel and PET film. DMEM extract containing rhCXCL13 was added to the lower layer to induce the migration of bone marrow MSCs in the experimental group. Pure DMEM medium was added to the lower layer in the control group. After culture for 6 h, the un-migrated cells on the upper surface of the membrane were swabbed. The migrated cells were fixed with methanol for 20 min, stained with crystal violet. The number of migrated cells was counted in the lower chamber of the filter member under 400× optical microscope in the upper, lower, left, right and central visual field of the microscope. Boyden chamber without Matrigel gel was set as the positive control group to calculate cell migration rate. The cell migration rate was the percentage of number of trans-membrane cells in the experimental group and number of trans-membrane cells in the positive control group. 6 h of culture was selected as the detection point. The changes of bone marrow MSCs migration rate in the experimental group and blank group were compared with the positive control group.

2.5. In vitro differentiation induction and treatment grouping of MC3T3-E1 cells

A piece of rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite, a piece of rhCXCL13-loaded hollow HA microspheres/CS composite, a piece of rhBMP-2-loaded hollow HA microspheres/CS composite and a piece of hollow HA microspheres/CS composite were soaked in EP tube containing 5 mL PBS and stored in 37 °C thermostat. The supernatant was centrifuged every 3 days to measure the rhBMP-2

and rhCXCL13 concentration, and replaced with 5 mL PBS. 10% FBS and 1% double resistance were added to the α -MEM medium and incubated in the 37 °C incubator containing 5% CO₂. In order to promote MC3T3-E1 cells differentiation, 50 μ g/mL ascorbic acid and 5 mM sodium glycerol-phosphatate were added to the medium, and the solution was changed every 3 days. MC3T3-E1 cells were divided into 5 groups (3 replicates in each group) as follows: blank control group, hollow HA microspheres/CS composite group, rhBMP-2-loaded hollow HA microspheres/CS composite group, rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group and rhCXCL13-loaded hollow HA microspheres/CS composite group. Composite material extraction solution was added in a certain proportion during liquid exchange, and equal volume of PBS solution was added in the control group.

2.6. Activity detection of MC3T3-E1 cell treated with composite

Cell activity detection was performed as follows: [1] Those cells in good growth state and logarithmic growth stage were counted for preparation of cell suspension; [2] Cells (2×10^4 /per well) were cultured in 96-well plates and pre-wetted. Each group contained three parallel samples; [3] Cells were induced and treated as required; [4] Cells were cultured for 7, 14 and 21 days in 37 °C thermostat; [5] When the cells grown to the specified time point, the culture medium containing 10% CCK8 was directly configured and added in the form of liquid exchange. After incubation for 1 h at 37 °C, the supernatant was transferred to the 96-well plate. The absorbance at 450 nm was determined by the microplate assay.

2.7. Detection of ALP activity and calcium salt deposition in MC3T3-E1 cells

In order to evaluate cell differentiation ability, ALP activity and calcium salt deposition were tested respectively after 7, 14 and 21 days of after composite treatment in MC3T3-E1 cells. ALP activity was detected by alkaline phosphatase assay Kit. Cell samples were lysed and stored at -80 °C (avoiding repeating freezing and thawing). The 96-well plate was provided with blank control well, standard sample well and sample well. The dosage in the standard sample well was 4, 8, 16, 24, 32 and 40 μ L, respectively. The dosage in the sample well was 5 μ L. The mixture was whisked gently by the tip and incubated for 5 min at 37 °C. 100 μ L reaction termination solutions was added to per well to terminate the reaction. The absorbance was measured at 405 nm. For calcium salt deposition detection, the MC3T3-E1 cells were fixed with paraformaldehyde for 10 min. The slides were washed with PBS three times and then stained with alizarin red S for 3 min. The slides were rinsed with distilled water quickly and took photographs under the microscope. Generally, orange-red staining represents the positive result of calcium salt deposits. The positive staining was determined (showed by OD value) by decolorization enzyme-lable assay.

2.8. Animal models of bone injury

15 New Zealand White male rabbits, all 6 months old with a weight range of 2.0–2.8 kg (average 2.5 kg). All animals were injected with ketamine hydrochloride through the ear vein. The left radius of each animal was prepared for surgery. A 2 cm longitudinal skin incisions was made, and space between extensor and flexor muscle groups were dissected to expose the radial bone. 15 mm lengths of segmental bone defect were created in the middle of the radius shaft using a delicate orthopedic saw. Following surgery, the animals were administered penicillin (intramuscular injection) for 3 days, and monitored daily for regular day-to-day activity, food intake, and clinical signs of infection. Before composite implantation, the defect site was irrigated with physiological saline. All animals were divided into 5 groups of 3 each. Group 1 was blank control group, group 2 received administration of hollow HA microspheres/CS composite, group 3 received

administration of rhBMP-2-loaded hollow HA microspheres/CS composite, group 4 received administration of rhCXCL13-loaded hollow HA microspheres/CS composite and group 5 received administration of rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite into the manipulated bone defect site. Subsequent serum ALP test, X-ray scoring, three-point bending test, histopathological evaluation, RT-PCR and WB were performed 30, 60 and 90 days after composite implantation. All animals were killed by CO₂ inhalation. Our animal studies were approved by the Jiangxi Provincial People's Hospital Affiliated to Nanchang University and were in accordance with the Guidelines for Care and Use of Experimental Animals. In addition, all animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

