


SCIENTIFIC ARTICLE

Preparation of Coralline Hydroxyapatite Implant with Recombinant Human Bone Morphogenetic Protein-2-Loaded Chitosan Nanospheres and Its Osteogenic Efficacy

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Objective: Spinal fusion is one of the most common surgical interventions for spine reconstruction. Despite the efforts to promote osteogenesis after spinal fusion, osteogenesis after spinal fusion remains a clinical challenge and new methods are still needed. The bone morphogenetic protein-2 (BMP-2) is a widely reported factor that can facilitate the osteogenesis in spinal fusion. In previous research, we found that the delivery of chitosan nanospheres could promote the effects of BMP-2 on osteogenic activity. The coralline hydroxyapatite (CHA) is one of the most frequently used implants in bone fusion. However, up to now no study has focused on the osteogenic efficacy of the CHA composite with recombinant human BMP-2 (rhBMP-2)-loaded chitosan nanospheres. This study aimed to investigate the effects of the CHA implant with rhBMP-2-loaded chitosan nanospheres on osteogenesis in spinal fusion.

Methods: The rhBMP-2-loaded microspheres and CHA composite (rhBMP-2 microspheres/CHA) were prepared and were used for implantation of the rats. All SD rats were divided into four groups: the rhBMP-2 microspheres/CHA composite group (containing 0.5 mg rhBMP-2), the rhBMP-2-loaded CHA (rhBMP-2/CHA) composite group (containing 0.5 mg rhBMP-2), the blank CHA group, and the negative control group. The microsphere morphology was scanned and analyzed using a scanning electron microscope. Micro-computed tomography examination and three-dimensional reconstruction were performed 4 weeks after the surgery. Hematoxylin and eosin staining was conducted for histological analysis. Both alkaline phosphatase (ALP) and calcium content were measured.

Results: The rhBMP-2-loaded CHA (rhBMP-2/CHA) composite was successfully prepared. Spherical regularity and a smooth and unwrinkled surface of the spheres were observed in all chitosan (CS)/rhBMP-2 microspheres. No side effects, infections, or abnormal behaviors were found in the animals. After 4 weeks of surgery, obvious new bone formation and bone fusion could be observed around the implant in both the rhBMP-2 microspheres/CHA composite group and the rhBMP-2/CHA composite group. No ectopic osteogenesis was found in the vertebral canal or other muscle tissues. After 4 weeks of implantation, in both the rhBMP-2 microspheres/CHA composite group and the rhBMP-2/CHA composite group, osteoid tissues could be found, and bone cells, bone marrow, and trabecular bone turned into mature sclerotic, obvious bone tissue formation could be also seen. Both ALP activity and calcium content in the rhBMP-2 microspheres/CHA composite group (6.52 ± 0.50 kat/g and 17.54 ± 2.49 μ g/mg) were significantly higher than in all other groups.

Conclusion: The composite with rhBMP-2-loaded CS nanospheres could enhance osteogenic efficacy and increase the ALP activity and calcium content. These results might provide a novel method for osteogenesis in spinal fusion and offer new insight into the role of BMP-2 in osteogenesis.

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Introduction

Spinal fusion is one of the most common surgical interventions for spine reconstruction, and is widely applied in a variety of spine conditions, including degeneration, deformity, tumors, and trauma¹⁻³. Osteogenic ability after surgery is an important index for the evaluation of spinal fusion⁴. Many methods are reported to be of benefit in promoting osteogenesis after spinal fusion. Lin *et al.* report that the PLGA/ β -TCP composite scaffold incorporating salvianolic acid B could promote bone fusion by angiogenesis and osteogenesis in a rat spinal fusion model⁵. Johnson (2011) found that oxysterols had the ability to promote osteogenesis, protect lipogenesis, and induce spinal fusion⁶. In recent years, spinal instrumentation has been developed to enhance osteogenesis and fusion⁷. However, despite the studies that have been produced, osteogenesis after spinal fusion remains a clinical challenge and new methods are still needed to enhance the efficacy of osteogenesis after spinal fusion.

