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*Original Article*

# BMP-2 promotes fracture healing by facilitating osteoblast differentiation and bone defect osteogenesis

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**Abstract:** Objective: To investigate the role of bone morphogenetic protein-2 (BMP-2) in promoting fracture healing in animal models. Methods: Mouse models with muscle bag heterotopic osteogenesis (HO) were divided into a HO control group (not implanted with 250 μg rhBMP-2 bone repairing material), and a HO observation group (implanted with 250 μg rhBMP-2 bone repairing material); while rat models with bone defect (BD) were divided into a BD control group (not implanted with 250 μg rhBMP-2 bone repairing material) and a BD observation group (implanted with 250 μg rhBMP-2 bone repairing material). At 4 weeks after HO establishment, the new bone formation at the operation site was observed through visual inspections and X-ray scanning. The content of serum alkaline phosphatase (ALP) was detected by automatic biochemical analyzer. The formation of new bone at the operative sites was observed by Hematoxylin and eosin staining and Masson staining. At 0, 2, 4 and 8 weeks after operation, the growth of the defect area and its surrounding callus were observed by X-ray scanning. At 4 and 8 weeks after bone defect establishment in the mouse models, the histological changes and osteogenesis of the bone defect site were observed. Results: The heterotopic osteogenesis experiment showed that at 4 weeks after operation, the mass at the muscle bag in the HO observation group became larger in contrast to the HO control group. X-ray scanning showed that there was obvious irregular bone shadow at the back muscle bag of mice from the HO observation group. The content of serum ALP in the HO observation group was significantly higher than that in the HO control group (all $P<0.05$). The muscle pocket in the HO observation group showed higher ectopic osteogenic activity comparing with the HO control group. Histological staining showed that bone tissue structure was visible in the newly regenerated bone, forming bone trabeculae and bone marrow tissue. Under the microscope, a large number of osteoblasts arranged neatly in a cubic shape presented at the edge of the new bone, and there were bone lacunae formed, and the bone tissue was in a relatively mature stage. In the rat bone defect models, X-ray scanning showed that the high-density development area was further increased. There was a large amount of callus formation in the bone defect area of the BD observation group, while the BD control group still had no high-density development. At 8 weeks after operation, the high-density development area decreased, indicating that there was partial absorption of callus, while there was still no high-density development in the BD control group. The callus of the bone defect area in the BD observation group was reduced and the defect area was gradually repaired, while the bone defect in the BD control group was still obvious and the bone repair was not completed. Conclusions: BMP-2 could promote osteoblast differentiation and bone defect osteogenesis *in vivo*. Thus, it is worthy of clinical application.

**Keywords:** Bone morphogenetic proteins-2, heterotopic osteogenesis, bone defect, mice

## Introduction

Bone defects caused by factors such as bone tumors, trauma, and infection are a major challenge in the field of orthopedics, especially with symptoms such as fractures, segmental bone defects, bone nonunion, accompanying false joint formation, persistent pain and decreased or lost motor abilities, which seriously affect the quality of life and health of patients [1]. In clinical practice, many methods are used, including autologous bone grafting, allograft bone grafting, xenogeneic bone grafting and substitutes of biosynthetic materials. However, each treatment method has certain limitations. For example, although autologous bone grafting is recognized as the gold standard for repairing bone defects, its source is limited [2, 3].

Allogeneic bone grafting involves immune rejection, disease transmission, ethics, uncertain osteogenic ability and so on [4]. The impact of these factors on fracture healing is clear and affirmative. Although the artificial bone-tissue engineering materials developed in recent years have certain bone conduction and bone induction capabilities, their osteoinductive effect is poor, and the degradation rate and plasticity are not satisfactory [5]. Therefore, it does not fully meet the clinical demands. The above reasons have limited the clinical application of various bone grafting and biological substitutes. With the development of bone tissue engineering technology, the development and utilization of growth factors to treat bone defects has become a hot research topic in the field of orthopedics [6]. Scholars began to use biological factors to accelerate fracture healing, which provided new treatment methods and ideas for solving such clinical orthopedic problems.

