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Original Article

Novel functionalized BMP-2 polylactic acid nanospheres regulate the TGF-β pathway to promote hormone-related osteonecrosis repair

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Abstract: Objective: To investigate the role and mechanism of the TGF-β pathway in promoting hormone-related osteonecrosis repair in novel functionalized bone morphogenetic protein-2 (BMP-2) polylactic acid (PLA) nanospheres. Methods: Ninety-six 12-month-old, clean-grade, male Sprague-Dawley rats (200 g-230 g) were selected and divided into four groups: the control group (group A, n=24), the model group (group B, n=24), the intervention group (group C, n=24), and the BMP-2 group (group D, n=24). Results: Compared with group A, the bone lacuna rate in group B was decreased significantly, and the trabecular bone area rate in group B was increased significantly. There were no significant differences in the alkaline phosphatase (ALP) content on the first and second days in groups A, B, C or D (P>0.05). The ALP activity of the osteoblasts in group B was significantly higher than it was in group A. There were no significant differences in the number of alizarin red stained mineralized nodules between the first and second days in groups A, B, or C (P>0.05). The number of alizarin red stained mineralized nodules of the osteoblasts in group B was significantly lower than it was in group A. Compared with group A, the expressions of TGF-β and Smad in the osteoblasts in group B were significantly decreased. There were no significant differences between group D and group A (P>0.05). Conclusion: The novel functionalized BMP-2 PLA nanospheres regulate the TGF-β pathway to promote the repair of osteonecrosis, which may be achieved by bone cell regeneration.

Keywords: BMP-2 polylactic acid nanospheres, TGF-β pathway, osteonecrosis

Introduction

With the widespread use of corticosteroids, hormone-related osteonecrosis is also increasing. The most common type is femoral head necrosis, which causes pain in the hip joint and medial thigh and ultimately leads to osteoporosis and bone deformity [1, 2]. At present, no specific treatment is available clinically, and there are no specific therapeutic drugs [3]. Therefore, the aim of this study was to explore the mechanism of corticosteroid-related osteonecrosis and provide a theoretical basis and practical approach for the specific treatment of this disease [4, 5].

Bone morphogenetic protein-2 (BMP-2), one of the cell growth factors involved in fracture healing, can stimulate the proliferation and differentiation of bone cells and chondroblasts [6, 7]. BMP-2 regulates gene expression in bone cells and chondroblast nuclei during fracture repair to promote bone formation [8-10]. BMP-2 polylactic acid (PLA) nanospheres are encapsulated in the polylactide carrier of polymer materials using nanotechnology. When applied to the necrotic femoral head, BMP-2 is slowly absorbed and utilized by the damaged bone to prolong its efficacy and has the advantages of directional precision and good cell penetration [10, 11].

At present, there are many studies on the treatment of mandibular fracture with BMP-2 PLA nanospheres. The BMP-2 and fibroblast growth factor (FGF) nanospheres slow-release system promotes osteoblast proliferation and promotes alkaline phosphatase (ALP) activity and mineralization, but its molecular mechanism has not yet been explored. TGF-β and