Among the factors involved in the progress of osteogenesis, the bone morphogenetic protein-2 (BMP-2) is a widely reported factor that can facilitate the osteogenesis⁸⁻¹⁰. BMP-2 belongs to the BMP family, which are osteoinductive proteins originally identified in demineralized bone¹¹. Most BMP, including BMP-2, are proven to promote bone healing without bone tissue transferring¹². It is considered that BMP-2 can promote the healing process of segmental bone defects. Numerous studies also show BMP-2 can facilitate the osteogenic ability of bone marrow stromal cells (BMSCs)¹³. In many other studies, BMP-2 is reported to promote osteogenesis and bone regeneration. Kolk *et al.* show that both recombinant human BMP-2 (rhBMP-2) and plasmid DNA of BMP-2 can facilitate the bone regeneration behavior¹⁴. Xie *et al.* demonstrate that mineralized short nanofibers coupled with BMP-2 peptides promote the alveolar bone regeneration¹⁵. Despite the osteogenic ability, the application of BMP-2 is limited due to its short circulation half-life¹⁶. BMP-2 easily dilutes and interacts with enzymes in blood, which further leads to its inactivation, if injected alone by intravenous injection¹⁷. Injection of BMP-2 can also cause side effects like the burst effect, resulting in the soft tissue hematoma and bone absorption phenomenon¹⁸. Thus, to extend the circulation time and to reduce possible side effects, it is necessary to develop appropriate delivery systems for BMP-2.

In recent decade, diverse local delivery systems are reported to enhance the bioavailability of BMP-2, such as the chitosan (CS) nanospheres¹⁹. Bouyer *et al.* reported an adaptable polymeric scaffold that could deliver tunable doses of BMP-2 and induced volumetric bone regeneration²⁰. Loozen *et al.* (2019) compared inclusion of bone progenitor cells with non-osteogenic target cells in gene delivery constructs and found that non-viral gene delivery of BMP-2 was

a potential method to induce transgene expression²¹. Hettiaratchi (2020) found that heparin-mediated delivery of BMP-2 improved spatial localization of bone regeneration²². In our previous research, we demonstrated that the CS/rhBMP-2 microsphere delivery system could remarkably enhance the induction and promotion effects of rhBMP-2 in ectopic osteogenesis²³. However, the effects of CS/rhBMP-2 microsphere on spinal fusion are still unknown.

For the implant system, it was found that the polyelectrolyte multilayer film coating loaded with BMP-2 on titanium and PEEK implants could facilitate the bone growth; however, a high dose of BMP-2 might result in localized and temporary bone impairment²⁴. The combination of the BMP-2 delivery system and implants is also reported in several studies. Teng *et al.* show that biomimetic coating incorporated with BMP-2co could enhance the peri-implant osteogenesis for zirconia implants²⁵. Another study demonstrated that micro-porous polyetheretherketone implants decorated with BMP-2 *via* phosphorylated gelatin coating could remarkably enhance the cell adhesion and osteogenic differentiation²⁶. Among the delivery and implant systems, the coralline hydroxyapatite (CHA) implant is one of the most commonly used in bone fusion^{27, 28}. Day *et al.* showed that the human umbilical cord mesenchymal stem cells could envelope the surface of the CHA/calcium carbonate micro-particles and had high osteogenic differentiation capability²⁹. It was also found that by incorporating BMP-2 into a biomimetic coating, the biocompatibility and osteogenicability of CHA could be improved³⁰. However, up to now no study has focused on the osteogenic efficacy of the CHA composite with rhBMP-2-loaded chitosan nanospheres.

In the present study, we aimed to: (i) prepare the CHA implant with rhBMP-2-loaded chitosan nanospheres; (ii) evaluate its osteogenic efficacy in spinal fusion model; and (iii) demonstrate the potential use of the CHA implant with rhBMP-2-loaded chitosan nanospheres in spinal fusion treatment. We demonstrated for the first time that the CHA implant with rhBMP-2-loaded chitosan nanospheres could enhance osteogenic efficacy and increase the alkaline phosphatase (ALP) activity and calcium content. These results could provide a novel method for osteogenesis in spinal fusion and provide a deeper understanding of the CS/rhBMP-2 system.

Methods and Materials

Preparation of Chitosan Blank Microspheres and Chitosan/Recombinant Human Bone Morphogenetic Protein-2 Microspheres

The preparation of CS blank microspheres and CS/rhBMP-2 microspheres was performed as reported in a previous

study²³. Briefly, CS with a concentration of 1.52 mg/mL, pH5.4, was obtained by mixing CS (molecular weight, 50,000–190,000; Aladdin Reagent, Shanghai, China), 1% (v/v) acetic acid, and NaOH solution. The mixture was then filtered and stirred for 1 h at room temperature. Then, 0.5 mg/mL thiamine pyrophosphate solution was added to form the nanospheres. The mixture was then stirred for another 30 min and centrifuged for 15 min at 25,000 g at room temperature. The precipitate was washed and dried under cryogenic conditions with reduced pressure.