Bone morphogenesis protein (BMP) is a growth factor that induces and promotes the formation of bone tissues [7]. It can also induce bone marrow mesenchymal stem cells to differentiate into chondroblasts and osteoblasts [8]. BMP is mainly involved in the early response to bone tissue injury or trauma [9]. So far, more than 40 BMP family members have been isolated and cloned through genetic recombination technology, of which BMP-2 and BMP-7 are widely used. BMP-2 especially is currently recognized as the strongest osteogenic growth factor in the BMP family, with strong osteogenic ability and significant ability to promote bone marrow mesenchymal stem cells to differentiate into osteoblasts [10]. However, the role of BMP-2 in promoting bone growth and accelerating fracture healing still needs to be confirmed, especially *in vivo*. In addition, the natural source of BMP-2 is limited, and its biological activity is difficult to fully exert, which cannot fully meet clinical demands. At present, recombination human BMP-2 (rhBMP-2) has been generated through genetic recombination technology. However, the roles of rhBMP-2 in inducing new bone in ectopic and repairing bone defects *in vivo* remain to be elucidated. In this study, muscle bag heterotopic osteogenesis (HO) and bone defect mouse models were established successfully to investigate the application of exogenous bone growth factor rhBMP-2 in promoting fracture healing. The results of this study can provide experimental basis and research direction for further clarifying the molecular mechanism of BMP-2 in the treatment of bone defects and provide a research foundation for the application of rhBMP-2 bone repairing materials.

## Methods

### *Establishment of animal models*

This study was approved by the Animal Ethics Committee of Shandong Provincial Hospital (Approval No. 2022-114) and it was performed according to the guidelines issued by the Chinese Association for Laboratory Animal Sciences. A total of 16 male specific-pathogen free (SPF) Institute of Cancer Research (ICR) mice aged 8-10 weeks and weighted 20-22 g and 16 male SPF Sprague Dawley (SD) rats aged 8-10 weeks and weighted 250-300 g were purchased from the Animal Centre of Shandong Provincial Hospital. These animals were kept in an environment with a room temperature of 22±1°C, a relative humidity of 45-75% and a 12 h light-dark cycle. ICR mice and SD rats were fed and watered ad libitum.

Before operation, SD rats and ICR mice were fasted overnight. 2% sodium pentobarbital (50 mg/kg) was applied for anesthetization. The bone defects in the SD rats were established as follows: The fur in the left leg was sheared after routine disinfection, a 2 cm long incision was made on the skin along the long axis of the femur in hind leg. The skin, fascia, muscles and periosteum were dissected in sequence. After the femur was completely exposed, a 6-8 mm long full-thickness bone defect was performed near 1/3 of the femoral head using a drilling machine, reaching deep into the bone marrow cavity. For SD rats in the control group, they were directly sutured with no material implanted (n=8). The implantation containing 250 μg rhBMP-2 bone repairing material (Hangzhou Jiuyuan Gene Engineering Co., Ltd.) was applied in the BD observation group (n=8) before the muscles and skin were sutured sequentially. The ICR mouse model of heterotopic osteogenesis (HO) in mice with muscle bag was established as follows: The fur in the left leg was shaved after routine disinfection, and then a 1 cm long incision was made on the skin. After skin incision, the lacuna in the lateral intramus-

cular spaces was separated and the muscle bag was exposed, establishing a muscle bag implantation model. For the mice in HO control group, they were directly sutured with no material implanted (n=8). The implantation containing 250 μg rhBMP-2 bone repairing material was applied in the HO observation group (n=8).

At different time points, these animals were sacrificed by sudden exposure to air containing 70% $CO_2$ in the clear and clean euthanasia chamber and followed by cervical dislocation. Following euthanasia, the tissues were harvested, and the blood was collected.