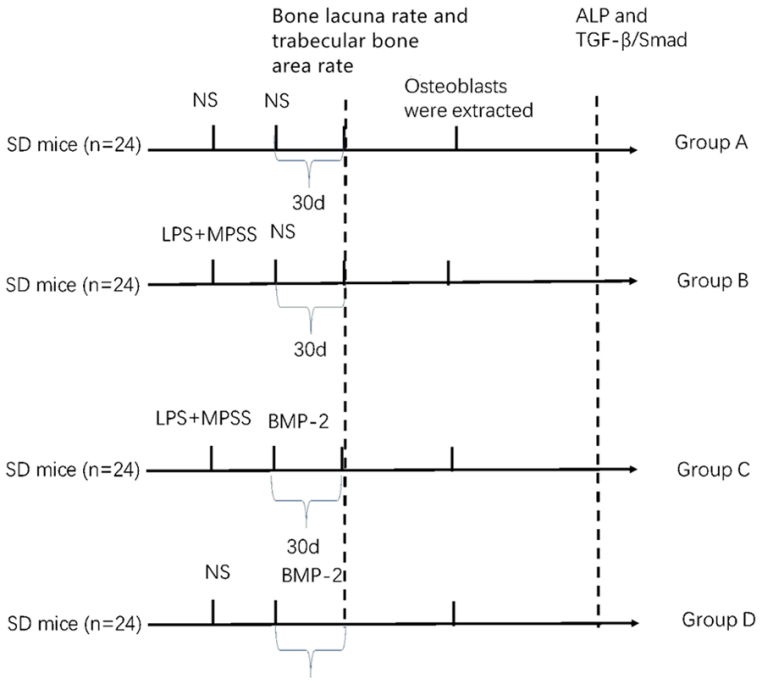

Figure 1. Experimental grouping and process.

BMP-2 both decrease when osteonecrosis occurs, yet both increase in the healing of fractures, and they play different roles in the different stages of fracture healing. BMP-2 content increases significantly in early bone induction, and TGF-β plays an important role in later bone formation and maturation [12, 13]. BMP-2 can recruit and induce mesenchymal cells to differentiate into irreversible cells at an early stage, increase the TGF-β factor, and form osteoblasts under the positive feedback of the TGF-β factor and regenerate continuously. Therefore, in this study, a hormone-related osteonecrosis model was constructed to explore the effect of BMP-2 PLA nanospheres on osteoblasts and the important molecules in the signaling pathway, so as to provide more methods for the clinical treatment of corticosteroid-related osteonecrosis.

Materials and methods

Animals and grouping

Ninety-six healthy, 12-month-old, male Sprague-Dawley (SD) rats weighing 200 g-230 g were purchased. The license number was SCXK (Su) 2009-0002. Rat feed and bedding were purchased from the Qinglongshan Animal Test Center, Nanjing. All rats maintained their biological rhythms: they were fed in separate cages (one per cage) with a constant room temperature (20°C-25°C) and circadian light rhythm control (8:00-20:00). All the rats were given adaptive feeding for one week before the experiment. The rats were divided into four groups: the control group (group A, n=24), the model group (group B, n=24), the intervention group (group C, n=24) and the BMP-2 group (group D, n=24). See **Figure 1**. All the experimental procedures were approved by the Research Commission on Ethics of The First Affiliated Hospital of Fujian Medical University. The research related to animal use complied with all the relevant national regulations and institutional policies for the care and use of animals.

Reagents and materials

Main reagents and materials: lipopolysaccharide (batch number: L3024; Sigma Company, USA), methylprednisolone injection (batch number: 1437009; Sigma Company, USA), ALP ELISA kit (batch number: 150303; Shanghai Xitang Biotechnology Co., LTD., China), TGF-β antibody (batch number: 3711; Cell Signaling Technology Company, USA), Smad antibody (batch number: 12470; Cell Signaling Technology Company, USA), β-actin antibody (batch number: 4970T; Cell Signaling Technology Company, USA). The BMP-2 PLA nanospheres were synthesized by solid phase synthesis to embed BMP-2 into the PLA nanospheres to prepare composite scaffolds [14].

Methods

The rats in groups B and C were injected with lipopolysaccharide (100 mg/kg) for 14 consecutive days, methylprednisolone (8 mg/kg) intramuscularly on the 14th day and 6 days successively to prepare a model of steroid-induced avascular necrosis of the femoral head (SANFH) [15]. At the first injection of methyl-

prednisolone, group C began an intramuscular injection of novel functionalized BMP-2 PLA nanospheres [2 μg/(kg·d)] for 30 days, while group D received the same dose of BMP-2 PLA nanospheres at the same time for 30 days. Groups A B were injected with the same amount of normal saline at each time point. The conditions of the animal model in groups B and C were observed. Criteria for successful modeling: narrowing of the joint space, thinning of the bone cortex, decreased bone density, low density shadow in the bone defect area, sparse and disorganized trabeculae bone.

Extraction of femoral osteoblasts: the rats were killed after they were anesthetized, their bilateral femoral heads were removed, their covered muscles and soft tissues were removed, the femoral heads were exposed, and the femoral heads were extracted and immersed in PBS. The femoral heads were cut and mixed with 0.25% trypsin for 15 min, followed by 0.1% collagenase II digestion for 1 h, and finally fetal bovine serum was added to stop the tissue digestion. The osteoblasts were collected after the centrifugation and suspended in a diluted medium. The cells were planted in a culture flask and cultured in a 5% CO_2 incubator at 37°C.