2.9. ALP test and X-ray scoring of radius in animal models

In order to evaluate the bone repair and osteogenesis in animal, serum ALP test and X-ray scoring were performed. For ALP test, blood samples were collected from the marginal artery of the external ear of the rabbit. Serum ALP activity was detected by alkaline phosphatase assay Kit. The assay method was consistent with that in the MC3T3-E1 cells. For X-ray scoring of radius, radial radiographs were performed to observe callus formation within and between groups of composite implantation. The animal in each group was anesthetized intraperitoneal injection with alpha salon (10 mg/mL) and fixed on the operating table. The white light window was opened in the X-ray machine. The work station was moved so that the surgery limb of the animal was put in the central projection window. Height between surgical limb of the animal and operation table was measured. The ray intensity was adjusted according to the height between surgical limb of the animal and operation table on the computer. X-ray photographs of radius were obtained by pressing the manual button twice.

2.10. Three-point bending test

In order to evaluate the mechanical stability of the regenerated bones, specimens from each group at the time points of 30, 60 and 90 days were subjected to a three-point bending test by using a mechanical testing facility (Model HY-0230, Shanghai Heng Yi Precision Instrument, Shanghai, People's Republic of China) at ambient temperature and humidity [26]. In brief, specimens were positioned on two supports spaced 18 mm apart, and the bending load was applied at the midpoint of the specimen at a constant displacement rate of 1 mm/min to break. The data generated in the measurement were automatically recorded.

2.11. RT-PCR and WB in animal models

In order to test the osteogenic capability in molecular level, the mRNAs and proteins in the animal model were extracted using standard histological techniques. The mRNA expression of osteogenic marker genes including Cbfa1, Osterix, OPN, OC, BSP and Coll was tested. The mRNAs expression quantity was presented as log₂ (Fold change). It is known that runt related transcription factor 2 (Runx2) is important transcription factors in the process of osteoblast and osteoclast differentiation, cartilage cells mutation and extracellular matrix secretion and bone growth. In this study, the protein expression of Runx2 was also tested. The β -actin was used for internal reference.

2.12. Statistical analysis

All quantitative data were analyzed with SPSS22 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were carried out using Student's *t*-test analysis. Statistical significance was presented as *P* value < 0.05.

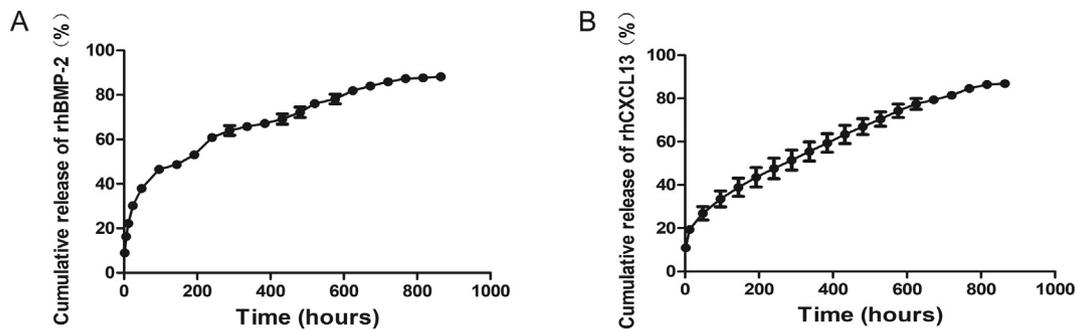


Fig. 1. A Cumulative release of rhBMP-2 from hollow HA microspheres/CS composites into PBS. B Cumulative release of rhCXCL13 from hollow HA microspheres/CS composites into PBS.

3. Results

3.1. RhBMP-2 and rhCXCL13 were released effectively in vitro

The rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites were soaked and centrifuged to obtain the supernatant for concentration test of rhBMP-2 and rhCXCL13. The concentration of rhBMP-2 and rhCXCL13 released at selected time points from hollow HA microspheres/CS composites into the surrounding PBS was showed in Fig. 1A and Fig. 1B, respectively. As showed in Fig. 1, rhBMP-2 and rhCXCL13 were mainly released within the first 48 h. The cumulative release of rhBMP-2 at 2, 6, 12, 24 and 48 h was 8.97%, 16.31%, 22.24%, 30.23% and 37.93%, respectively. After the third day, the cumulative release rate slowed down. On the 35th day, the cumulative release reached to 87.73% (Fig. 1A). The cumulative release of rhCXCL13 at 2, 6, 12, 24 and 48 h was 10.95%, 14.51%, 19.36%, 23.36% and 26.84%, respectively. On the 35th day, the cumulative release reached to 84.38% (Fig. 1B).

3.2. RhBMP-2 promoted the growth of rat bone marrow MSCs cells

RhBMP-2 sustained release solution from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites was used to culture rat bone marrow MSCs for detection of ALP activity (Fig. 2). From Fig. 2, we could see that the microspheres carrying rhBMP-2 could significantly promote the growth of bone marrow MSCs, and the effect was more significant with the extension of time. After culturing for 4 and 7 days, the microspheres carrying rhBMP-2 could better promote cell proliferation and differentiation than the control group, which suggested that the hollow HA microspheres/CS composites had good controlled release effect.

