# Treatment of osteonecrosis of femoral head with BMSCs-seeded bio-derived bone materials combined with rhBMP-2 in rabbits

XIAO Zeng-ming 肖增明, JIANG Hua 江华 *, ZHAN Xin-li 詹新立, WU Zhen-guo 武振国 and ZHANG Xing-lin 张兴琳

**Objective:** To evaluate the effect of autologous bone marrow mesenchymal stem cells (BMSCs) seeded bio-derived bone materials (BBM) combined with recombinant human bone morphogenetic protein-2 (rhBMP-2) in repairing defect of osteonecrosis of femoral head (ONFH).

**Methods:** Early-stage osteonecrosis in the left hip was induced in 36 adult New Zealand white rabbits (provided by the Animal Center of Guangxi Medical University, Nanning, China) after core decompression and delivery of liquid nitrogen into the femoral head. Then the animals were divided into three groups according to the type of implants for bone repair: 12 rabbits with nothing (Group Ⅰ, the blank control group), 12 with BBM combined with rhBMP-2 (Group Ⅱ), and 12 with BMSCs-seeded BBM combined with rhBMP-2 (Group Ⅲ). At 4, 8, and 12 weeks after surgery, X-ray of the femoral head of every 4 rabbits in each group was taken, and then they were killed and the femoral heads were collected at each time point, respectively. Gross observation was made on the femoral heads. After hematoxylin and eosin staining, Lane-sandhu scores of X-ray and bone densitometry were calculated and the histomorphometric measurements were made for the new bone trabeculae.

**Results:** At 12 weeks after surgery, two femoral heads collapsed in Group Ⅰ, but none in Group Ⅱ or Group Ⅲ. X-ray examination showed that the femoral heads in Group Ⅰ had defect shadow or collapsed while those in Group Ⅱ had a low density and those in Group Ⅲ presented with a normal density. Histologically, the defects of femoral heads were primarily filled with no new bone but fibrous tissues in Group Ⅰ. In contrast, new bone regeneration and fibrous tissues occurred in Group Ⅱ and only new bone regeneration occurrd in Group Ⅲ. Lane-sandhu scores of X-ray, bone mineral density and rate of new bone in trabecular area in Group Ⅲ were higher significantly than those of the other two groups.

**Conclusions:** Our findings indicate a superior choice of repairing the experimental defect of ONFH with BMSCs-seeded BBM combined with rhBMP-2.

**Key words:** ***Engineering tissues; Osteogenic repair; Bone marrow mesenchymal stem cells; Bone morphogenetic protein; Bio-derived bone materials***

*Chin J Traumatol 2008; 11(3):165-170*

Reconstructing trabecular and subchondral bones after osteonecrosis of the femoral head continues to be a challenging problem for orthopedists.[1] For the potentiality of bone marrow mesenchymal stem cells (BMSCs) to differentiate into various types of cells and the osteoinductive capability of recombinant human bone morphogenetic protein-2 (rhBMP-2), many researchers use them for bone tissue engineering materials. In this study, we use the tissue engineering bones of BMSCs seeded onto an absorbable scaffold (bio-derived bone materials, BBM) combined with rhBMP-2 to repair defects after experimental osteonecrosis of the femoral head in rabbits.

Department of Orthopedics, First Affiliated Hospital, Guangxi Medical University, Nanning 530021, China (Xiao ZM, Jiang H, Zhan XL, Wu ZG and Zhang XL)

*Corresponding author: Tel:86-771-5350189, 13978837575, E-mail: JHGL007@163.com

This study was supported by the Natural Science Foundation of Guangxi Zhuang Autonomous Region (No. 0135044).

## METHODS

### Preparation of BBM combined with rhBMP-2 and BMSCs-seeded BBM combined with rhBMP-2

**Isolation and culture of BMSCs of rabbits** Autologous bone marrow was aspirated from the iliums of the rabbits, which took a 2-week quarantine period before surgery. Karyocytes were obtained with plastic adherence methods. BMSCs were collected from the marrow blowouts by centrifugation at 800 r/min for 5

minutes, resuspended in Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (Gibco Company, USA) containing 10% FBS, 50 μg L-Glutamine, 100 U penicillin G and 100 μg streptomycin per milliliter culture media in a humidified atmosphere of 5% carbon dioxide at 37 $^0$C, and finally transferred to 50-cm$^2$ flasks at 4×10$^4$ cells/cm$^2$. The growth of cells was observed under an inverted microscope, and the culture media was changed every two days. After 5-7 days, the first generation cells were transferred to another flask by 0.25% trypsin digestion, which obtained the second generation cells. After culture for 1-2 weeks, the third generation cells were obtained and identified by surface markers of CD34, CD44, CD105 and CD166 for the source of *in vivo* experimant.