*Detection of serum alkaline phosphatase levels*

The serum levels of alkaline phosphatase (ALP) in ICR mice with heterotopic osteogenesis in the muscle bag were compared between the HO control group and HO observation group. Four weeks after operation, venous blood was collected from these mice, and the serum was isolated through centrifugation at 2500 r/min for 20 minutes. The serum level of ALP was detected by the fully automatic biochemical analyser (Type: Cobas c311, Roche). The test was conducted according to the instructions.

*Hematoxylin and eosin (H&E) staining of the operative sites*

According to the previous report [11], paraffin slices of the operative sites from the HO control group and HO observation group were dewaxed and rehydrated, then stained with hematoxylin (Beyotime Biotechnology, China) for 1 min. After washing for 3 min, these slices were destained with hydrochloric acid alcohol. Then, after washing for 3 min, these slices were fast stained with eosin (Beyotime Biotechnology, China). Finally, after washing for 3 min, these slices were dehydrated, cleaned, and cover-slip mounted using neutral balsam.

*Masson's staining of the operative sites*

The pieces of operative sites in mice fixed in 10% neutral buffered formalin were dehydrated in an increasing ethanol series. Then, they were embedded in paraffin and cut (5-μm thick) using a microtome. Subsequently, they were mounted on glass slides and heated at 40°C for 30 minutes to attach to sections. Then, the sections were dewaxed using xylene and rehydrated using a decreasing ethanol series for one minute. The distilled water was used to wash these sections. Finally, these tissue slices were stained in Masson's trichrome staining reagents following the manufacturer's protocol, according to the previous studies [12]. All tissues were detected by measuring fibrosis under the microscope at 2× and 10× magnification.

*Statistical method*

SPSS 22.0 software (IBM Company, USA) was used for data analyses. The measured data were presented in Mean ± standard deviation (SD). The comparison of inter-group was conducted by One-way ANOVA analysis followed by LSD-t test among more than three groups. The independent-samples t test was used between two groups. The enumeration data was presented in the form of [n (%)]. $\chi^2$ partition test was used for pairwise comparison of the enumeration data. $P<0.05$ was considered with statistical significance.

## Results

*Observations of the HO effects in mice muscle bag of the HO control and HO observation group*

At 4 weeks after operation, the mass at the muscle bag of mice in the observation group became larger in contrast to the HO control group, and in the HO observation group, some mice could not straighten and walk normally, but all mice were in good condition and their diets and activities were normal. X-ray scanning showed that there was more obvious irregular bone shadow at the muscle bag in the hind leg of mice in HO observation group compared to the HO control group. The mice were sacrificed and the muscle bag was exposed. This showed that heterotopic osteogenesis in the HO observation group was generated, which was similar with the results of X-ray scanning, as shown in **Figure 1**.

*Comparison of serum ALP levels between the HO control and HO observation group*

As shown in **Figure 2**, the results showed that the serum ALP level in the mice from the HO observation group was significantly higher than that in the mice from the HO control group ($P<0.05$), suggesting that the muscle pocket of the mice in the HO observation group showed higher ectopic osteogenic activity.