RhCXCL13 promoted the migration of rat bone marrow MSCs cells.

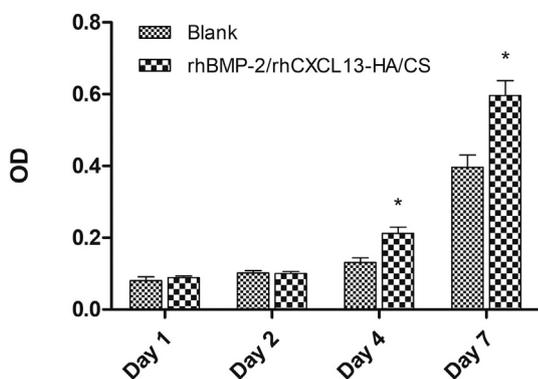


Fig. 2. Effect of rhBMP-2 released from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites on ALP activity of rat bone marrow MSCs cells. * represents significance of ALP activity in each treatment group ($P < 0.05$) compared with the blank control group.

RhCXCL13 sustained release solution from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites was used to culture rat bone marrow MSCs for detection of cell migration rate (Fig. 3). From crystal violet staining, we could see that the invasive cell number of bone marrow MSCs in the rhCXCL13 group was more than that of blank group (Fig. 3A). The cell migration rate of bone marrow MSCs in the control and experimental group was $(5.90 \pm 1.41)\%$ and $(13.39 \pm 5.88)\%$, respectively (Fig. 3B). It was can be seen that rhCXCL13 in the microspheres had good activity and was not inactivated during composites loading and subsequent release.

3.3. Activity of MC3T3-E1 cell was not affected by the composite

In order to test whether the loaded composites affect the state of MC3T3-E1 cell, CCK-8 assay kit was used to detect cell activity at 7, 14 and 21 days (Fig. 4). As showed in Fig. 4, there was no significant difference in cell proliferation rate among five groups. This indicated that the loaded composites had no effect on growth of MC3T3-E1 cell.

3.4. ALP activity was increased in MC3T3-E1 cells

ALP can appropriately reflect the degree of proliferation and activity of bone and chondrocytes, which can be regarded as an important indicator of bone regeneration. In this study, ALP activity was tested after 7, 14 and 21 days of composite treatment in MC3T3-E1 cells (Fig. 5). After treatment for 7, 14 and 21 days, rhBMP-2 can significantly promote the ALP activity in the rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group.

3.5. The calcium salt deposition was observed in MC3T3-E1 cells

In fact, calcium salt deposition is also an evaluation indicator of osteoblast differentiation, except ALP. In the present study, the calcium salt deposition was also tested after 7, 14 and 21 days of composite treatment in MC3T3-E1 cells (Fig. 6). In Fig. 6A, the calcium salt deposition (orange-red staining) was most significantly observed in the rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group on day 21. The statistical analysis in Fig. 6B showed that rhBMP-2 could significantly promote the calcium salt deposition in the rhBMP-2-loaded hollow HA microspheres/CS composite group and rhCXCL13-loaded hollow HA microspheres/CS composite group on day 14 and rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group on day 21.

3.6. ALP activity was increased in animal models

We have tested the ALP activity after 7, 14 and 21 days of composite

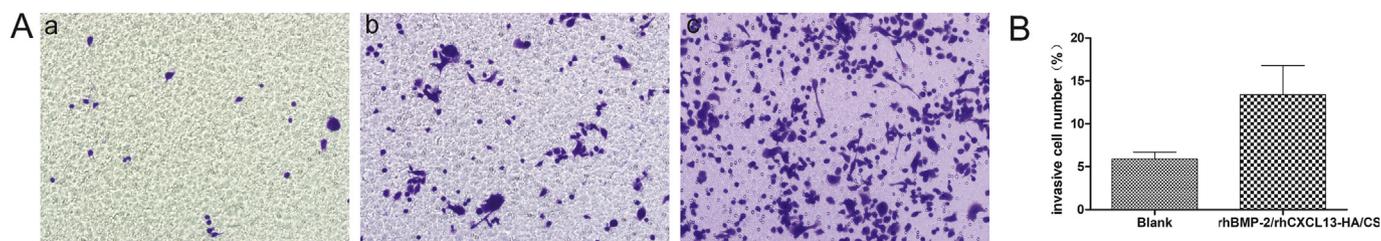


Fig. 3. Effect of rhCXCL13 released from rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composites on the migration of rat bone marrow MSCs cells. A: Bone marrow MSCs stained with crystal violet in cell migration test of by the Boyden chamber method. a: blank group; b: experimental group; c: positive control group B: Statistic analysis of invasive cell number of bone marrow MSCs in cell migration test of by the Boyden chamber method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