**Assembly of BMSCs combined with rhBMP-2**

According to a previous report,[4] BBM was made in size of 2 mm×2 mm×10 mm, which was processed with physical and chemical methods (partially being deproteinized) from rabbit bones. At the ratio of 1 mg:1 ml, rhBMP-2 powder (Biosynthesis Biotechnology Company, Beijing, China) was dissolved in the compound of hydrochloric acid (4 mol/L) and Calcium Chloride (5 mol/L), kept at 4$^0$C for 12 hours. The BBM was suspended in solution of rhBMP-2 in a vacuum chamber for 12 hours at 4$^0$C, then dialysed for 48 hours and gradually cryodesiccated. After identification of *in vitro* BMSCs, 5×10$^6$ cells were resuspended in 2 ml culture medium and then seeded onto scaffolds combined with rhBMP-2, at a density of 5×10$^6$ per graft. Tissue constructs were placed in a humidified incubator at 37$^0$C for 24 hours to allow cells to attach to the scaffolds. The medium was changed every 3 days. The BMSCs-seeded grafts were cultured for a period of 1 week before implantation. The assembled grafts were analyzed by light microscopy and electron microscopy, which confirmed that the cells had adhered to the scaffolds combined with rhBMP-2.

**Surgical procedures of establishment of animal model and grouping**

A total of 36 6-month-old New Zealand white rabbits (weighing 2.0-2.5 kg of either sex, provided by the Animal Center of Guangxi Medical University, Nanning, China) were employed in this study. Under general anesthesia, defects of osteonecrosis of the left femoral head were established in all the animals.[2,3] Briefly, all the animals were anesthetized with a combination of Ketamine (80 mg/kg body weight) and 5% pentobarbital (1 g/kg body weight) intraperitoneally. With the rabbit in the prone position, the back of left hind limb was shaved and treated with antiseptic solution and sterilly draped. The hip joint was exposed through lateral incision. The articular capsule was opened and the femoral head was exposed, but not cutting the ligamentum teres to dislocate the hip joint. And at 1 cm distant from the greater trochanter, a hole was made in the direction of the femoral head (with the hole diameter of 2 mm) along the articular cartilage. And the cancellous bones were shaved with a special curet along the inner side of the hole till the articular cartilage, which accounted for about 50% of the total volume of the femoral head. Then fluid nitrogen was used to freeze the femoral head for about 5 minutes. After rewarming, BBM was implanted.

The defect sites were left unfilled in 12 control rabbits (Group Ⅰ), filled with BBM combined with rhBMP-2 in 12 rabbits (Group Ⅱ), and filled with BMSCs-seeded BBM combined with rhBMP-2 in the other 12 rabbits (Group Ⅲ). Two animals died during operation and two were died of diarrhea after operation, who were replaced to maintain the individual group size. The incision was closed in two layers. The animals were maintained on a soft diet for 48 hours. A regular diet was resumed 3 days postoperatively.

**Radiological analysis**

The rabbits underwent standardized serial radiography of the left femoral head at 4, 8, and 12 weeks after operation. To quantify the information of rate of bone formation available in these serial radiography, bone repair was observed based on Lane-Sandhu radiographic criteria.

**Histological examination and computerized morphometric analysis**

After X-ray photographs, 4 rabbits from each group were killed under general anesthesia at intervals of 4, 8 and 12 weeks. After sacrifice, the left femoral head was harvested and taken for bone densitometry. Each sample was measured for 4 times. The bone mineral density (BMD) of the femoral head was tested with single-energy photon densitometric analyser. Mean values were obtained. After bone densitometry, the specimens were fixed in 10% buffered formalin for 24

hours, decalcified in ethylenediamine tetraacetic acid, embedded in paraffin, and cut into 5-μm-thick coronal sections. The sections were stained by hematoxylin and eosin (HE) to evaluate the presence of bone formation. These sections were photographed to provide histological images of the specimens. To choose five vision fields of these specimens under 20×20 magnification randomly, the rate of new bone trabecular area (the ratio of the areas of new bone trabecula to the total areas of bone trabecula) was calculated with Leica Qwin image analysis system.

