

Effect of recombinant human bone morphogenetic protein delivered by chitosan microspheres on ectopic osteogenesis in rats

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Abstract. In the present study, the effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) delivered by chitosan (CS) microspheres on ectopic osteogenesis was investigated in a rat model. rhBMP-2-loaded CS microspheres and blank CS microspheres were prepared. A total of 24 male Sprague Dawley rats were divided into 4 groups with 6 rats in each group: The CS/rhBMP-2 group, the rhBMP-2 group, in which rhBMP-2 was directly implanted (rhBMP-2 dose in either group, 1 mg), the CS blank group and the control group. X-ray was performed at 4 weeks after ectopic osteogenesis surgery and micro-computed tomography (CT) examination was scheduled at 1, 2, 3 and 4 weeks after the surgery to determine ectopic osteogenesis in the different groups. Histological analysis, and determination of alkaline phosphatase (ALP) activity and calcium content were also performed. The mean diameter of the osteoid tissues was 1.1 ± 0.3 cm (range, 0.8-1.4 cm) in the CS/rhBMP-2 group, which was significantly bigger than that in the rhBMP-2 group (0.3 ± 0.1 cm; range, 0.1-0.4 cm) at 4 weeks after the surgery. X-ray analysis and micro-CT scan indicated that the area of high-density tissues and the radionuclide intensity, as well as bone volume in the 3-dimensional reconstruction were greatest in the CS/rhBMP-2 group, followed by those in the rhBMP-2 group. All parameters, including bone mineral density, tissue mineral density, tissue mineral content and bone volume fraction, were significantly higher in the CS/rhBMP-2 group at 3 and 4 weeks after the surgery,

compared with those in the rhBMP-2 group. The histological analysis, ALP activity analysis and determination of calcium content revealed that the CS/rhBMP-2 system had the greatest ability to induce osteoblast differentiation. In conclusion, the CS/rhBMP-2 microsphere delivery system significantly enhanced the induction and promotion effects of rhBMP-2 regarding ectopic osteogenesis. The present study enhances the basic data available for future application of the CS/rhBMP-2 microspheres delivery system and provides a deeper understanding of the role of BMP-2 in bone regeneration.

Introduction

Since bone tissue engineering has provided an efficient tool for bone regeneration, numerous studies have focused on associated effects and the underlying mechanisms (1). Several different growth factors have been indicated to stimulate bone growth (2), collagen synthesis (3) and fracture repair (4), and have thus attracted the attention of scholars regarding their effects on bone regeneration.

Among the factors, bone morphogenetic proteins (BMPs) have been proven to facilitate bone healing without bone tissue transfer (5). Studies have indicated that BMP-2, a protein of BMP family, has important roles in bone generation-associated processes, including osteoblastic differentiation (6), the healing process of segmental bone defects (7) and the capacity of bone marrow stromal cells (BMSCs) to undergo osteogenesis (8), and has been approved by the Food and Drug Administration of the USA for clinical use in the orthopedic and dental fields (9). However, large amounts of BMP-2 are required to achieve clinically significant bone regeneration and BMP-2 is easily inactivated by dilution or interaction with enzymes in the blood if applied alone by intravenous injection (10-12). To enhance the bioavailability of BMP-2, numerous local delivery systems have been assessed (13), among which the Chitosan (CS) delivery system was proven efficient and enhanced the osteogenic activity (14). Certain studies also reported that BMP-2 promotes ectopic osteogenesis (15,16). However, few studies have focused on the effects of BMP-2 delivery systems, e.g. CS-based delivery systems, on processes of ectopic osteogenesis.

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To the best of our knowledge, it has remained elusive whether the CS/human recombinant (rh)BMP-2 microsphere delivery system influences the ectopic osteogenesis process. The present study aimed to investigate the effect of rhBMP-2 delivered by CS microspheres on ectopic osteogenesis in rats. It provides basic data for future application of the CS/rhBMP-2 microsphere delivery system and a deeper understanding of the roles of BMP-2 in bone regeneration.

Materials and methods

Preparation of CS blank microspheres and CS/rhBMP-2 microspheres. The preparation of CS blank microspheres and CS/rhBMP-2 microspheres was performed using a procedure modified from a previous study (17), namely 42 ml thiamine pyrophosphate solution was added instead of DS and ZnSO₄. In brief, to prepare CS blank microspheres, a certain amount of CS (molecular weight, 50,000-190,000; Aladdin Reagent Co. Ltd., Shanghai, China) was dissolved in 1% (v/v) acetic acid and the pH was adjusted to 5.4 by addition of NaOH solution to obtain a mixture with a CS concentration of 1.52 mg/ml. The mixture was then filtered through a membrane with 0.22 μm pores and 100 ml of the filtrate was stirred for 1 h at room temperature, followed by addition of 42 ml thiamine pyrophosphate solution (0.5 mg/ml). When the nanospheres formed, the solution turned from clear to an emulsion and the reaction was continued until no alteration appeared. After completion of the reaction, the mixture was stirred for another 30 min at room temperature and centrifuged for 15 min at 25,000 x g at room temperature. The precipitate was washed with water and dried under cryogenic conditions with reduced pressure.