The CS/rhBMP-2 microspheres were prepared by using 100 mg dried CS blank microspheres and 5 mg rhBMP-2, followed by addition of 25 mL double-distilled water, stirring and centrifugation for 15 min at 25,000 × g at room temperature.

Characterization of Chitosan/Recombinant Human Bone Morphogenetic Protein-2 Microspheres

The microsphere morphology was scanned and analyzed using a scanning electron microscope (S-4800; Hitachi, Tokyo, Japan) and a laser diffraction particle size analyzer (N5; Beckman Coulter, Brea, CA, USA). All experiments were performed in triplicate.

Preparation of Recombinant Human Bone Morphogenetic Protein-2-Loaded Chitosan Nanospheres and Coralline Hydroxyapatite Composite

To obtain the rhBMP-2-loaded microspheres and CHA composite (rhBMP-2-microspheres/CHA), an appropriate amount of the microspheres was added to 10 mL double distilled water, before stirring for 5 min at room temperature. Then the CHA artificial bone (pore size 100–250 μm, porosity rate 80%, produced by Guangdong Key Laboratory of Orthopedics and Implant Materials, China) was immersed into the suspension and the water was removed under negative pressure at 15 °C to form the rhBMP-2-loaded chitosan nanospheres and CHA composite. For preparation of rhBMP-2-loaded CHA composite (without CS microspheres), rhBMP-2 with a concentration of 1.2 mg/mL was added to the CHA artificial bone dropwise, and the water was removed under negative pressure at 15 °C. The CHA artificial bone composite was sterilized by irradiation of 3000 Gy ⁶⁰Co and stored at 4°C prior to the experiments.

Animals, Treatment and General Observation

A total of 24 male Sprague Dawley (SD) rats were provided by the Experimental Animal Center of Southern Medical University (Guangzhou, China); they were 4 weeks old and weighted 120–140 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. The protocol of the present study was approved by the authors' affiliation.

All rats were divided into four groups, with six rats in each group: (i) the rhBMP-2-microspheres/CHA composite

group (containing 0.5 mg rhBMP-2); (ii) the rhBMP-2-loaded CHA (rhBMP-2/CHA) composite (containing 0.5 mg rhBMP-2); (iii) the blank CHA group; and (iv) the negative control group, which was implanted with the same size gelatin sponge instead of microspheres.

The rats were anesthetized with 50 mg/kg ketamine and 5 mg/kg hydrochloride diazepam. Then the rats were fixed on the operation table. The hair on their backs was cut off and the skin was incised. After the L₅ and L₆ transverse processes of spine were exposed, the right inferior margin of L₅ and the right upper margin of L₆ were worn away using a high-speed grinding drill. Then the composite was implanted between the L₅ and L₆ transverse processes. The incision was sutured and penicillin (800 × 10³ U/d) was used for protection from infection for the first 3 days after surgery.

Four weeks after the surgery, the rats were observed for side effects, infections, or abnormal behaviors. The palpation method was used to detect the fusion position and the joint instability.

Micro-Computed Tomography Assessment

The micro-computed tomography (CT) examination and three-dimensional (3D) reconstruction were performed 4 weeks after the surgery using a micro-CT apparatus for animals (Little Chalfont, UK). The formation of a continuous bone bridge was considered successful fusion.

Histology

For histological analysis, the animals were killed at 4 weeks after the surgery and tissues around the implants were obtained and fixed in formalin buffer. Hematoxylin and eosin (H&E) staining was applied to observe the histologic morphology of the tissues using an inverted microscope.

Measurement of Alkaline Phosphatase Activity

The implants were taken out 4 weeks after the surgery. For each animal, 0.5 g of tissue around the implants was weighted and washed with deionized water. The ALP activity was determined using an ALP Detection Kit (cat. no. A059-2; Nanjing Jiancheng Biotechnology, Nanjing, China) according to manufacturer's protocols.

Measurement of the Calcium Content

The centrifugal precipitate was collected and digested, and the calcium content of the tissues was determined using an Atomic absorption spectrophotometer (i7500; Hitachi) applying the following formula: calcium content = calcium content (μg)/sample wet weight (mg).