**Statistical analysis**

All the data were presented as mean ± standard deviation ($\overline{x}$ ± SD). The data were analyzed with SPSS 13.0 software using ANOVA for the Lane-sandhu scores of X-ray, the rate of new bone trabecular area and BMD among Groups Ⅰ, Ⅱ, and Ⅲ at 4, 8, and 12 weeks after operation. A *P*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

**Detection of BMSCs**

The surface makers of BMSCs (CD34, CD44, CD105 and CD166) were detected by flow cytometry. The results were CD34(-), CD44(+), CD105(+) , and CD166(+).

**Observation of BMSCs-seeded materials combined with rhBMP-2**

The BMSCs were combined with BBM tightly with a favourable morphology as shown by scanning electron microscope.

**Radiographic findings**

The serial radiographs obtained immediately after surgery and at 4, 8, 12 weeks after surgery revealed obvious difference among the three groups. We quantified these difference by Lane-sandhu X-ray scores. The results of these scores are summarized in Table 1. At 4 weeks after surgery, the femoral head defect showed low density of radiography in all groups, but the place where implanted BBM showed high density of radiography and clear interface between the bones and the neighbouring tissues in Groups Ⅱ and Ⅲ. At 8 weeks after surgery, the low density of radiography was also clear in the defect of Group Ⅰ, where cystic degeneration occurred. BBM was decreased in Group Ⅱ, where the density of radiography was higher than that of 4 weeks. Especially, the materials of Group Ⅲ were smaller and the density was higher than those of Group Ⅱ at the same time point. At 12 weeks, the low density of the defect was also obvious and small area of scleritization occurred around the defect. The interface between the bones and BBM was indistinct in Group Ⅱ. In Group Ⅲ, the BBM was absorbed obviously, and the image of new bones was clear, thus the density of the femoral head was highest in all three groups at the same time point (Fig.1). As shown in Table 1, Lane-sandhu scores indicated a statistically significant difference in the three groups.

**Histological findings**

At 4 weeks after surgery, histological evaluation of the femoral head defects of Group Ⅰ demonstrated hyperplasia of fibres and low ossification. The defects of Group Ⅱ were filled with few new bones. Osteogenesis was more obvious in the defects of Group Ⅲ (Fig.2), and new bones grew into the BBM. At 8 weeks, immature osteotylus filled in the defects of Group Ⅰ, which was surrounded by fibrous tissues. New bones grew in the defects, but the interface between the materials and the bones was clear in Group Ⅱ. The areas of new bone growth increased and the interface between the materials and the bones was indistinct in Group Ⅲ (Fig.3). At 12 weeks, histological examination revealed that the femoral head defects of Group Ⅰ had a histological appearance of fibrosis, whereas the defects were filled with new bones in Groups Ⅱ and Ⅲ. Especially, newly-formed bones were filled in the defects in Group Ⅲ, and the implanted BBM was extensively degradated, thus the reconstructed bones were made of hard and well-formed bones that were well-incorporated into the surrounding native bones (Figs. 4 and 5). As shown in Table 2, computerized morphometric analysis demonstrated a statistically significant difference in bone regeneration in femoral head defects in Group Ⅲ (20.5% ± 1.36% at 4 weeks, 30.8% ± 2.46% at 8 weeks and 63.2% ± 2.36% at 12 weeks) compared with Groups Ⅰ and Ⅱ (3.94% ± 0.28% and 10.6% ± 1.14% at 4 weeks, 7.12% ± 1.26% and 17.1% ± 1.17% at 8 weeks, and 10.24% ± 0.32% and 31.1% ± 1.13% at 12 weeks,respectively, *P*<0.05).

**Bone densitometric findings**

At 8 and 12 weeks after operation, the difference of BMD among the three groups was statistically signifi-

Chinese Journal of Traumatology 2008; 11(3):165-170

cant ($P$<0.05), in order of Group III>Group II >Group I (Table 3).