To prepare the CS/rhBMP-2 microspheres, 100 mg dried CS blank microspheres and 5 mg rhBMP-2 were added to 25 ml double-distilled water, followed by stirring for 30 min at 4°C and subsequent centrifugation for 15 min at 25,000 x g at room temperature. The liquid supernatant was then collected and dried to obtain CS/rhBMP-2 microspheres.

Characterization of CS/rhBMP-2 microspheres. The microsphere morphology and grain diameter were analyzed using a scanning electron microscope (S-4800; Hitachi Ltd., Tokyo, Japan) and a laser diffraction particle size analyzer (N5; Beckman Coulter, Brea, CA, USA). The shape and size of microspheres were determined and the grain diameter was calculated. All experiments were performed in triplicate.

Determination of the entrapment efficiency and drug loading ratio. As described previously (16), the drug loading ratio and entrapment efficiency of CS/rhBMP-2 nanoparticles were calculated by using an ELISA kit (cat. no ELH-BMP2-1; RayBiotech Inc., Norcross, CA, USA). All experiments were performed in triplicate. The optical density of the supernatant was determined at 450 nm according to the protocol for the ELISA kit.

In vitro sustained-release profile and degradation. The measurement of the *in vitro* sustained-release profile was in accordance with that of a previous study by our group and another study (17,18). In brief, 50 mg CS/rhBMP-2

microspheres were immersed in 2 ml PBS (pH 7.4), followed by agitation in a water bath oscillator. The solution was centrifuged for 15 min (25,000 x g) at room temperature and 100 μl supernatant was taken every 3 days at 6, 12, 18, 24, 48 and 72 h. Each time the supernatant was obtained, 100 μl PBS was added and the solution was agitated in a water bath oscillator. The experiments lasted for 30 days. The content of rhBMP-2 was measured using the ELISA kit as described above.

For measurement of the degradation of the CS/rhBMP-2 microspheres, 50 mg microspheres (weight, m₀) was added in a centrifuge tube with 2 ml PBS (pH 7.4), followed by agitation in a water bath. Every 3 days, 3 tubes were randomly selected and the microspheres in each tube were dried for determining the weight (m_i). The experiment lasted for 45 days and the *in vitro* degradation was calculated as $D = (m_0 - m_i) / m_0 \times 100\%$. All experiments were performed in triplicate.

Animals and treatment. A total of 24 male Sprague Dawley (SD) rats were provided by the Experimental Animal Center of Southern Medical University (Guangzhou, China). The age of all rats was 6 weeks and their weight was 130-160 g. The rats were kept under a 12-h light/dark cycle and at a constant temperature (23-25°C) and relative humidity (70%). All animals were housed in micro-isolator cages with free access to food and water according to the Guide for the Care and Use of Laboratory Animals of China (19). Any effort was made to avoid any unnecessary pain for the animals. The protocol of the present study was approved by the Institutional Animal Care Committee at General Hospital of Southern Theater Command, People's Liberation Army (Guangzhou, China).

All rats were divided into 4 groups with 6 rats in each group: The CS/rhBMP-2 group, which was treated with microspheres loaded with 1 mg rhBMP-2, the rhBMP-2 group in which 1 mg rhBMP-2 was directly implanted, the CS blank group, which was treated with unloaded microspheres of the same size and the control group, which was implanted with a gelatin sponge of the same size. All microspheres were sterilized by irradiation of 3,000 Gy⁶⁰Co and stored at 4°C prior to the experiments.

Ectopic osteogenesis experiment and measurement. All SD rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg/kg). A 1.5-cm incision was made on the left leg of each rat and the muscle bag model of quadriceps femoris was generated. The abovementioned materials (microspheres or rhBMP-2) were implanted into the muscle bag and the wound was then sutured. After the surgery, all rats were intraperitoneally injected with 4x10⁵ units of penicillin every day for 3 days. The hardness of the tissue around the implant was analyzed by palpation every day.