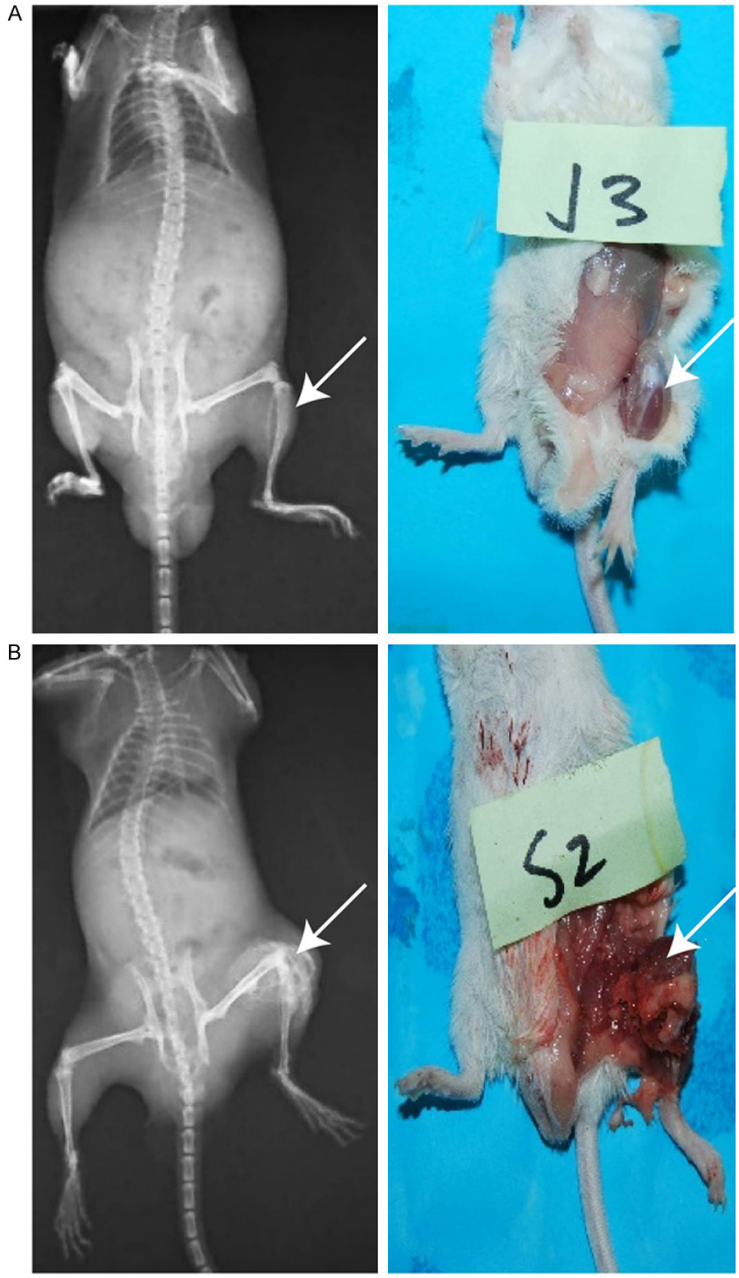


**Figure 1.** The heterotopic osteogenesis effects were compared between the HO control group and HO observation group. White arrows indicates the location of heterotopic osteogenesis. A: The heterotopic osteogenesis effects in the HO control group. B: The heterotopic osteogenesis effects in HO observation group. HO: heterotopic osteogenesis.

*Comparison of histological observation results between the HO control and HO observation group*

At the 4th week after the operation, the mice were sacrificed, and the new heterotopic bone was separated. These new heterotopic bones were stained with H&E and Masson staining, respectively, as shown in **Figure 3**. In the H&E staining of the HO observation group, bone tissue structure was found in the new bone in contrast to the HO control group, forming bone trabeculae and myeloid tissue. Some bone trabeculae were connected to each other to form a grid structure, and the bone marrow cavity was penetrated. In a field of local magnification, a large number of osteoblasts, neatly arranged in a cubic, were found at the edge of the newly formed bone, and there were formed bone lacunae, indicating that the bone tissue was in a relatively mature stage.

The Masson staining was used for differential staining of collagen fibers and muscle fibers. Under the conditions of Masson staining, muscle fibers presented as red and collagen fibers were green or blue. Compared with the HO control group, there was a significant production of collagen bone matrix in the newly formed bone in the HO observation group, which was different from the surrounding red stained muscle fiber tissues.