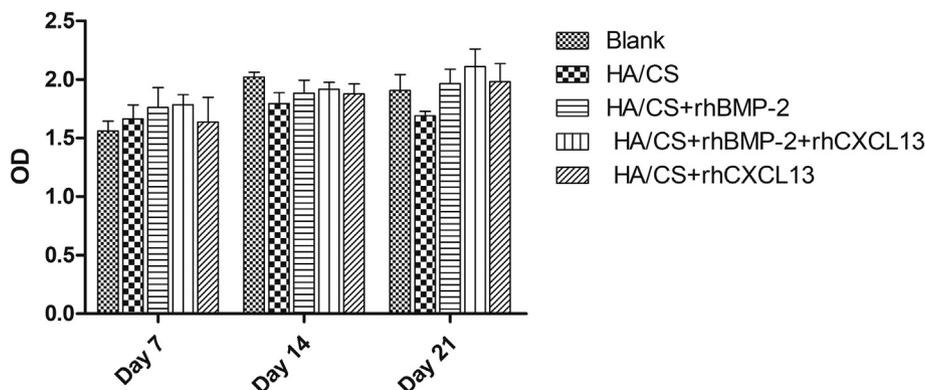


Fig. 4. Cell activity detection of MC3T3-E1 cell treated with composite. MC3T3-E1 cells were divided into 5 groups as follows (3 replicates in each group): Blank, HA/CS, HA/CS + rhBMP-2, HA/CS + rhBMP-2 + rhCXCL13 and HA/CS + rhCXCL13.

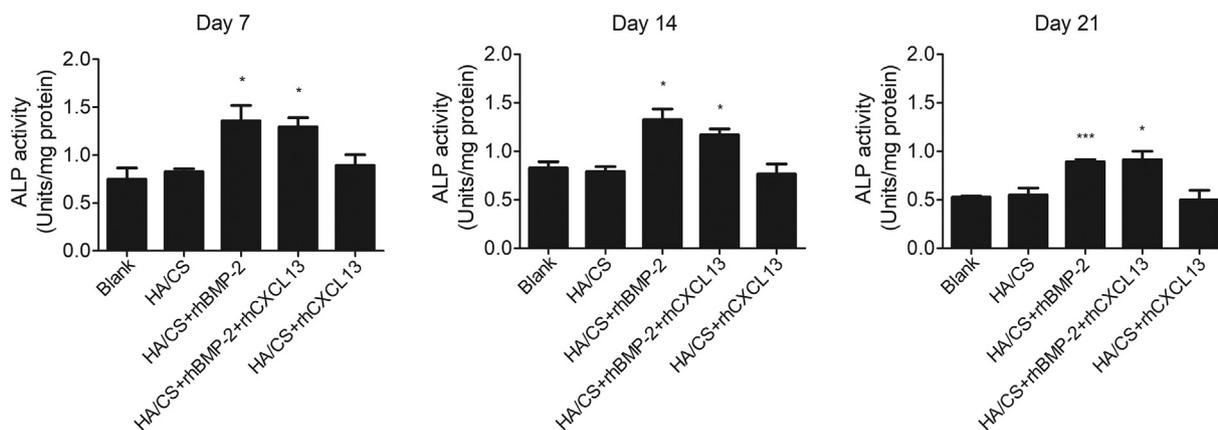


Fig. 5. ALP activity in MC3T3-E1 cells after 7, 14 and 21 days of composite treatment. * and *** represent significance of ALP activity in each treatment group ($P < 0.05$ and $P < 0.001$) compared with the blank control group.

treatment in MC3T3-E1 cells, similarly, we further test the ALP activity in the animal models after 30, 60 and 90 days of composite implantation (Fig. 7). Compared with the control group, the ALP activity was significantly increased in rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group on day 30, 60 and 90. It is suggested that the bone and chondrocyte hyperplasia was active in above two groups, especially in rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group.

3.7. New bone formation was observed in X-ray of the radius in animal models

X-ray radiography in Fig. 8 showed new bone formation in each

group. Bone callus was observed in rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group, but was rarely seen in other groups. Medullary cavities between the two ends of the fractured region were mostly connected in rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group. On day 90, the group of rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite achieved complete healing with extensive bone formation and medullary cavity recanalization.

Flexural strength of the radius was increased in animal models. The flexural strength of the newly regenerated bones was examined by biomechanical test (three-point bending test) with results shown in Fig. 9. Maximum load values in rhBMP-2-loaded hollow HA