Statistical Analysis

The measurement data are expressed as the mean ± SD. 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 20.0 (SPSS, Chicago, IL, USA).

Results

Preparation and Characterization of Chitosan/Recombinant Human Bone Morphogenetic Protein-2 Microspheres

The characterization of CS/rhBMP-2 microspheres is shown in Fig. 1. Similar to our previous research, spherical regularity and a smooth and unwrinkled surface of the spheres was observed in all CS/rhBMP-2 microspheres, indicating successful preparation of the CS/rhBMP-2 microspheres.

General Observation

Observation and palpation of the rats were undertaken. No side effects, infections, or abnormal behaviors were found in the animals. Using palpation, we found that at 4 weeks after surgery, the fusion position of all rats in the rhBMP-2 microspheres/CHA composite group and the rhBMP-2/CHA composite group showed good fusion, with no joint instability observed.

Micro-Computed Tomography Evaluation for Fusion of the Coralline Hydroxyapatite Composite

To evaluate fusion of the CHA composite, micro-CT and 3D reconstruction were performed. Four weeks after surgery, obvious new bone formation and bone fusion could be observed around the implant in both the rhBMP-2 microspheres/CHA composite group and the rhBMP-2/CHA composite group (Fig. 2). No ectopic osteogenesis was found in the vertebral canal or other muscle tissues. No new bone formation or bone fusion was observed in the CHA blank group or the negative group. This result suggested that rhBMP-2 significantly facilitated the bone fusion of the CHA composite.

Histological Assay

Based on H&E staining, after 4 weeks of implantation, in both the rhBMP-2 microspheres/CHA composite group and the rhBMP-2/CHA composite group, osteoid tissues could be found, and bone cells, bone marrow, and trabecular bone turned into mature sclerotic; obvious bone tissue formation