*Comparison of the bone repair results in the bone defect model between the BD control and BD observation group*

X-ray scanning was used to observe the bone repair conditions at the operation site in SD rats, focusing on the growth of the operation area and its surrounding callus at 0, 2, 4 and 8 weeks after operation in both groups.

As shown in **Figures 4** and **5**, at one week after the surgery, X-ray scanning revealed that there were obvious bone defects in the right leg of both groups. Two weeks after the surgery, it was found that there was some high-density imaging at the bone defect site in the BD observation group, indicating the formation of a bone callus, while there was no high-density imaging or callus formation in the BD control group.

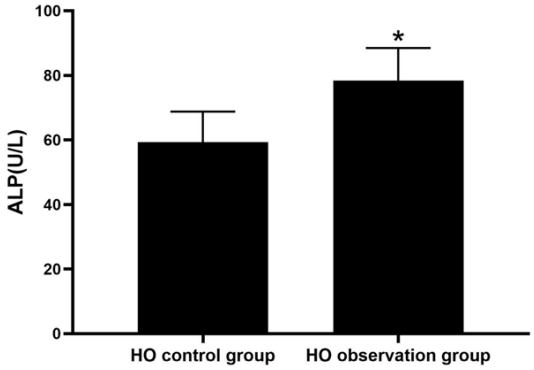


**Figure 2.** Comparison of the serum ALP level between HO control and observation group. ALP: Alkaline phosphatase.

Four weeks after the surgery, it showed that the high-density imaging area was further increased and the bone defect area was blurry in the BD observation group, indicating a large amount of callus formation. However, there was still no high-density imaging in the BD control group. The operation site of SD rats was dissected, and the observed results were consistent with the imaging results of X-ray scanning. The bone defect area in the BD observation group was surrounded by a large amount of callus, while in control group, only a small amount of callus was generated, and the bone defects were still significant. At the 8$^{th}$ week after the surgery, it was found that the high-density imaging area in the BD observation group was decreased, indicating partial absorption of bone callus, while in the BD control group there was still no high-density imaging. The results in BD model were consistent with the imaging results of X-ray scanning. The bone callus in the bone defect area of the BD observation group were decreased, and the bone defect area was gradually repaired, while SD rats from the BD control group still had significant bone defects and did not complete bone repairs.

## Discussion

Bone morphogenesis protein (BMP), as a member of TGF-β growth factors superfamily [13], is widely presented in the bone matrix and could promote the differentiation of mesenchymal stem cells into osteoblasts [14, 15]. Necrosis and absorption at the fracture end could release endogenous BMP. The amount of bone formation was positively proportional to the concentration of BMP and negatively proportional to the diffusion distance. Bone regeneration always occurs between the bone ends. When the distance between the bone ends was beyond the critical value, the amount and distribution of endogenous BMP does not induce chemotaxis well. Therefore, the application of exogenous BMP may make up for the deficiency of endogenous BMP. However, extracting BMP from different animals or human bone tissues is not only complicated, but also inefficient. Therefore, using genetic engineering technology to produce recombinant human bone morphogenesis protein is the key for wide application of BMP in clinical practice. Research has revealed that recombinant BMP plays a similar role as BMP extracted from bone tissue in inducing osteogenesis [16]. Both could induce local undifferentiated mesenchymal cells to differentiate into chondrocytes and form cartilage. With the invasion of blood vessels, endochondral osteogenesis occurs in the cartilage area, and the new bone gradually matures.

The recombinant human BMP-2 (rhBMP-2) has been widely recognized for its clinical efficacy for over 20 years [19-21]. rhBMP-2 has been considered as a promising bone inducing material that has been approved by the Food and Drug Administration (FDA) in the US for clinical treatment. Similar products have also been launched in China. The “Bone Youdao” (Hangzhou Jiuyuan Gene Engineering Co., Ltd.) is a bone repair material, which takes rhBMP-2 as the main active ingredient and contains 250 μg BMP-2 protein per each sample. Previous study had validated the biological activity of this bone material through the cell experiments [17]. In this study, the bone repair material rhBMP-2 was applied, and the role of BMP-2 in promoting fracture healing in animal models was investigated.