X-ray imaging was performed at 4 weeks after the surgery and micro-computed tomography (CT) examination was performed at 1, 2, 3 and 4 weeks after the surgery to determine the ectopic osteogenesis in the different groups of animals. The grafts were evaluated using a micro-CT apparatus from GE Healthcare (Little Chalfont, UK) with the following parameters: 80 kV, 0.6 mm, 80 μA and exposure time, 3,000 msec. The grafts were evaluated for osteogenesis capacity based on

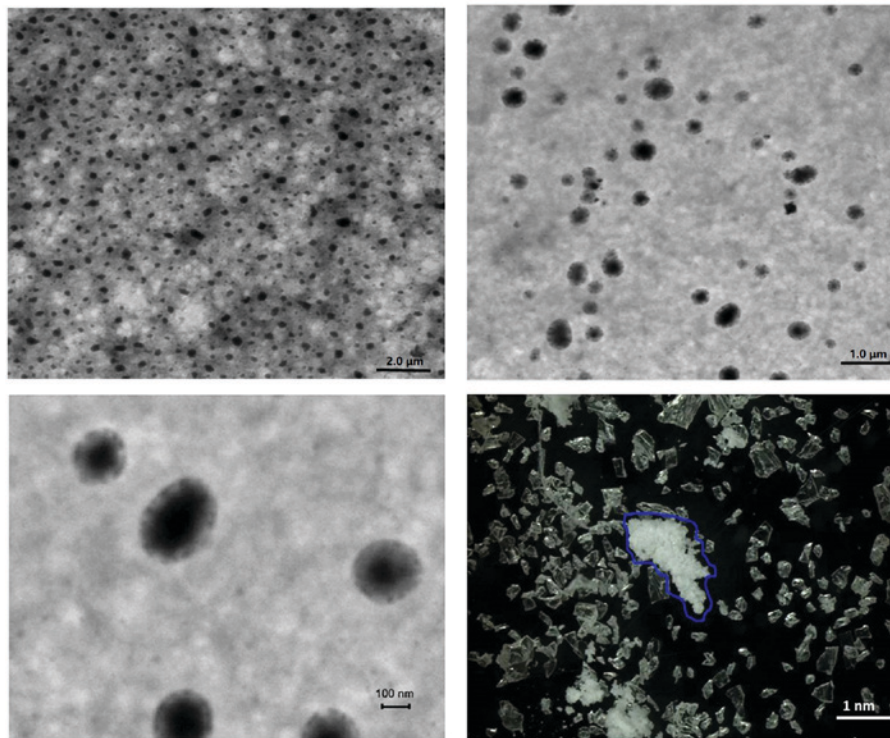


Figure 1. Morphology of CS/rhBMP-2 microspheres. CS/rhBMP-2 microspheres featured spherical regularization, dispersed evenly, and the surface of the whole sphere was smooth and unwrinkled, with rhBMP-2 in the spheres. The blue line refers to the protein in the nanosphere. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

the following morphometric indices: Bone mineral density (BMD), tissue mineral density (TMD), tissue mineral content (TMC) and bone volume fraction (BVF).

Histology. The animals were sacrificed at 4 weeks after the surgery. Tissues around the implants were obtained and fixed in formalin buffer. The samples were subjected to H&E staining and the histologic morphology in each group was observed under an inverted microscope.

Alkaline phosphatase (ALP) activity analysis and determination of the calcium content. The implants were taken out at 4 weeks after the surgery. For each animal, 0.5 g tissue around the implants was obtained, ground and washed with deionized water. Cell Lysis solution (RIPA lysis buffer; Beyotime Institute of Biotechnology, Haimen, China) was added and the mixture was centrifuged at 16,000 \times g for 30 min at 4°C. ALP activity was determined using an ALP Detection Kit (cat. no. A059-2; Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) according to manufacturer's protocols. The calcium content of the tissues was determined using an Atomic absorption spectrophotometer (i7500; Hitachi Ltd.) by using the following formula: Calcium content=calcium content (μ g)/sample wet weight (mg).

Statistical analysis. The measurement data are expressed as the mean \pm standard deviation. Comparison between two groups was performed using the Student's t-test. Comparison among three or more groups was performed using one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

All calculations were made using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Characterization of CS/rhBMP-2 microspheres. First, the physicochemical properties of the CS/rhBMP-2 microspheres were determined. As presented in Fig. 1, analysis of microsphere morphology indicated that the CS/rhBMP-2 microspheres demonstrated spherical regularity and the surface of the spheres was smooth and unwrinkled, with rhBMP-2 contained in the spheres. The grain diameter analysis indicated that the grain diameter of CS/rhBMP-2 microspheres ranged from 58.8 to 955 nm, with the diameters being mainly distributed within the 142-531 nm range (79.3%; Fig. 2). The mean diameter of the CS/rhBMP-2 microspheres was 230 nm. The entrapment efficiency for CS/rhBMP-2 microspheres was determined to be $66.9 \pm 4.6\%$ and the drug loading ratio was $33.4 \pm 2.3 \mu\text{g}/\text{mg}$.