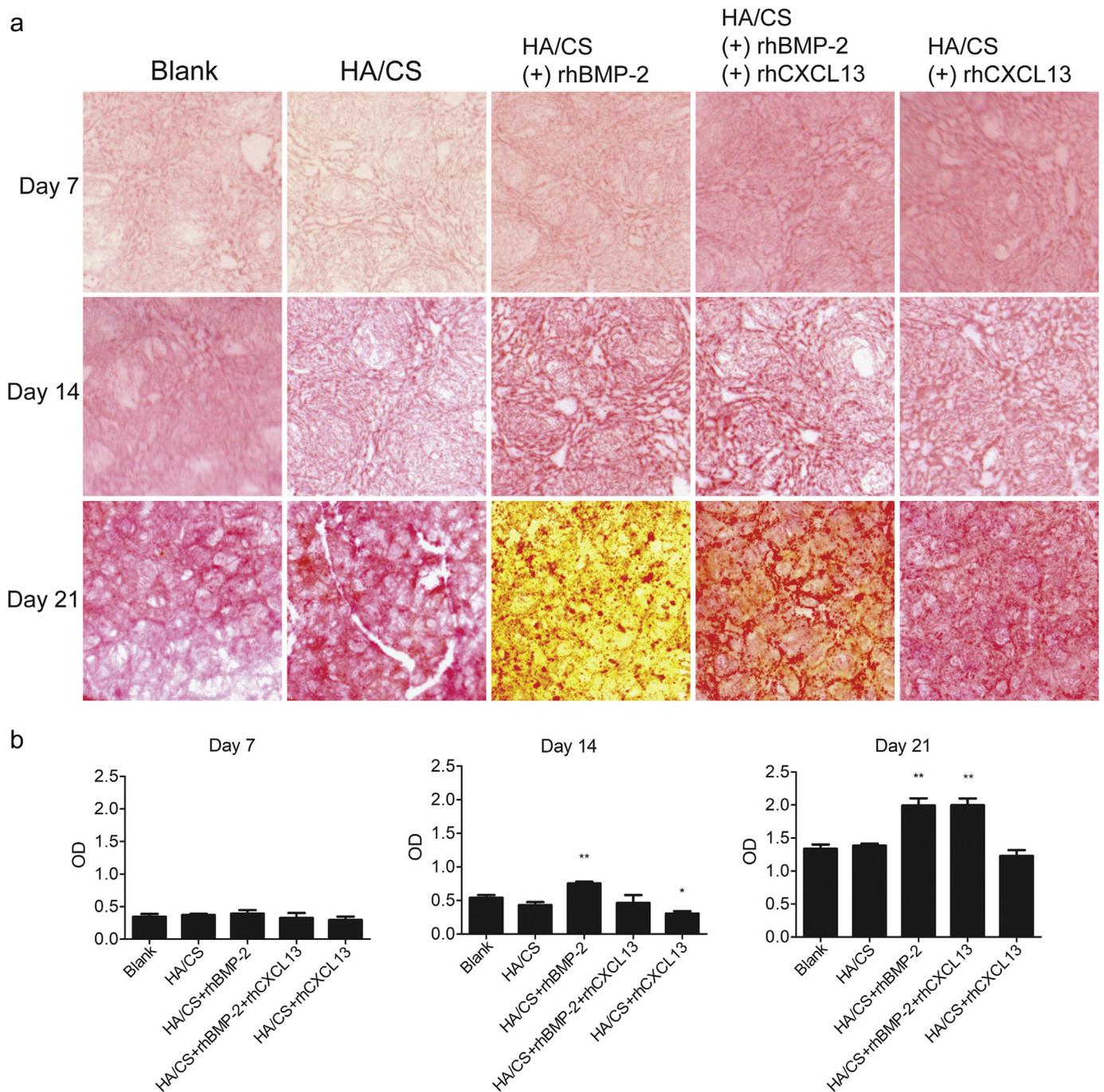


Fig. 6. Calcium salt deposition levels in MC3T3-E1 cells after 7, 14 and 21 days of after composite treatment.

A: Alizarin red S staining of MC3T3-E1 cells after 7, 14 and 21 days of after composite treatment

B: Calcium salt deposition detection in MC3T3-E1 cells after 7, 14 and 21 days of after composite treatment. * and ** represent significance of calcium salt deposition in each treatment group ($P < 0.05$ and $P < 0.01$) compared with the blank control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microspheres/CS composite group, rhCXCL13-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group continuously increased during the time course of 30 to 90 days. At each time point, maximum load value in rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group was always higher than in other groups.

3.8. The expression of bone formation related molecules was up-regulated in RT-PCR and WB

In order to test the mRNA expressions of osteogenic markers including Cbfa1, Osterix, OPN, OC, BSP and Coll, RT-PCR was performed in the animal model (Fig. 10). The mRNA expressions of Cbfa1, Osterix, OPN, OC, BSP and Coll in animal model were all significantly up-regulated in both rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group after 30, 60 and 90 days of composite implantation.

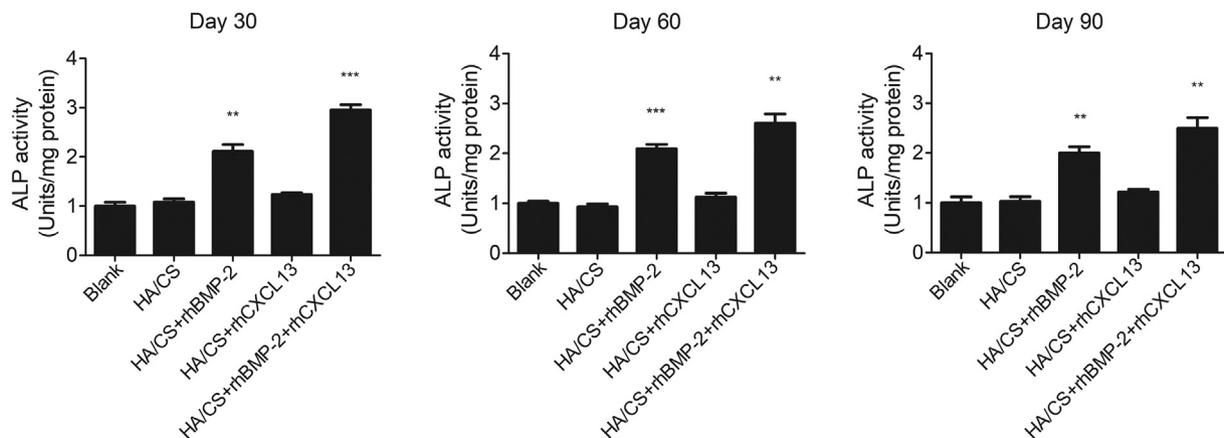


Fig. 7. ALP activity in animal models after 30, 60 and 90 days of composite treatment. ** and *** represent significance of ALP activity in each treatment group ($P < 0.01$ and $P < 0.001$) compared with the blank control group.