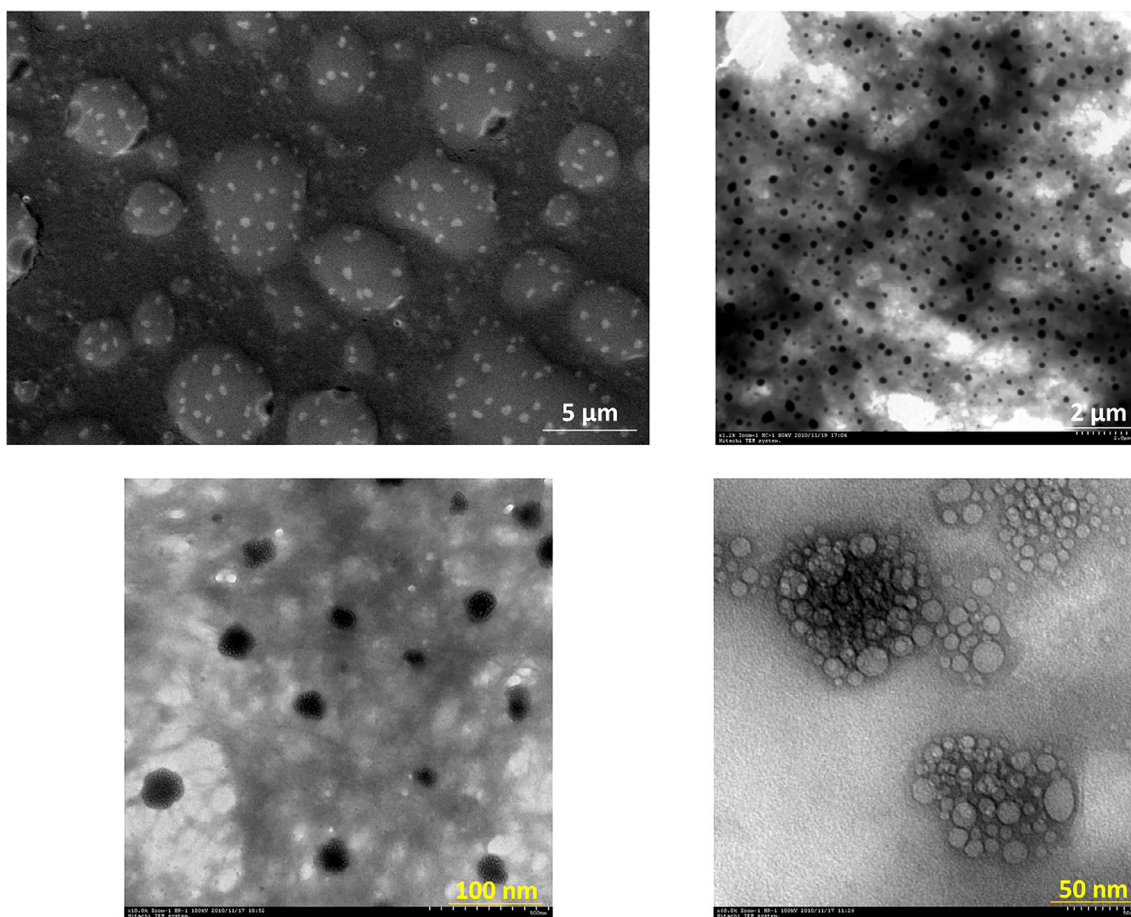


Fig 1 Characterization of chitosan (CS)/recombinant human bone morphogenetic protein-2 (rhBMP-2) microspheres. The CS/rhBMP-2 microspheres under transmission electron microscopy (TEM). Spherical regularity and smooth and unwrinkled surface of the spheres were observed in all microspheres.