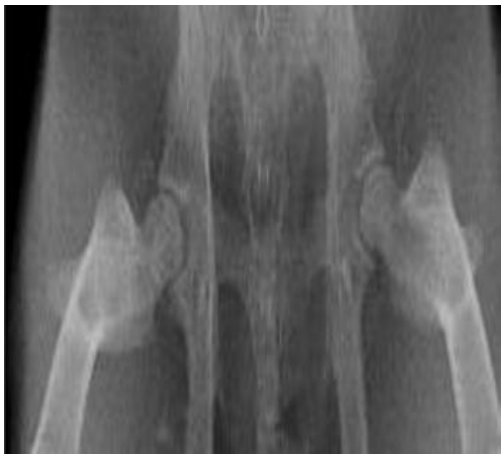

**Fig.1.** Radiograph of femoral head from Group III in 12 weeks illustrates that the hip joint space is normal and the articular cartilage is not collapsed.

**Table 1.** Lane-sandhu scores of X-ray ( $\bar{x}$ ± $s$, scores)

| Groups | Time after operation (weeks) | | |
|---|---|---|---|
| | 4 | 8 | 12 |
| I | 0.34 ± 0.19 | 1.06 ± 0.35 | 2.02 ± 0.32 |
| II | 1.21 ± 0.26 | 2.40 ± 0.23 | 4.31 ± 0.24 |
| III | 3.81 ± 0.24 | 6.50 ± 0.68 | 9.44 ± 0.32 |
| $t$ | 2.712 | 2.739 | 2.893 |
| $P$ value | <0.05 | <0.05 | <0.05 |

**Table 2.** Rate of trabecular area of new bones ( $\bar{x}$ ± $s$, %)

| Groups | Time after operation (weeks) | | |
|---|---|---|---|
| | 4 | 8 | 12 |
| I | 3.94 ± 0.28 | 7.12 ± 1.26 | 10.24 ± 0.32 |
| II | 10.6 ± 1.14 | 17.1 ± 1.17 | 31.1 ± 1.13 |
| III | 20.5 ± 1.36 | 30.8 ± 2.46 | 63.2 ± 2.36 |
| $t$ | 2.541 | 2.603 | 3.010 |
| *P value* | *<0.05* | *<0.05* | *<0.05* |

**Table 3.** Bone mineral density ( $\bar{x}$ ± $s$, g/cm$^2$)

| Groups | Time after operation (weeks) | | |
|---|---|---|---|
| | 4 | 8 | 12 |
| I | 0.565 ± 0.083 | 0.587 ± 0.068 | 0.582 ± 0.032 |
| II | 0.589 ± 0.074 | 0.617 ± 0.057 | 0.629 ± 0.053 |
| III | 0.613 ± 0.078 | 0.643 ± 0.046 | 0.692 ± 0.063 |
| $t$ | 1.782 | 2.631 | 2.744 |
| $P$ value | *<0.05* | *<0.05* | *<0.05* |

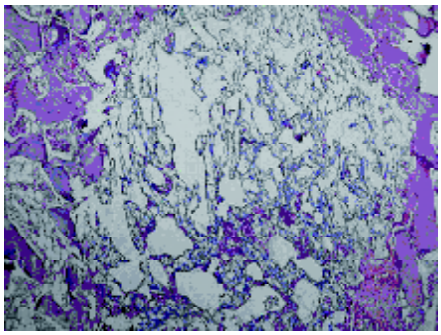

**Fig.2.** Histological view of a hemisected specimen from Group III in 4 weeks shows that new bones grow toward bio-derived bones, which are degradating slowly ( HE × 400).

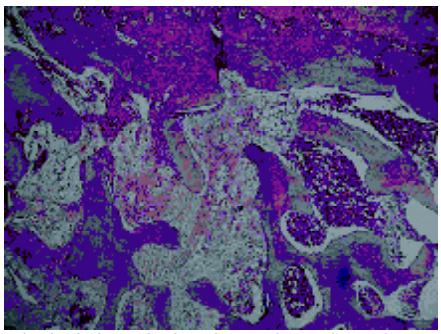

**Fig.3.** Histological view of a hemisected specimen from Group III in 8 weeks shows that the areas of new bone increase and bio-derived bone is degradating gradually. The interface between the materials and bones is indistinct (HE × 400).