At present, the research on BMP-2 focuses on *in vivo* study [18]. The muscle bag heterotopic osteogenesis model is a commonly used animal model to evaluate the osteogenic activity of BMP-2 bone repair materials *in vivo*. This is because muscle tissue contains skeletal myosatellite cells and muscle derived stem cells, which have the ability to differentiate into muscle, bone and fat tissue in the induction environment [14], and the blood supply in skeletal muscle is rich, which is conducive to cell proliferation and differentiation [19]. In this study, a mouse model of muscle bag ectopic osteogen-

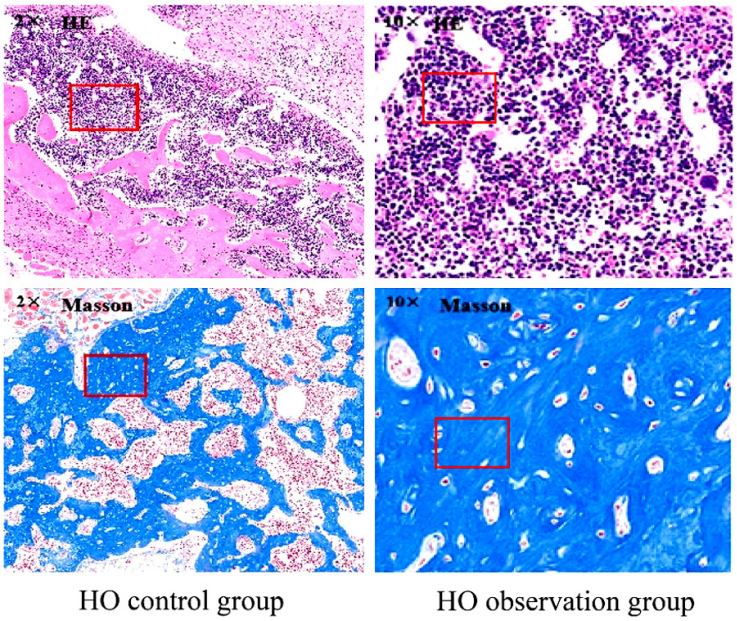


**Figure 3.** The histological staining results of new heterotopic bone using H&E and Masson staining in the HO control group and the HO observation group. H&E: Hematoxylin and eosin. HO: heterotopic osteogenesis.