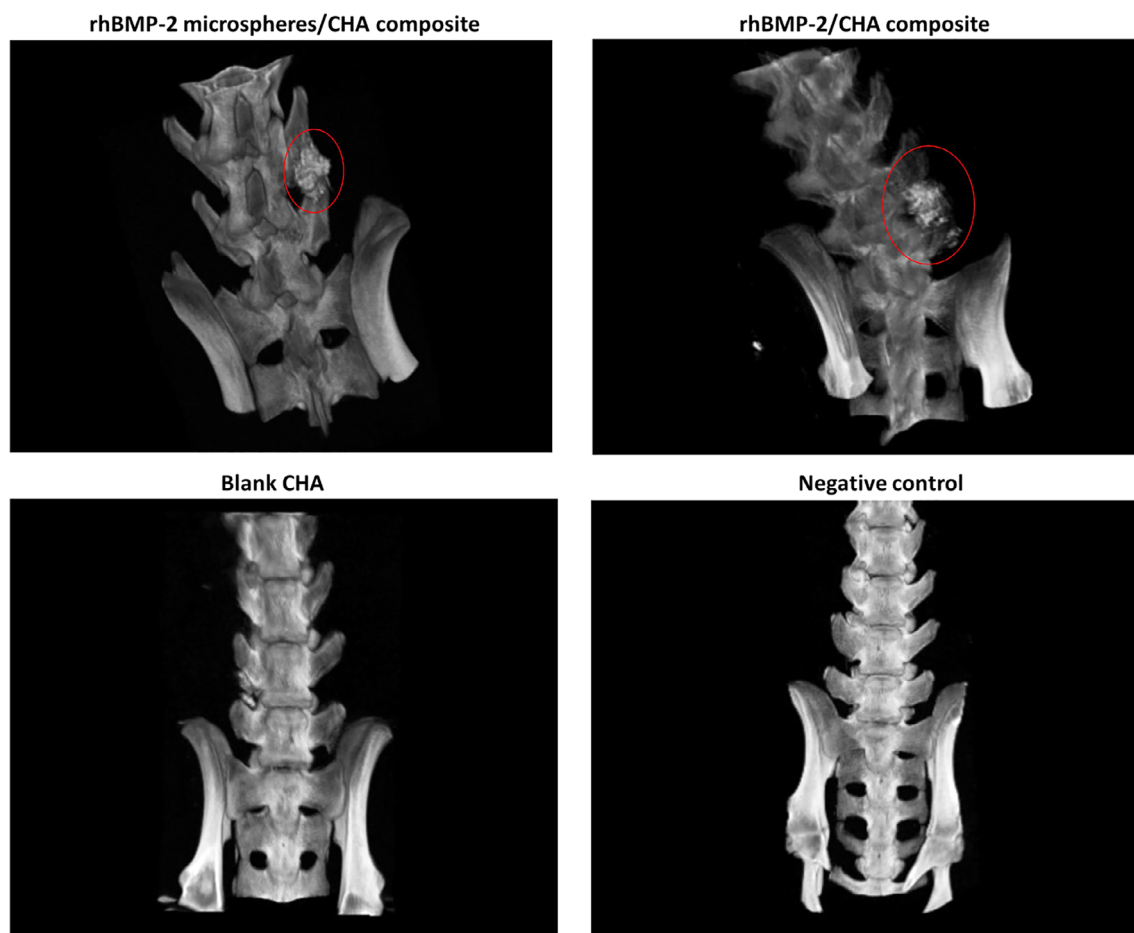


Fig 2 Micro-computed tomography (CT) evaluation for fusion of the coralline hydroxyapatite (CHA) composite. CT and three-dimensional reconstruction for different groups. The red cycle showed the fusion place of the implant. New bone formation and bone fusion could be observed around the implant in both the recombinant human bone morphogenetic protein-2 (rhBMP-2) microspheres/CHA composite group and the rhBMP-2/CHA composite group. No ectopic osteogenesis was found in the vertebral canal or other muscle tissue $\times 5$.

could be also seen (Fig. 3). However, no bone tissue formation was observed around the implants in the CHA blank group and the negative group, further indicating the promotion of rhBMP-2 for bone fusion of the CHA composite.

Measurement of Alkaline Phosphatase Activity and Calcium Content

Finally, we evaluated the ALP activity and calcium content in rats of different groups. As shown in Table 1, both ALP activity and calcium content in the rhBMP-2 microspheres/CHA composite group were significantly higher than in all other groups ($P < 0.05$). The rhBMP-2/CHA composite group also showed remarkably higher values of ALP activity and calcium content than the CHA blank and the negative groups ($P < 0.05$). These results suggested that the rhBMP-2 microsphere-loaded CHA composite had the best ability for spinal fusion.

Discussion

The osteogenesis after spinal fusion remains a challenge in clinic. In the present study, we demonstrated for the first time that rhBMP-2-loaded chitosan nanospheres could facilitate the spinal fusion process without any side effects.

The effect of BMP-2 on bone fusion has already been reported in many studies. Salehi (2018) reported that BMP-2 could induce bone formation in spinal fusion and anti-inflammatory peptides could promote the effect³¹. Park *et al.* also demonstrated that BMP-2 could enhance the early bone formation in spine fusion using a rat ovariectomy osteoporosis model³². Some other studies also reported that application of BMP-2 in spinal fusion might also cause ectopic osteogenesis³³ and might be associated with increased risk of cancer, which is still controversial³⁴. However, up to now, no study has focused on the spinal fusion effect of rhBMP-2-loaded chitosan nanospheres. In this research, we successfully prepared CHA composite with rhBMP-2-loaded chitosan