Histological observation

The rats were killed after being anesthetized, their bilateral femoral heads were removed, their covered muscles and soft tissues were removed, and the femoral heads were exposed. The femoral heads were treated with a paraffin section and observed with a high power lens. The positive number of bone lacunae in each section (positive = more than 50 osteocytes at 5 observation points) was analyzed and the bone lacuna rate was calculated.

Western blot

The bone tissue TGF-β expression was measured, the bone tissue protein was extracted at a low temperature, and the protein concentration was determined using the BCA method. The protein concentration of each sample was adjusted according to the quantitative results of the protein, and 10 μL of a denatured sample was added. Then 12% SDS-AGE was used to separate the samples, and we transferred the membranes and sealed them, and β-actin (1:2,000), TGF-β (1:1,000), and Smd (1:1,000) primary antibodies were added dropwise, and left at 4°C overnight. Next we washed the membrane, and then the secondary antibody (1:1,000) was incubated for 2 h at room temperature. After we washed the membrane, a highly sensitive ECL chemiluminescent reagent was applied to the front side of the membrane, and the exposure time was determined according to the fluorescence brightness of the membrane. The results were analyzed with Quantity One gel quantitative software. The expressions of TGF-β and the Smad protein in each group were compared using mean optical density and the ratio of the target protein to the internal reference for β-actin.

Statistical analysis

The SPSS 22.0 software package was used for the statistical analysis. The measurement data were expressed as the mean ± standard deviation (mean ± sd). One-way ANOVA was used for the comparison among three groups, and the comparisons between two groups used SNK tests. There is a significant difference at $P<0.05$.

Results

BMP-2 polylactic acid nanospheres can improve bone lacuna and the trabecular bone area rates

Compared with group A, the bone lacuna rate in group B was decreased significantly, but the rate in group C was significantly improved compared with the rate in group B, but it was still decreased compared with the rate in group A (all $P<0.05$). There was no significant difference between group D and group A ($P>0.05$). Compared with group A, the trabecular bone area rate in group B was significantly increased, and the rate in group C was significantly improved compared with the rate in group B, but it was still increased compared with the rate in group A (all $P<0.05$). There was no significant difference between group D and group A ($P>0.05$). The results indicated that BMP-2 PLA nanospheres can improve bone lacuna and trabecular bone area rates. See **Figure 2**.

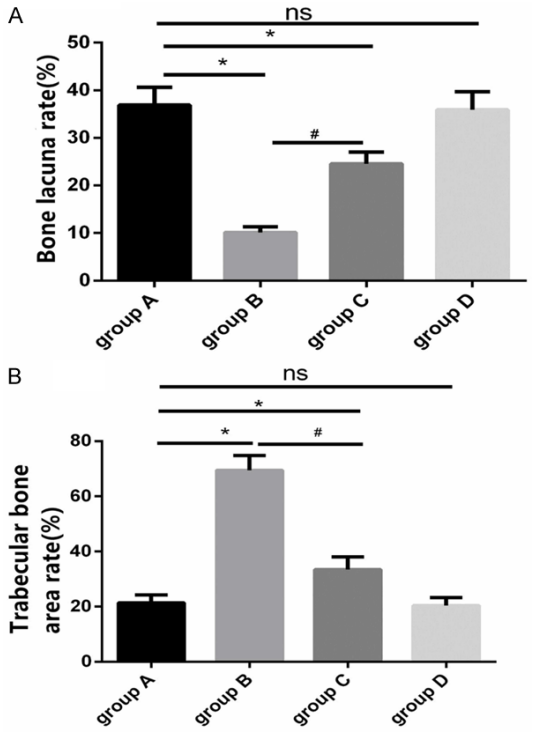

Figure 2. Comparison of the trabecular bone area and the bone lacuna rates among the three groups. A. Bone lacuna rate (%); B. Trabecular bone area rate (%). *P<0.05 VS. group A; #P<0.05 VS. group B; ns: VS. group A P>0.05.