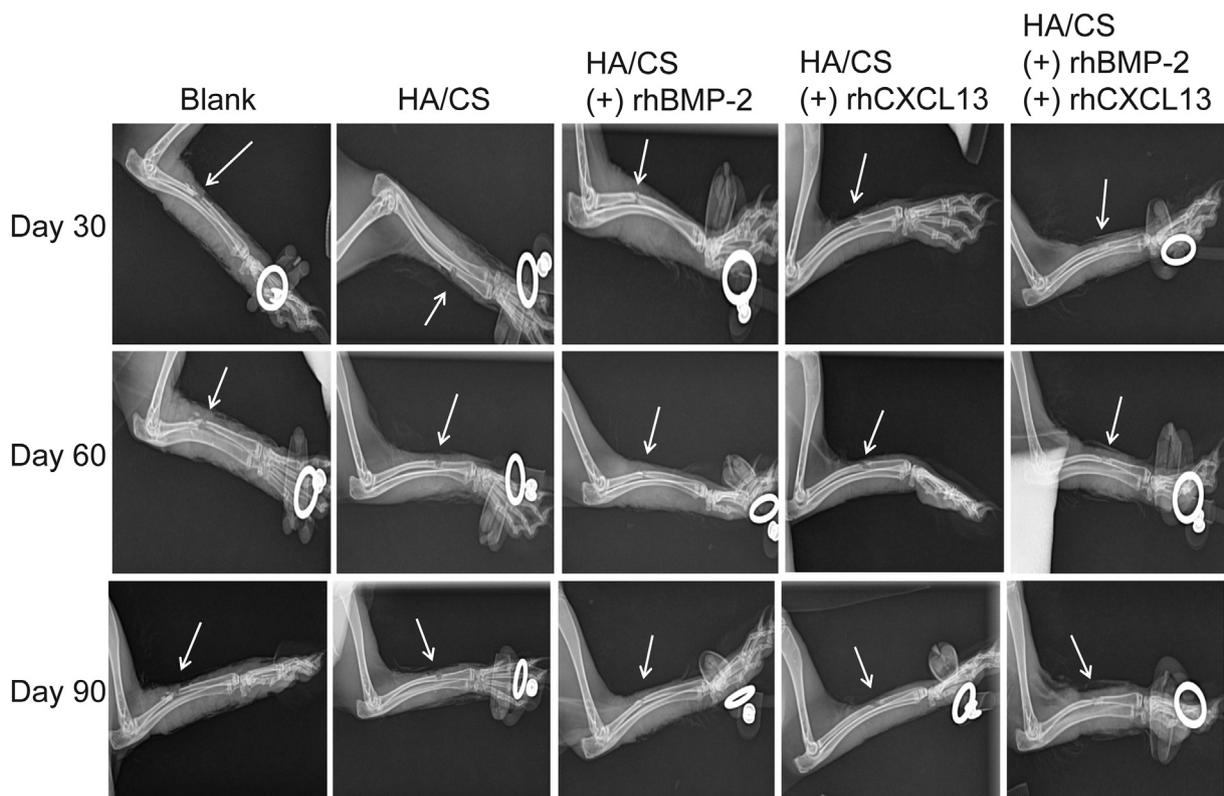


Fig. 8. Representative radiographs of the defect sites at different days after composite implantation. Arrow represented the defect site.

In addition, the protein expression of Runx2 was also tested (Fig. 11). Compared with the control group, the expression of Runx2 in rhBMP-2-loaded hollow HA microspheres/CS composite group and rhBMP-2/rhCXCL13-loaded hollow HA microspheres/CS composite group was increased with time.

4. Discussion

In this study, we showed that hollow HA microspheres/CS composite could support high release capacity for rhBMP-2 and rhCXCL13. Moreover, rhBMP2 and rhCXCL13 are continuously released from loaded hollow HA microspheres/CS composite over 30, which was manifested as obvious release effect. Lutichau et al. [27] has demonstrated that CXCL13 had good chemotaxis activity on MSCs by Boyden

chamber method. This suggested that directional migration effect of CXCL13 on MSCs can induce MSCs to move towards the injury site, which is crucial for reversing the stem cell conversion crisis and promoting the bone repair process [25]. Therefore, in order to investigate whether rhCXCL13 affect the migration of rat bone marrow MSCs cells, the rhCXCL13 sustained release solution from the composites was used to culture rat bone marrow MSCs in this study. Our result showed that the invasive cell number of bone marrow MSCs in the rhCXCL13 group was more than that of the blank group. Moreover, the cell migration rate of bone marrow MSCs in the control and experimental group was $(5.90 \pm 1.41)\%$ and $(13.39 \pm 5.88)\%$, respectively. This indicated that rhCXCL13 had the tendency that could promote the migration of rat bone marrow MSCs cells. In addition, we also found that rhBMP2 sustained release solution from the composites also could significantly