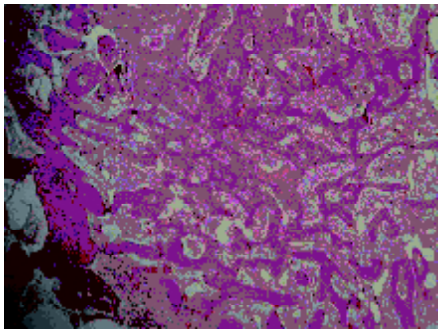

**Fig.4.** Histological view of a hemisected specimen from Group III in 12 weeks shows that bio-derived bones are extensively degradated and the reconstructed bones consist of hard and well-formed bones (HE × 400).

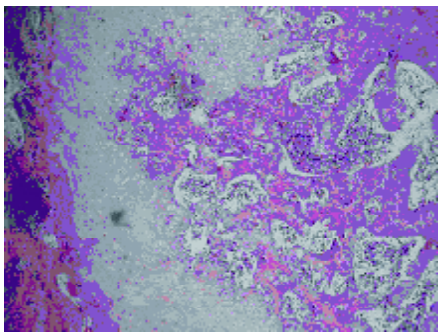

**Fig.5.** Histological view of a hemisected specimen from Group III in 12 weeks shows that new bone trabeculae and endochondral bones ingrow into degradated bio-derived bones (HE × 400).

## DISCUSSION

In the advanced stage, hip arthroplasty is a successful treatment for osteonecrosis of the femoral head, but the long-term results may be less than optimal.[5] Especially in young adults or in the early stage, the joint-preserving treatment is more important. Core decompression of the hip is a common joint-preserving treatment. Replacement of the necrotic bone at an early stage to promote osteogenesis as well as to heal subchondral bone lesions may delay or prevent the progression of the disease.[6] Bone-grafting is one of the most effective methods to enhance the bone repair but has associated problems and limitations.[7] Autogenous bone-grafting is a gold standard now, but its supply is limited and donor-site complications will occur. Allografts are useful, but its shortcomings are immunogenicity and potentiality to transmit disease. We use BMSCs-seeded BBM combined with rhBMP-2 substituting autografts or allografts to implant into the defect of osteonecrosis of the femoral head, simulating clinically core decompression with bone-grafting.

In accordance with the methods of Takaoka et al[2] and Tang et al[3], left-hip early-stage osteonecrosis was induced in adult rabbits after core decompression and delivery of liquid nitrogen into the femoral head. In this study, at 4 weeks after operation, X-ray examination of Group Ⅰ demonstrated low density of the femoral head and cystic degeneration. Histological examination indicated a little vacuity bone lacuna and myelofibrosis in the defect. At 12 weeks after operation, the defect did not repair by itself. We find that the animal model in this experiment is consistent with the early-stage human osteonecrosis of the femoral head and its advantage is not to interfere the osteogenesis repair of bone graft after replacement of necrotic bone, which is important to evaluate the simulative bone-grafting treatment of osteonecrosis of the femoral head. Mont et al[8] indicated that it is an important model for study of osteonecrosis because it has structural compromise and is similar to treat osteonecrosis by bone-grafting.

With the development of tissue engineering technique, the importance of BMSCs and BMP is gradually realized. BMSCs mainly exist in bone marrow, and it is condsidered as one of the best seed cells because of the potentiality of differentitation into bones, cartilages, fats, muscles, tendons, coriums and nerve cells.[9] BMP-2 is one of major osteoinductive proteins in the family of BMPs. Cloning the complementary DNA encoding human BMP-2 sequence allows the manufacture of large quantities of highly purified rhBMP-2 with consistent biological activity.[10, 11] Herngiou et al[12] reported that a decrease of osteogenic stem cells was present in the bone marrow of some of patients with osteonecrosis of the femoral head, which indicated that osteonecrosis was associated with a decrease in progenitor cells in the proximal femur.