In vitro sustained-release profile and degradation. As presented in Fig. 3A, the release of rhBMP-2 lasted for 30 days and was in consistency with biphasic dynamics. The initial phase was a fast drug release phase with a cumulative drug release rate of $45.9 \pm 3.8\%$ at the first 48 h and a cumulative drug release rate of $66.0 \pm 4.4\%$ at the first 6 days. The posterior phase was a slow release phase with a cumulative drug release rate of $80.8 \pm 4.8\%$ at 15 days and a cumulative drug release rate of $90.1 \pm 3.6\%$ at 30 days.

The degradation assay indicated that the weight of the microspheres gradually decreased and the degradation rate

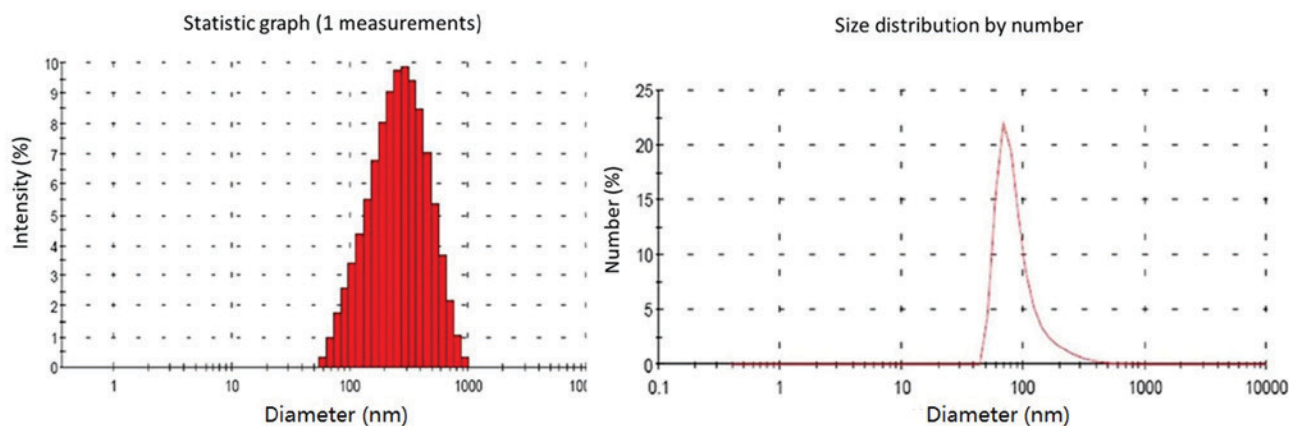


Figure 2. Grain diameter analysis by a laser diffraction particle size analyzer. Grain diameters of CS/rhBMP-2 microspheres ranged from 58.8 to 955 nm, mainly distributed within the 142-531 nm range (79.3%). The mean diameter for CS/rhBMP-2 microspheres was 230 nm. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

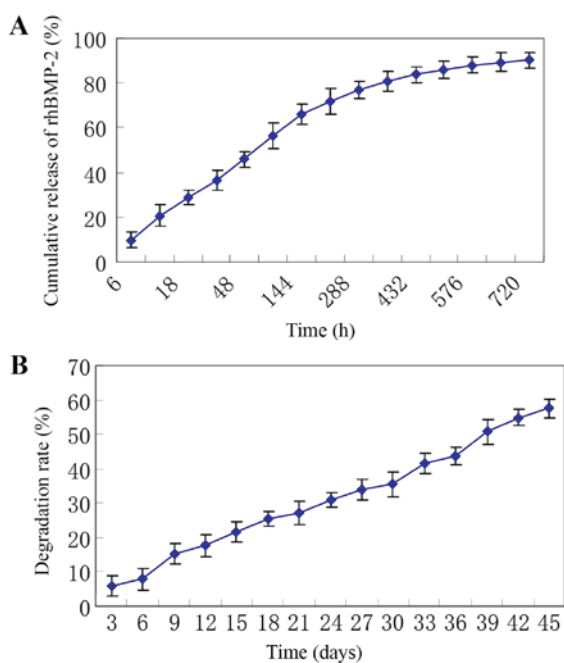


Figure 3. (A) *In vitro* sustained release profile. The release of rhBMP-2 lasted for 30 days. (B) Degradation analysis indicated that the weight of the microspheres gradually decreased and the degradation rate increased with time. rhBMP-2, recombinant human bone morphogenetic protein-2.

increased with time, with a degradation rate of $21.5 \pm 3.0\%$ at 15 days, $35.6 \pm 3.6\%$ at 30 days and $57.6 \pm 2.8\%$ at 45 days (Fig. 3B).

General observations in the ectopic osteogenesis experiment.