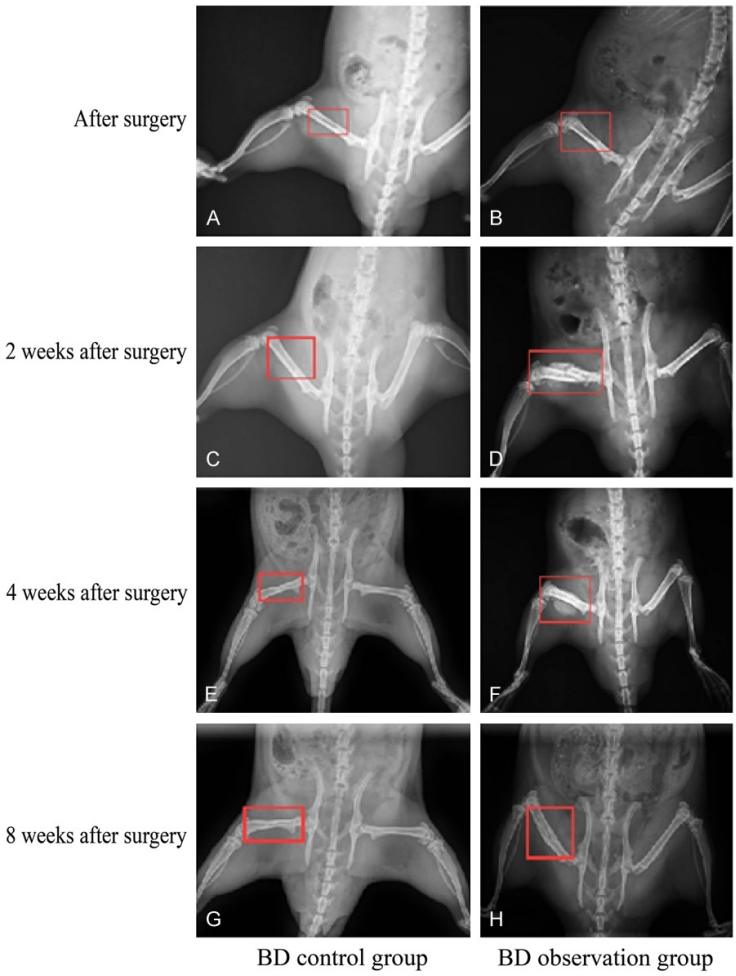


**Figure 4.** The X-ray scanning results of bone defects in the rat models at different time points after surgery in the BD control group and the BD observation group. A: The operation site at first week after surgery in the BD control group; B: The operation site at first week after surgery in the BD observation group; C: The operation site at second week after surgery in the BD control group; D: The operative site at second week after surgery in the BD observation group; E: The operation site at 4th week after surgery in the BD control group; F: The operation site at 4th week after surgery in the BD observation group; G: The operation site at 8th week after surgery in the BD control group; H: The operative site at 8th week after surgery in the BD observation group. BD: bone defect.

esis was established to verify the osteogenic effect of BMP-2. Through X-ray scanning and direct observation after dissection, it was found that there were no abnormalities or new bone formation at the surgical site in the control group, while there was significant bone tissue formation at the surgical site in the observation group. In order to evaluate the quality of newly formed bone, histological staining was performed on the newly formed bone, and the bone mass and trabeculae were analyzed. Bone trabeculae are the extension of cortical bone within cancellous bone, forming an irregular 3D network structure in the bone marrow cavity, which supports hematopoietic tissue. Their microstructure is closely related to the bone quality. Therefore, the microstructure analysis of bone trabeculae is very important in bone analysis. Through H&E and Masoon staining, the formation of bone trabeculae and myeloid tissue can be observed on histological sections, and a large number of osteoblasts were gathered at the edge of new bone, which were in accordance with the report from Shi et al. [20].

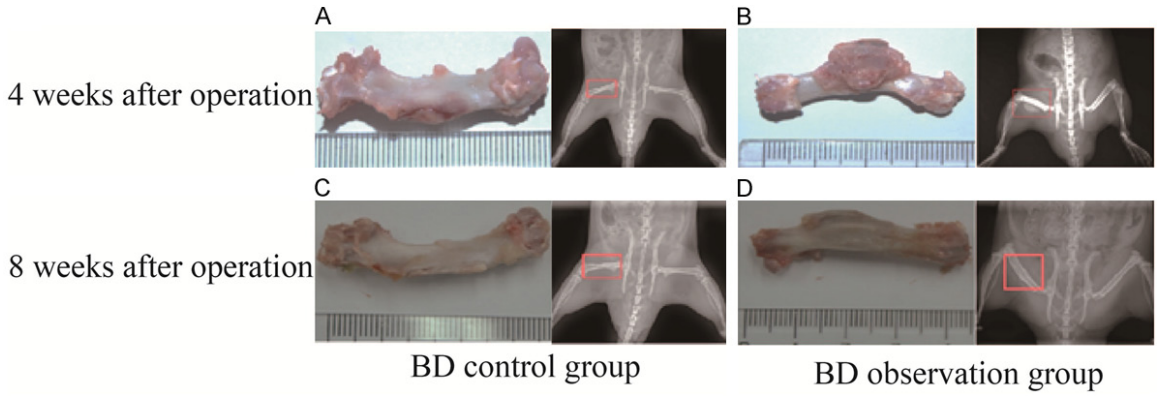


**Figure 5.** The comparison of anatomical results at the operative sites in rat models with bone defects between the BD control group and the BD observation group. A: The operative sites at 4th week after surgery in the BD control group; B: The operation sites at 4th week after surgery in the BD observation group; C: The operation sites at 8th week after surgery in the BD control group; D: The operation sites at 8th week after surgery in the BD observation group. BD: bone defect.