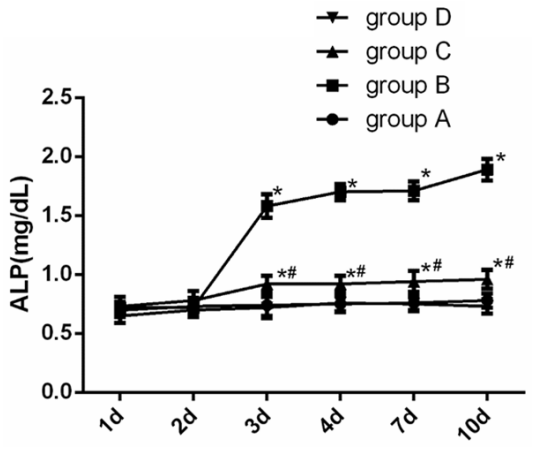

Figure 3. The ALP activity expression of the osteoblasts. ALP: alkaline phosphatase. *P<0.05 VS. group A; #P<0.05 VS. group B.

BMP-2 polylactic acid nanospheres can improve the alkaline phosphatase activity of osteoblasts

There were no significant differences in the alkaline phosphatase (ALP) content between the first and second days in groups A, B, and C (P>0.05). The ALP activity of the osteoblasts in group B was significantly increased compared to group A, and the activity in group C was significantly improved compared with the activity in group B, but it was still higher than the activity in group A (all P<0.05). There was no significant difference between group D and group A (P>0.05). See **Figure 3**.

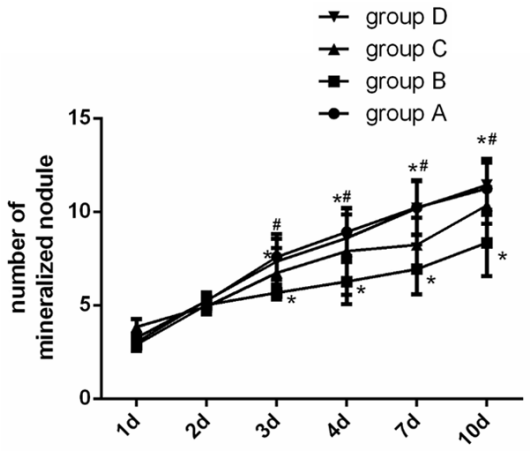

Figure 4. Comparison of the number of alizarin red stained mineralized nodules among the three groups. Number of mineralized nodules: number of alizarin red stained mineralized nodules. *P<0.05 VS. group A; #P<0.05 VS. group B.

BMP-2 polylactic acid nanospheres can improve alizarin red stained mineralized nodules

There were no significant differences in the number of alizarin red stained mineralized nodules between the first and second days in groups A, B and C (P>0.05). The number of alizarin red stained mineralized nodules of osteoblasts in group B was significantly decreased compared to group A, and the number in group C was significantly improved compared with the number in group B, but it was still lower than the number in group A (all P<0.05). There was no significant difference between group D and group A (P>0.05). See **Figure 4**.

BMP-2 polylactic acid nanospheres inhibit the expression of TGF-β and the Smad protein

Compared with group A, the expression of TGF-β and Smad protein in osteoblasts in group B was significantly decreased, while that in group C was significantly improved compared