After the surgery, the rats were returned to their cages with free access to food and water, and the surgical wounds healed well in all animals. After 3 weeks, the area around the implants became hard in the CS/rhBMP-2 group; however, the other groups exhibited no obvious change. After 4 weeks, the area around the implants became hard in the CS/rhBMP-2 group and in the rhBMP-2 group, and osteoid tissues were found in each of these two groups at the area around the implants; however, no such osteoid tissues were present in the CS blank and the control groups.

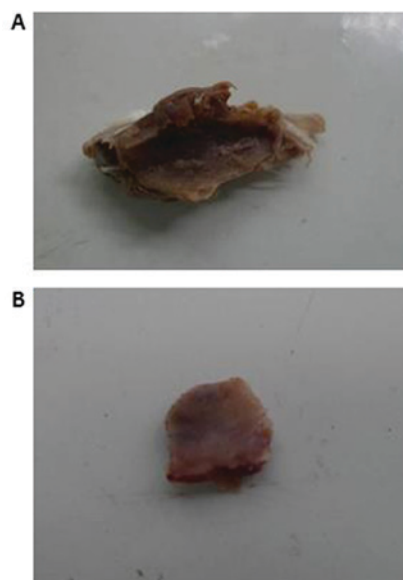


Figure 4. Osteoid tissues from the CS/rhBMP-2 and rhBMP-2 groups. (A) Osteoid tissues from the CS/rhBMP-2 group; (B) osteoid tissues from the rhBMP-2 group. The mean diameter of the osteoid tissues in the CS/rhBMP-2 group (1.1 ± 0.3 cm) was significantly bigger compared with that in the rhBMP-2 group (0.3 ± 0.1 cm). rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

The mean diameter of the osteoid tissues was 1.1 ± 0.3 cm (range, 0.8-1.4 cm) in the CS/rhBMP-2 group, which was significantly bigger than that in the rhBMP-2 group (0.3 ± 0.1 ; range, 0.1-0.4 cm; $P=0.013$; Fig. 4). X-ray analysis also demonstrated that the area of high-density tissues was largest in the CS/rhBMP-2 group, followed by the rhBMP-2 group. However, no high-density tissue was observed in the CS blank and the control group (Fig. 5).

Micro-CT scan and 3-dimensional (3D) reconstruction.

To further investigate the effect of CS/rhBMP-2 on ectopic osteogenesis, micro-CT scan and 3D reconstruction were performed. As presented in Fig. 6, no ectopic osteogenesis was observed in the CS blank and the control group. However, in the CS/rhBMP-2 and rhBMP-2 groups, obvious ectopic

Table I. ALP and Ca²⁺ content in each group (n=6).

Group	ALP activity (katal/g)	Ca ²⁺ contents (μg/mg)
CS/rhBMP-2	1.94±0.35 ^{a,c}	5.20±1.42 ^{a,c}
rhBMP-2	1.48±0.56 ^{b,c}	3.80±1.40 ^{b,c}
CS blank	0.20±0.07	0.19±0.08
Control	0.18±0.06	0.20±0.08

^aP<0.05 compared with the rhBMP-2 group; ^bP<0.05 compared with the CS blank group; ^cP<0.05 compared with the Control group. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan; ALP, alkaline phosphatase.