Previous studies showed that the effect of BMP-2 on bone repair required a balance in release and retention of growth factors, such that the released BMP-2 could act as a chemoattractant to the surrounding cells [21], and the retained BMP-2 could promote the bone formation [22]. It has been well established that BMP-2 requires a suitable delivery vehicle to be effective [23], because it is important that the protein should be retained at the site of implantation for a certain time period. Previous studies have demonstrated a positive correlation between the retention of rhBMP-2 upon implantation and the osteoinductive activity in the muscle bag heterotopic osteogenesis model in rats [24]. Some studies reported that many delivery mediums including collagen and alginate provided poor control over BMP-2 localization *in vivo*, the implantation of bone material rhBMP-2 used in this study was demonstrated to prolong the BMP-2 bioactivity [25], indicating that adequate BMP-2 release was achieved. In this study, considerable heterotopic bone formation in muscle bag treated animals with the rhBMP-2 was observed, similar to what has been showed previously in this model [26].

Other research demonstrated that BMP-2 was essential in osteoblast differentiation, and it was considered to have the potential to improve the therapeutic effect of fracture healing in patients [27]. Some studies found that BMP could stimulate the transcription of core binding factor-A1 (Cbfα1), a transcription factor that could activate the production of osteoblast specific genes, such as alkaline phosphatase (ALP), osteopontin, bone sialoprotein, Collagen type I-alpha 1 and osteocalcin to induce bone marrow mesenchymal stem cells (BMSCs) to differentiate into osteoblasts [28]. In this study, the results showed that the level of ALP in the HO observation group was significantly more than that in the HO control group. Previous studies showed rhBMP-2 dose-dependently induced the activity of ALP in C2C12 cells, which was able to specifically convert the differentiation pathway of the clonal myoblastic cell line into that of osteoblast lineage [29]. The ALP was found in several tissues from the whole body and the maximum concentration of ALP was showed in the cells that comprise the liver and bone. Moss et al. reported that the ALP examination had long been applied in the diagnosis of bone problems such as osteomalacia and rickets. Above all, ALP activity could serve as an index for quantifying rhBMP-2 bioactivity. The results of ALP detection in this study also indirectly reflected the osteogenic activity induced by rhBMP-2.

To further validate the bone repair and osteogenic induction effect of BMP-2, a mouse bone defect model was established. A 6-8 mm long full-thickness bone defect was prepared near 1/3 of the femoral head using a drilling machine, reaching deep into the bone marrow cavity, and BMP-2 repair materials were implanted. The results showed good bone defect repair ability in the observation group in

contrast to the control group. Both imaging detection and animal anatomy showed that there was a large amount of callus formation in the bone defect area in the observation group, with significant bone repair effects. However, the control group had no callus formation in the bone defect area, and the defect area was difficult to heal, which is similar with other studies [30].

However, there are still some limitations in this research as a single-center study, with small sample and animal experiments. This study did not involve human experiments, which may be superior to animal experiments. The specific mechanism was not explored as to whether the role of BMP-2 in promoting bone formation explain is achieved by a certain signal transduction. In the future, a multicenter and more deep study with human experiments is required for further confirmation.

In summary, BMP-2 exhibits good osteoinductive and osteogenic activity *in vivo*. It can be used as a scaffold for bone tissue engineering to repair bone injuries and promote bone healing.

## Disclosure of conflict of interest

None.

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