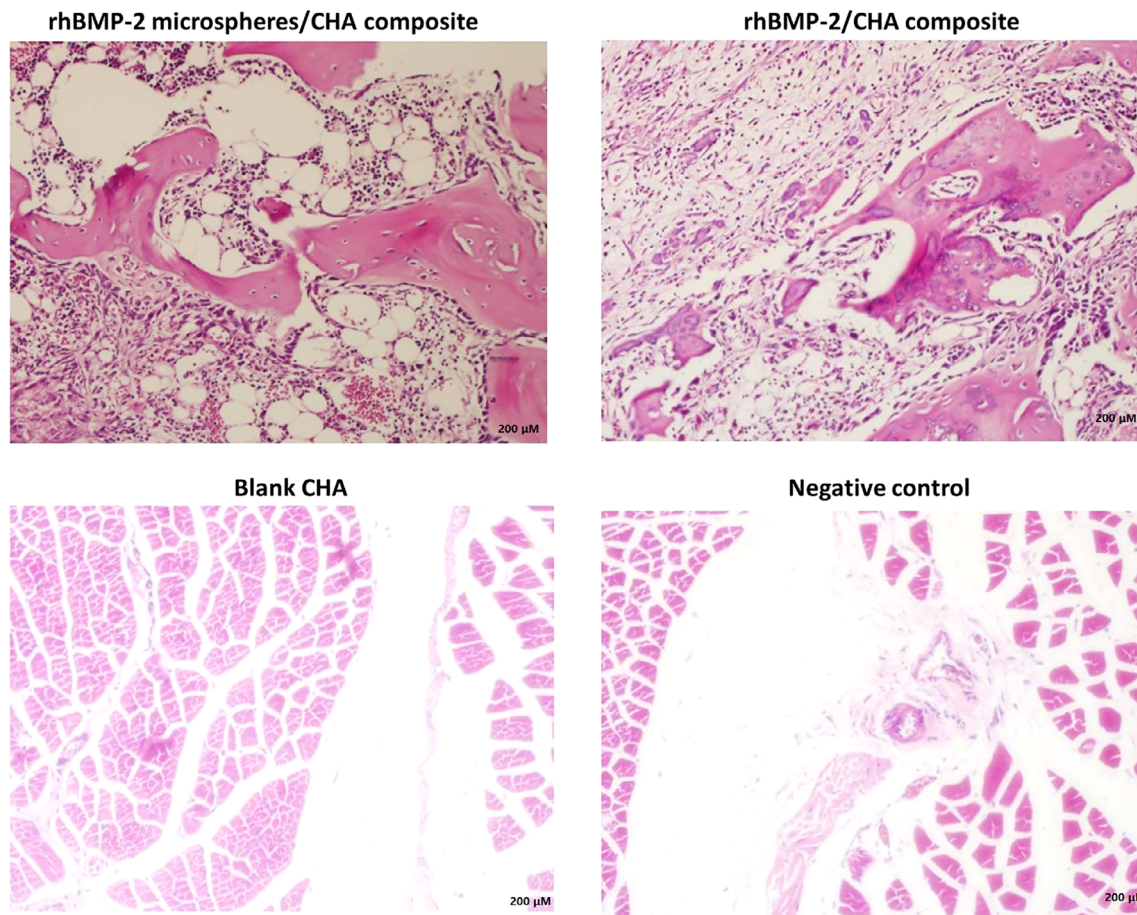


Fig 3 Histological assay. Hematoxylin and eosin staining for tissues around the implant in different groups. Osteoid tissues, bone cells, bone marrow, trabecular bone turning into mature sclerotin, and bone tissue formation could be seen in both the recombinant human bone morphogenetic protein-2 (rhBMP-2) microspheres/coralline hydroxyapatite (CHA) composite group and the rhBMP-2/CHA composite group. No bone tissue formation was observed around the implants in the CHA blank group and the negative group.

TABLE 1 ALP activity and calcium content in different groups (mean \pm SD)

Groups	ALP (kat/g)	Calcium ($\mu\text{g}/\text{mg}$)
rhBMP-2 microspheres/CHA composite	6.52 ± 0.50	17.54 ± 2.49
rhBMP-2/CHA composite	5.86 ± 0.35	13.97 ± 3.45
Blank CHA	5.86 ± 0.35	4.59 ± 1.15
Negative control	5.86 ± 0.35	4.37 ± 1.12
F-value	42.959	39.242
P-value	0.000	0.000

ALP, alkaline phosphatase; CHA, coralline hydroxyapatite; rhBMP-2, recombinant human bone morphogenetic protein-2; SD, standard deviation.

nanospheres and found that rhBMP-2-loaded chitosan nanospheres could facilitate the spinal fusion process.

Chitosan nanospheres are considered a good carrier for BMP-2. Refaat *et al.* demonstrated that binding with COMP could significantly enhance the efficacy of BMP and reduce the dose of BMP in spinal fusion³⁵. In a long-term study, authors found that long-term delivery of BMP-2 by heparin-conjugated PLGA nanospheres could improve the osteogenic efficacy of BMP-2³⁶. In our previous research, we also found that BMP-2-loaded chitosan nanospheres could enhance the ectopic osteogenic ability of rats, and chitosan nanospheres themselves did not change the ectopic osteogenesis ability²³. We also demonstrated that microspheres based on chitosan-dextran sulfate polyion complexes were a highly efficient vehicle for delivery of rhBMP-2 protein and could promote the osteogenesis, and chitosan nanospheres themselves did not influence the osteogenesis³⁷. All these results indicated that chitosan could enhance the effects of BMP-2

on osteogenesis. However, this is the first time we found that chitosan nanospheres could also enhance the promotion ability of rhBMP-2 in the spinal fusion process. The present study also has some limitations. The biomolecular mechanisms for the osteogenic effects of rhBMP-2-loaded microspheres and CHA composite are still unclear. More *in vitro* and clinical research is needed in the future to confirm our results.

In conclusion, we successfully prepared the CHA composite with rhBMP-2-loaded chitosan nanospheres and found that the composite with rhBMP-2-loaded chitosan

nanospheres could enhance osteogenic efficacy and increase the ALP activity and calcium content in an *in vivo* spinal fusion model. These results could provide a novel method for osteogenesis in spinal fusion.

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