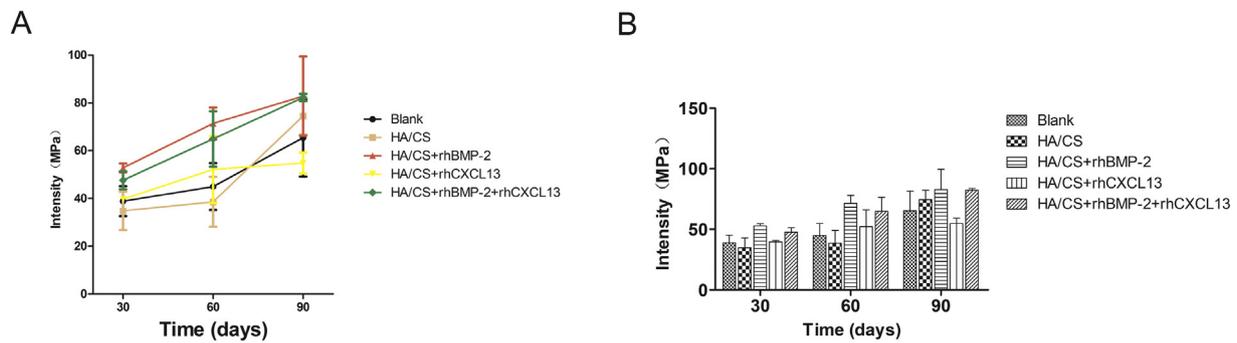


Fig. 9. The maximum flexural strength of defective radius in animal models after 30, 60 and 90 days of composite implantation. A: The line chart of the maximum flexural strength of defective radius. B: The statistical chart of the maximum flexural strength of defective radius.

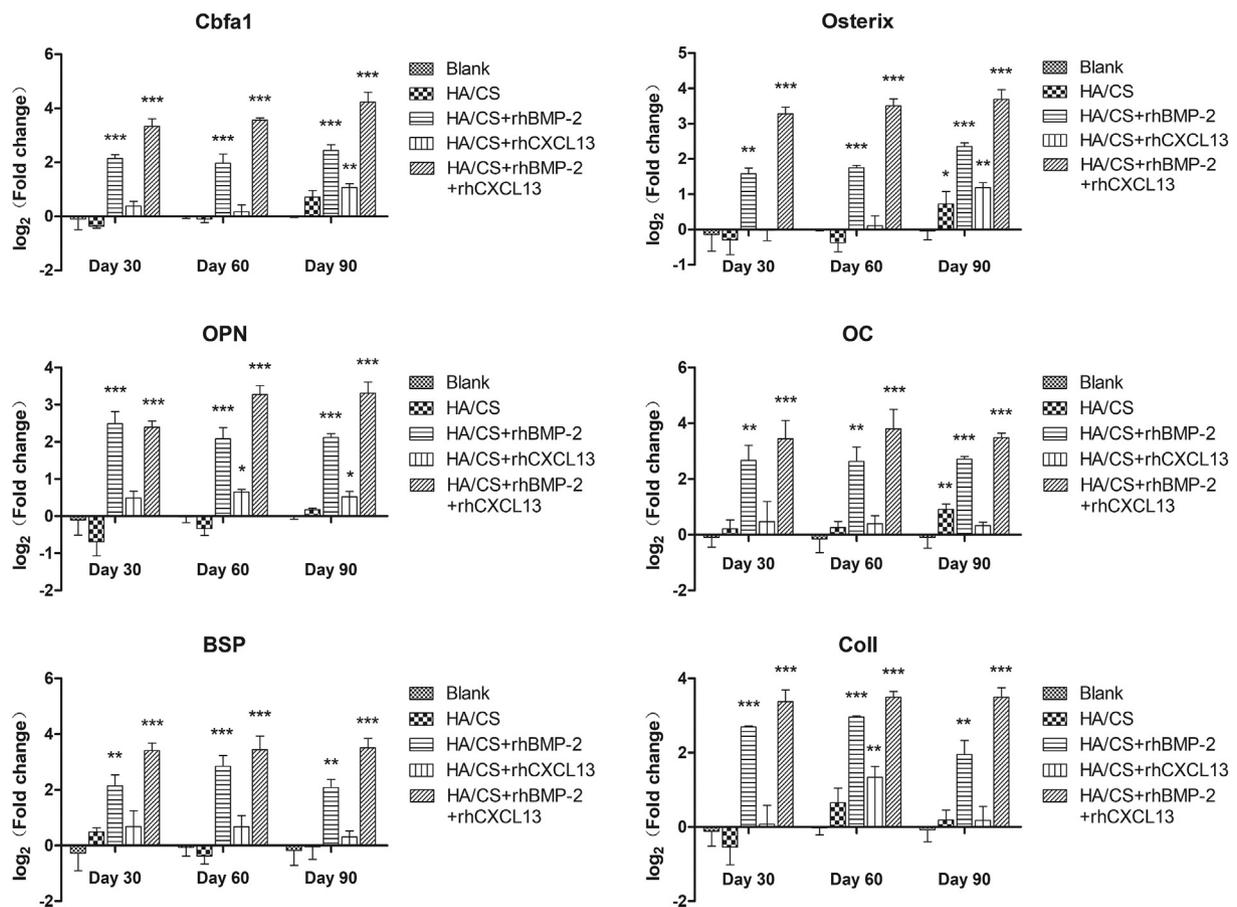


Fig. 10. The mRNA expressions of osteogenic markers including Cbfa1, Osterix, OPN, OC, BSP and Coll on day 30, 60 and 90 in the animal model. Log₂ (Fold change) > 0 and Log₂ (Fold change) < 0 represent up-regulation and down-regulation, respectively. *, ** and *** represent significance at each treatment group (P < 0.05, P < 0.01 and P < 0.001) compared with blank control group.

promote the proliferation and differentiation of bone marrow MSCs, and the effect was more significant with the extension of time. This indicated that rhBMP2 could remarkably improve the biological activity of bone marrow MSCs recruited by rhCXCL13 and play an important role in promoting cell proliferation, differentiation and tissue regeneration.