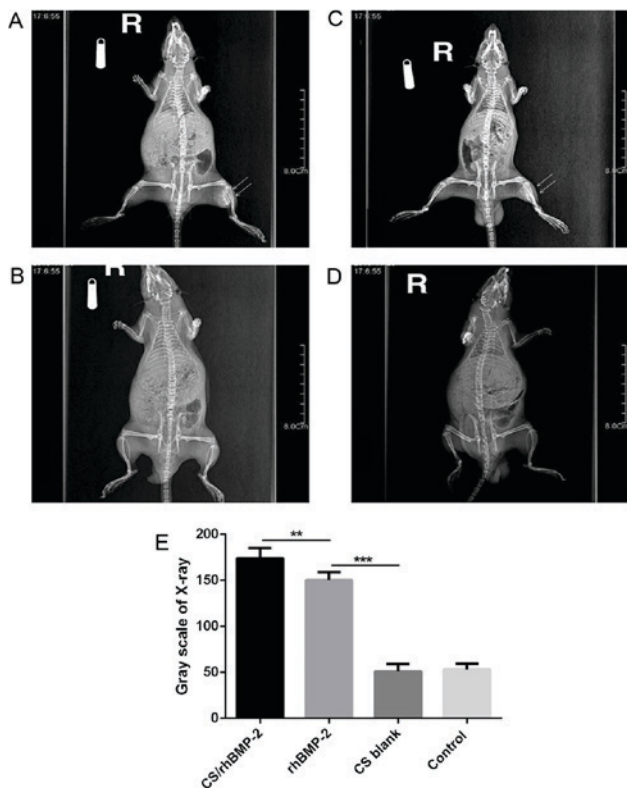


Figure 5. X-ray analysis of the different groups. (A) An area of high-density tissues (arrows) was obvious in the CS/rhBMP-2 group; (B) an area of high-density tissues (arrows) was present in the rhBMP-2 group, but it was smaller than that in the CS/rhBMP-2 group. (C and D) No high-density area was present in the CS blank and the control group. (E) X-ray analysis also indicated that the area of high-density tissues was the greatest in the CS/rhBMP-2 group, followed by the rhBMP-2 group. **P<0.01, ***P<0.001 as indicated. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

osteogenesis was observed with a higher bone density at the edge and a lower bone density at the center. Furthermore, the bone volume in the 3D reconstruction for the CS/rhBMP-2 group was obviously higher than those in the rhBMP-2 group. In addition, analysis of the micro-CT bone parameters revealed that at 3 weeks after surgery, all parameters, namely the TMD, BVF, BMD and TMC, were significantly higher in the CS/rhBMP-2 group compared with those in the rhBMP-2 group (P<0.05; Fig. 7).

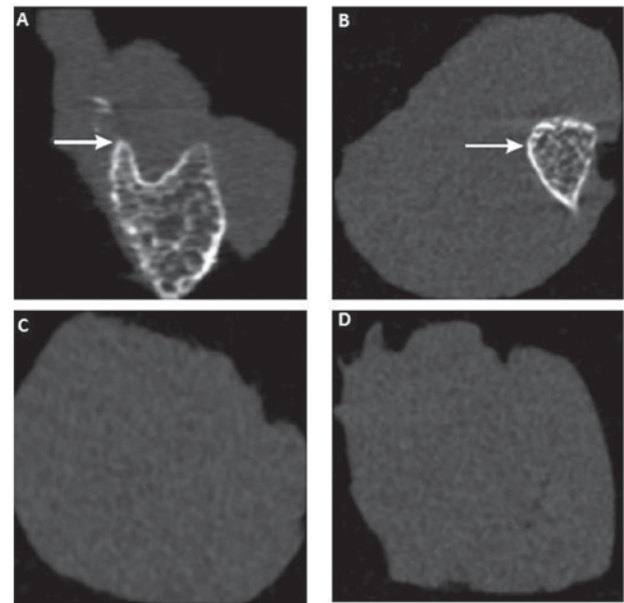


Figure 6. Micro-computed tomography scan for assessment of ectopic osteogenesis in the different groups at 4 weeks after surgery. (A) CS/rhBMP-2 group; (B) rhBMP-2 group; (C) CS blank group; (D) control group. The area of ectopic bone formation is indicated by white colour/arrows. In the CS/rhBMP-2 and rhBMP-2 groups, obvious ectopic osteogenesis was observed with a higher bone density at the edge and a lower bone density at the center. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

Histology. Histological analysis was performed to further confirm the ectopic osteogenesis in the different groups. The results indicated that in all groups, the implants were completely absorbed and no inflammatory or granulomatous tissues were present. In the CS/rhBMP-2 group, mature bone tissue was formed, and the bone cells, bone marrow and bone trabeculae had been converted into mature bone; the formation of a bone marrow cavity and myeloid material between the trabeculae was also observed (Fig. 8A). In the rhBMP-2 group, osteoid tissues were also present (Fig. 8B); however, they were not as mature as those in the CS/rhBMP-2 group. No osteoid tissues were observed in the blank and control groups (Fig. 8C and D).

ALP activity and calcium content. At last, the ALP and Ca²⁺ content was determined in each group. As presented in Table I, the ALP activity and the calcium content were significantly higher in the CS/rhBMP-2 and rhBMP-2 groups compared with those in the other 2 groups (P<0.05). Of note, the ALP activity and calcium content were also significantly higher in the CS/rhBMP-2 group compared with the rhBMP-2 group (P<0.05), indicating that the CS/rhBMP-2 system had the greatest ability to induce osteoblast differentiation.