BMSCs and cytokine therapy are effective for osteonecrosis of the femoral head.[13] Ectogenic BMSCs and BMP implanted in the osteonecrosis defect may improve the osteogenesis of BMSCs. However, BMSCs and BMP would flow away soon if without appropriate carriers. Thus we used BBM as carriers of BMSCs and BMP in this study. BBM combined with rhBMP-2 (Group Ⅱ) and BMSCs-seeded BBM combined with rhBMP-2 (Group Ⅲ) were implanted respectively in defects of osteonecrosis of the femoral head. At 4 weeks after operation, BBM started to degrade and few new bones formed in Group Ⅱ. At 12 weeks, majority of the materials was degraded. Histological observation did not detect any inflammatory reaction. The above-mentioned findings demonstrated that BBM combined with rhBMP-2 had good biocompatibility and degradation, but could not repair femoral head defects alone (without BMSCs). At 4 weeks, new bones and cartilages started to grow in Group Ⅲ. The main way of ossification was endochondral osteosis. Cartilage grew gradually and enchondral bone formation grew at the same time. At 12 weeks, a few new bone trabeculae and cavitas medullaris formed, and the defect was almost substituted by new bones in Group Ⅲ. The new bone volume and BMD in Group Ⅲ were significantly higher than those in Groups Ⅰ and Ⅱ.

During the process of stem cell evolvement, we consider that bio-derived bones and rhBMP-2 could not only induce osteoprogenitor cells to differentiate into osteoblasts, but also make mesenchymal stem cells endochondral ossification. Our findings indicate that BMSCs-seeded BBM combined with rhBMP-2 are capable of improving the quantity and quality of new bones to grow in the subchondral defects of the femoral head, and repairing early-stage osteonecrosis of the femoral head in rabbits. However, the relationship between meschymal stem cells and osteoblasts and mecha-

nism of BMP-2 regulation in molecular level are not clear yet now. Further investigation needs to be done.

## REFERENCES

1. Mont MA, Jones LC, Hungerford DS, et al. Nontraumatic osteonecrosis of the femoral head: ten years later. J Bone Joint Surg Am 2006;88(5):1117-1132.

2. Takaoka K, Yoshioka T, Hosoya T, et al. The repair process in experimentally induced avascular necrosis of the femoral head in dogs. Arch Orthop Trauma Surg 1981; 99(2) :109-115.

3. Tang TT, Lu B, Yue B, et al. Treatment of osteonecrosis of the femoral head with hBMP-2-gene-modified tissue-engineered bone in goats. J Bone Joint Surg Br 2007;89(1):127-129.

4. Lan X, Yang ZM, Ge BF, et al. Culture of osteoblasts on bio-derived bones. Chin J Traumatol 2005; 8(2):86-90.

5. Lieberman JR, Berry DJ, Mont MA, et al. Osteonecrosis of the hip: management in the 21st century. Instr Course Lect 2003;52:337-355.

6. Lieberman JR, Conduah A, Urist MR. Treatment of osteonecrosis of the femoral head with core decompression and human bone morphogenetic protein. Clin Orthop Relat Res 2004; (429):139-145.

7. Khan SN, Cammisa FP, Sandhu HS, et al. The biology of bone grafting. J Am Acad Orthop Surg 2005;13(1):77-86.

8. Mont MA, Jones LC, Elias JJ, et al. Strut-autografting with and without osteogenic protein-1: a preliminary study of a canine femoral head defect model. J Bone Joint Surg Am 2001;83 (7):1013-1022.

9. Jiang Y, Jahagirdar BN, Reinhard RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418(6893):41-49.

10. Burkus JK, Dorchak JD, Sanders DL, et al. Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine 2003; 28(4):372-377.

11. Sandhu HS, Khan SN. Recombinant human bone morphogenetic protein-2: use in spinal fusion applications. J Bone Joint Surg Am 2003;85 Suppl 3:89-95.

12. Hernigou P, Poignard A, Manicom O, et al. The use of percutaneous autologous bone marrow transplantation in nonunion and avascular necrosis of bone. J Bone Joint Surg Br 2005; 87(7):896-902.

13. Yang C, Yang S, Du J, et al. Vascular endothelial growth factor gene transfection to enhance the repair of avascular necrosis of the femoral head of rabbit. Chin Med J 2003;116(10): 1544-1548.

*(Received November 12, 2007)*
Edited by LIU Yang-e