It is well-known that the main osteogenesis indexes are ALP activity and calcium salt nodes. In order to evaluate the osteogenic capability, detection of ALP activity and calcium salt deposition were performed in MC3T3-E1 cells and animal models. In MC3T3-E1 cells, rhBMP2 can significantly promote the ALP activity in the rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded

hollow HA microspheres/CS composite group. In addition, rhBMP2 could significantly promote the calcium salt deposition in the rhBMP2-loaded hollow HA microspheres/CS composite group, rhCXCL13-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group. In animal models, the ALP activity was significantly increased in rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group. It is suggested that the bone and chondrocyte hyperplasia was active in above two groups, especially in rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group. In summary, our result indicated that rhCXCL13 could recruit and supplement sufficient number and vitality of seed

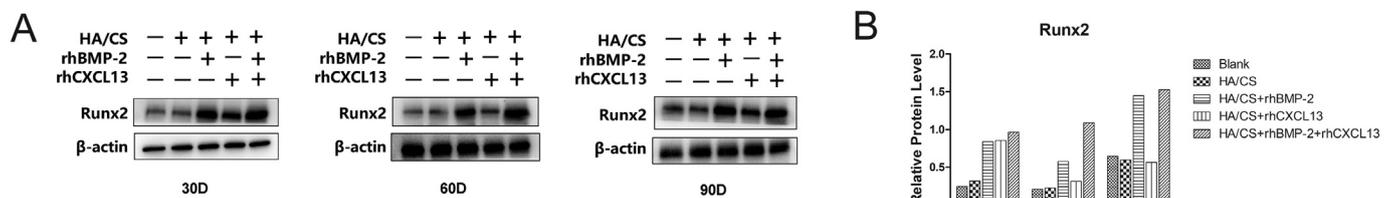


Fig. 11. The protein expressions of Runx2 on day 30, 60 and 90 of composite implantation in the animal model.

A: The chart of Runx2 protein bands.

β-actin is the internal reference; “+” and “-” represent treatment or no treatment, respectively. D: Days.

B: The statistical chart of Runx2 protein expression in 5 groups including Blank, HA/CS, HA/CS + rhBMP-2, HA/CS + rhCXCL13 and HA/CS + rhBMP-2 + rhCXCL13.

cells, such as bone marrow MSCs. Then, rhBMP2 would promote the proliferation and differentiation of these cells, and could better promote bone regeneration and repair of bone defects.

Beside ALP activity detection, we also performed the X-ray scoring of radius and flexural strength test in the animal model to further observe the capability of bone formation. In X-ray scoring of radius test, bone callus was observed in rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group, but was rarely seen in other groups. Furthermore, medullary cavities between the two ends of the fractured region were mostly connected in rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group. On day 90, the group of rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite achieved complete healing with extensive bone formation and medullary cavity recanalization. In the flexural strength test, maximum load values in rhBMP2-loaded hollow HA microspheres/CS composite group, rhCXCL13-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group continuously increased during the time course of 30 to 90 days. Moreover, the maximum load value in rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group was always higher than in other groups at each time point. Our result suggested that rhBMP2 and rhCXCL13 could promote bone formation and improve the bending resistance of bone tissue.

In the molecular level, we tested the mRNA expressions of osteogenic markers including Cbfa1, Osterix, OPN, OC, BSP and Coll and the protein expression of Runx in animal models. The mRNA expressions of Cbfa1, Osterix, OPN, OC, BSP and Coll were all significantly up-regulated in both rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group. In addition, the protein expression of Runx2 in rhBMP2-loaded hollow HA microspheres/CS composite group and rhBMP2/rhCXCL13-loaded hollow HA microspheres/CS composite group increase with time. This indicated that rhBMP2 and rhCXCL13 could promote osteoblasts differentiation and bone healing.

5. Conclusions

In a word, our study has demonstrated that the hollow HA microspheres/CS composite can serve as a bioactive and osteoconductive carrier for rhBMP2 and rhCXCL13 in bone regeneration, warranting further development of hollow HA microspheres/CS composite as potential bone graft substitutes. In addition, BMP2 may promote osteoblast differentiation of seed cells recruited by CXCL13, which would play an important role in large bone defects.

Acknowledgments

This study was funded by National Natural Science Foundation of

China of “Effect of non-Smad-dependent signaling pathway in the bone repairing of rhBMP-2/hollow HA microspheres/CS composite” (81560355) and “Hollow hydroxyapatite microspheres/chitosan composite as a sustained delivery vehicle for rhBMP-2/rhCXCL13 in the treatment of bone defects” (81560377).

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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