Discussion

In the field of bone tissue engineering, BMP-2 is a growth factor known to enhance osteogenesis. However, the circulation half-life of BMP-2 is rather short and the bone formation does not increase dose-dependently with increasing BMP-2 concentrations. To overcome the insufficiency of direct injection of

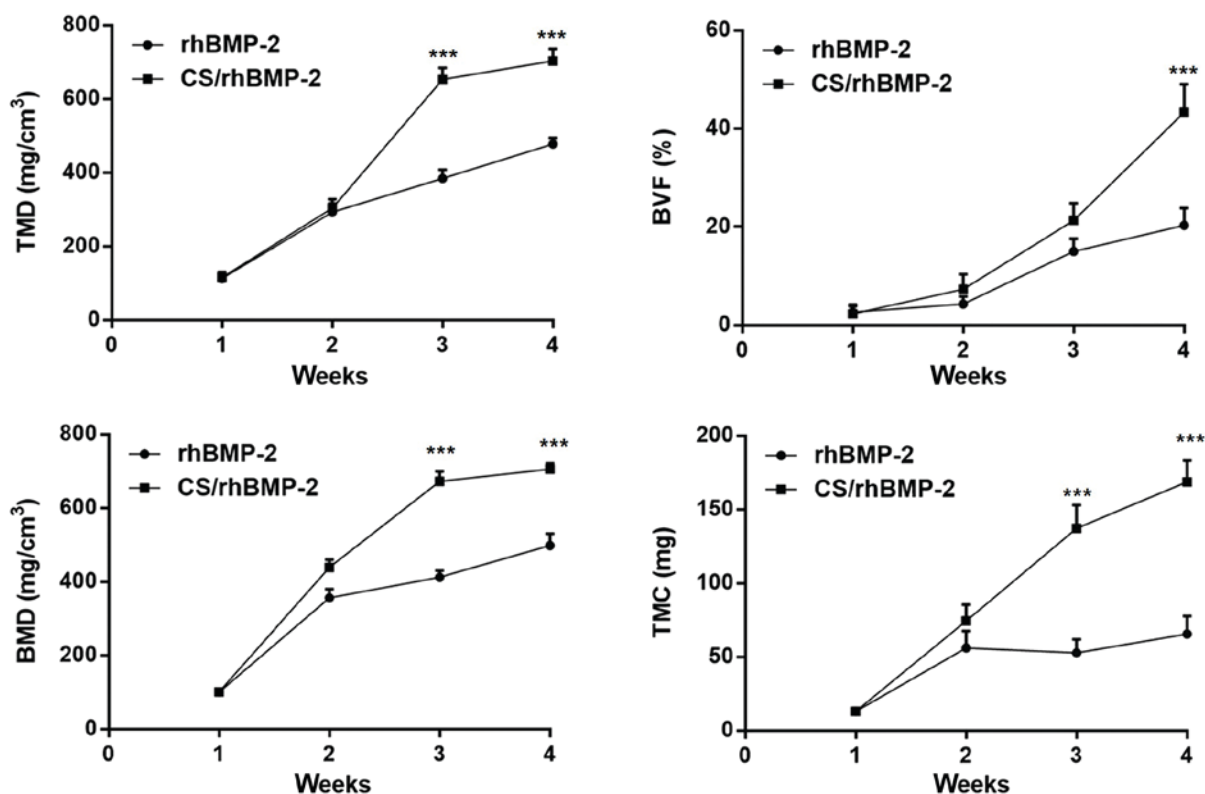


Figure 7. Bone parameters in the CS/rhBMP-2 and rhBMP-2 groups. At 3 weeks after the surgery, the TMD, BVF, BMD and TMC were significantly higher in the CS/rhBMP-2 group compared with those in the rhBMP-2 group. *** $P < 0.001$ vs. the rhBMP-2 group. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan; BMD, bone mineral density; TMD, tissue mineral density; BVF, bone volume fraction; TMC, tissue mineral content.