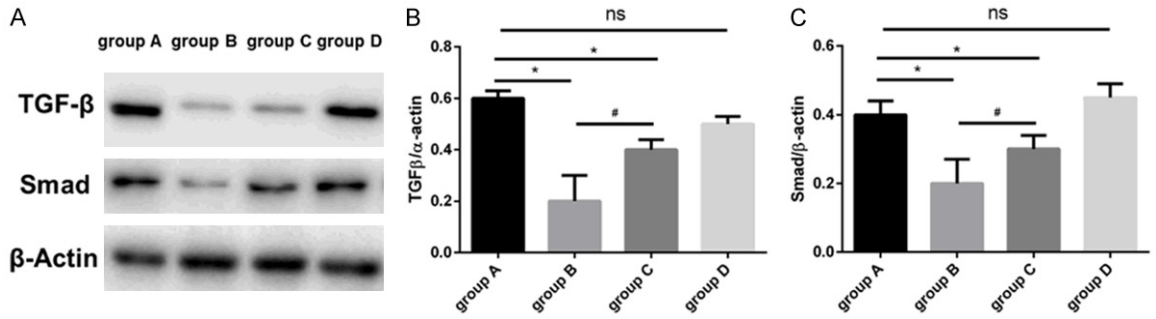

Figure 5. Comparison of the TGF-β and Smad protein expressions. A. Western blot bands of the four groups of TGF-β, Smad internal reference protein and β-actin protein; B. Gray scale analysis of TGF-β; C. Gray scale analysis of Smad protein. *P<0.05 VS. group A; #P<0.05 VS. group B; ns: VS. group A P>0.05.

with that in group B, but still decreased compared with that in group A (all P<0.05). There was no significant difference between group D and group A (P>0.05). See **Figure 5**.

Discussion

SANFH is usually a kind of aseptic necrosis caused by ischemic necrosis of the femoral head due to excessive use of corticosteroids [16, 17]. This is usually manifested as the bone lacuna rate decreases and the trabecular bone breaks, which eventually leads to osteoporosis [18]. In this experiment, the SANFH model was prepared using lipopolysaccharide combined with methylprednisolone. It was found that the bone lacuna rate was decreased and the trabeculae bone was significantly damaged, an indication that the model was successfully constructed. At present, in the treatment of clinically aseptic osteonecrosis, in addition to inhibiting the etiology, inhibiting trabecular bone injury is also an important treatment method. Current treatments include mesenchymal stem cell therapy, which can proliferate and differentiate into osteoblasts, repair damaged trabeculae bone, secrete protective cytokines, and stimulate the generation of cartilage and bone [1, 19]. In this study, BMP-2 PLA nanospheres were constructed to promote the formation of osteoblasts, which can repair and regulate growth factors and induce bone repair. Schofer et al. found that BMP-2 PLA nanospheres promote bone development and bone healing through FGF, which is consistent with the results of this experiment. A possible mechanism was to promote bone development by secreting transforming growth factor [20].

At present, BMP-2 PLA nanospheres are microsomes formed by adsorbing BMP-2 onto macromolecule compounds. Their carriers are starch microspheres or gelatin polysaccharide microspheres [21]. Currently, BMP-2 PLA nanospheres are mainly used for repairing mandibular fractures and repairing corticosteroid osteonecrosis and other bone injury diseases [22]. Liang et al. found that glucocorticoids can directly damage the osteocytes, resulting in cell necrosis. BMP-2 PLA nanospheres can slow down the killing sensitivity to hormones and have a repair effect on osteoblasts [23]. Huang et al. found that BMP-2 PLA nanospheres have no immunogenicity and have significant biological activity on rabbit osteoblasts, which can promote mandibular fractures and accelerate fracture repair in rabbits [24]. This experiment also found that BMP-2 PLA nanospheres can repair the development of osteoblasts.

TGF-β is involved in the dynamic balance process of cell growth and differentiation, as well as the growth and development of osteoblasts. Its ligands mainly include bone morphogenetic protein and growth differentiation factor [25]. When it binds to TGF-β, it catalyzes the phosphorylation of TGF-β. The Smad family of proteins transfer cell surface receptors to the nucleus and mediate the signal transduction of the TGF-β family members. In this experiment, it was found that the expression of the Smad protein decreased when BMP-2 binds to TGF-β [26]. Subramaniam et al. have also found that BMP-2 PLA nanospheres can activate TGF-β type II, promote TGF-β activation, and activate Smad proteins [27].

There are still some limitations to this study. Although BMP-2 PLA nanospheres inhibit hormone-related osteonecrosis through the TGF-β pathway, whether BMP-2 PLA nanospheres target TGF-β molecules to alleviate osteonecrosis has not been determined. In our future research, we will construct a molecular cell model that inhibits or overexpresses TGF-β to investigate the mechanism of BMP-2 PLA nanospheres in the treatment of hormone-related osteonecrosis at the gene level.

In conclusion, the novel functionalized BMP-2 PLA nanospheres regulate the TGF-β pathway to promote the repair of osteonecrosis, which may be achieved by bone cell regeneration.

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Disclosure of conflict of interest

None.

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