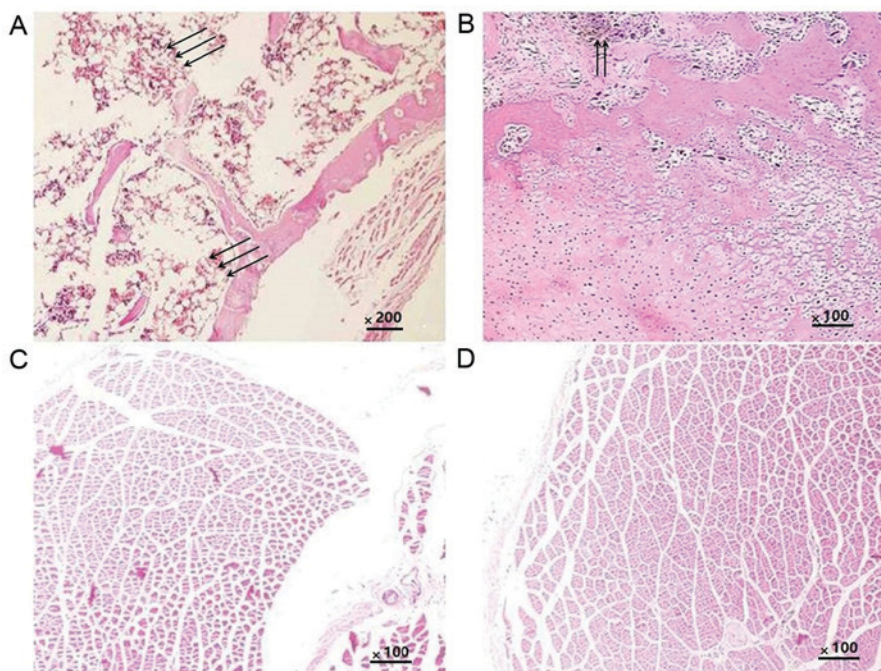


Figure 8. Histological analysis of different groups of rats. (A) CS/rhBMP-2 group; (B) rhBMP-2 group; (C) CS blank group; (D) control group (H&E; magnification, $\times 200$). In the CS/rhBMP-2 group, mature bone tissue was formed, the bone cells, bone marrow and bone trabeculae were converted into mature bone, and the formation of a bone marrow cavity and myeloid material between the trabeculae was also observed (arrows). In the rhBMP-2 group, osteoid tissues were also present (arrows); however, they were not as mature as those in the CS/rhBMP-2 group. rhBMP-2, recombinant human bone morphogenetic protein-2; CS, chitosan.

BMP-2, several novel BMP-2 local delivery systems have been reported, including injectable sonication-induced silk hydrogel (20), polyethylene glycol-coated albumin

nanoparticles (21) and CS-based 3D constructs (22). Studies have demonstrated that collagen sponges may be used as an adequate matrix to prolong the duration of BMP-2 residing in

the tissue to facilitate bone regeneration (23). Bhakta *et al* (24) reported that collagen and hyaluronan matrices may be used for delivery of BMP-2, which may help abrogate the adverse clinical effects associated with high-dose growth factor use.

Among the delivery systems, CS-based systems have attracted a large amount of attention. Yilgor *et al* (25) developed a sequential BMP-2/BMP-7 delivery system with CS-based scaffolds for bone tissue engineering, with which an enhanced ALP activity was achieved. Gan *et al* (26) designed a CS-based delivery system for BMP-2 and reported that it enhanced bone regeneration. However, whether the CS/rhBMP-2 microspheres delivery system is able to initiate ectopic osteogenesis has remained elusive. The present study aimed to investigate the effect of rhBMP-2 delivered by CS microspheres on ectopic osteogenesis of rats.

First, CS/rhBMP-2 microspheres were successfully prepared and their physicochemical properties were demonstrated. Subsequently, by using several analysis methods, including X-ray analysis, micro-CT scan and 3D reconstruction, histological analysis, ALP activity analysis and calcium content analysis, it was demonstrated that rhBMP induced ectopic osteogenesis in rats, and promoted osteoblast differentiation by enhancing ALP activity and the calcium content. Of note, the CS/rhBMP-2 microsphere delivery system significantly enhanced the induction of osteogenesis compared with that achieved with rhBMP-2 alone.

Several associated studies have focused on BMP-2 in processes of osteogenesis. Lee *et al* (15) studied the effect of dual treatment of stromal cell-derived factor (SDF)-1 and BMP-2 on ectopic and orthotopic bone formation and identified that BMP-2 induced ectopic and orthotopic bone regeneration; however, SDF-1 treatment did not enhance the effect. Ma *et al* (27) demonstrated that rhBMP-2 and basic fibroblast growth factor had a synergistic effect on ectopic osteogenesis in mice. Lai *et al* (28) indicated that application of rhBMP-2 sustained-release nanocapsules significantly promoted ectopic osteogenesis compared with the direct use of rhBMP-2. All of these studies were consistent with the present results.

In conclusion, in the present study, a CS/rhBMP-2 microsphere delivery system was prepared and used to investigate the effect of rhBMP-2 delivered by CS microspheres on ectopic osteogenesis in rats. The results demonstrated that rhBMP induced ectopic osteogenesis in rats, and promoted osteoblast differentiation by enhancing ALP activity and the calcium content. Of note, the CS/rhBMP-2 microsphere delivery system significantly enhanced the ectopic osteogenesis compared with the effects of rhBMP-2 on its own. The present study may provide basic data for the future application of the CS/rhBMP-2 microsphere delivery system and provide a deeper understanding of the roles of BMP-2 in bone regeneration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJX and WW conducted the experiments and analyzed the data. YJX wrote the manuscript and revised the manuscript. HX, QSY and XY conducted the experiments. LHL and JHW collected the data. YZ analyzed the data, revised the manuscript and approved the submission.

Ethics approval and consent to participate

The protocol of the present study was approved by the Institutional Animal Care Committee at General Hospital of Southern Theater Command, PLA (Guangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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