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Original Article

Effect of bone mesenchymal stem cells transplantation on the micro-environment of early osteonecrosis of the femoral head

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Abstract: Autologous implantation of bone mesenchymal stem cells (BMSCs) has achieved promising clinical efficacy for the treatment of early-stage osteonecrosis of the femoral head (ONFH). However, the underlying mechanisms are not completely elucidated. Here, we investigated the effect of BMSCs on the early ONFH in vitro and in vivo. In co-cultured system, primary BMSCs enhanced the activity and inhibited the apoptosis of primary OB. The concentrations of VEGF and BMP-2 in the co-cultured medium were significantly higher than those without co-culture. Importantly, BMSCs implantation increased OB, capillaries and VEGF and BMP-2 expressions of the necrotic areas of femoral head in the ONFH rabbits. In conclusion, our results indicated that BMSCs treated the early ONFH possibly through increasing OB and capillaries, as well as VEGF and BMP-2 expression in the femoral head. These results provided possible mechanisms for the treatment of early-stage ONFH with BMSCs transplantation.

Keywords: BMSCs, ONFH, OB, VEGF, BMP-2

Introduction

ONFH is a common ailment characterized by necrosis of bone trabecular and bone marrow [1]. Currently, many methods including conservative treatment and surgeries have no definitive effect [2, 3]. Recently, BMSCs have been widely used in ONFH treatment, and the results of clinical studies indicated that this treatment was safe and effective [4-6].

BMSCs are regarded as potential seed cells for bone tissue engineering [7], and have differentiated into osteoblasts (OB) to restore trabecular bone [8]. Moreover, BMSCs can secrete large amounts of growth factors and angiogenic factors [9], such as VEGF and BMP-2. VEGF is essential for bone formation and repair during the osteogenesis process [10-12]. BMPs could induce osteogenesis in the femoral head with necrosis and stimulate the formation of new bones [13]. Additionally, BMSCs can be easily isolated from bone, and auto-implantation of BMSCs does not induce any immunological rejection.

Though treatment of early-stage ONFH with autologous BMSCs implantation achieved a promising outcome, its specific mechanisms remain elusive. Therefore, we explored the effect of BMSCs on OB in vitro and investigated the effect of BMSCs implanted into the ONFH rabbit. Our study will enhance the understanding of the mechanisms of early-stage ONFH treatment with BMSCs transplantation.

Materials and methods

Animals

Thirty New Zealand white rabbits were obtained from the Animal Center at Xi'an Jiaotong University. Animals were allowed to acclimatize to a new controlled environment. All animal

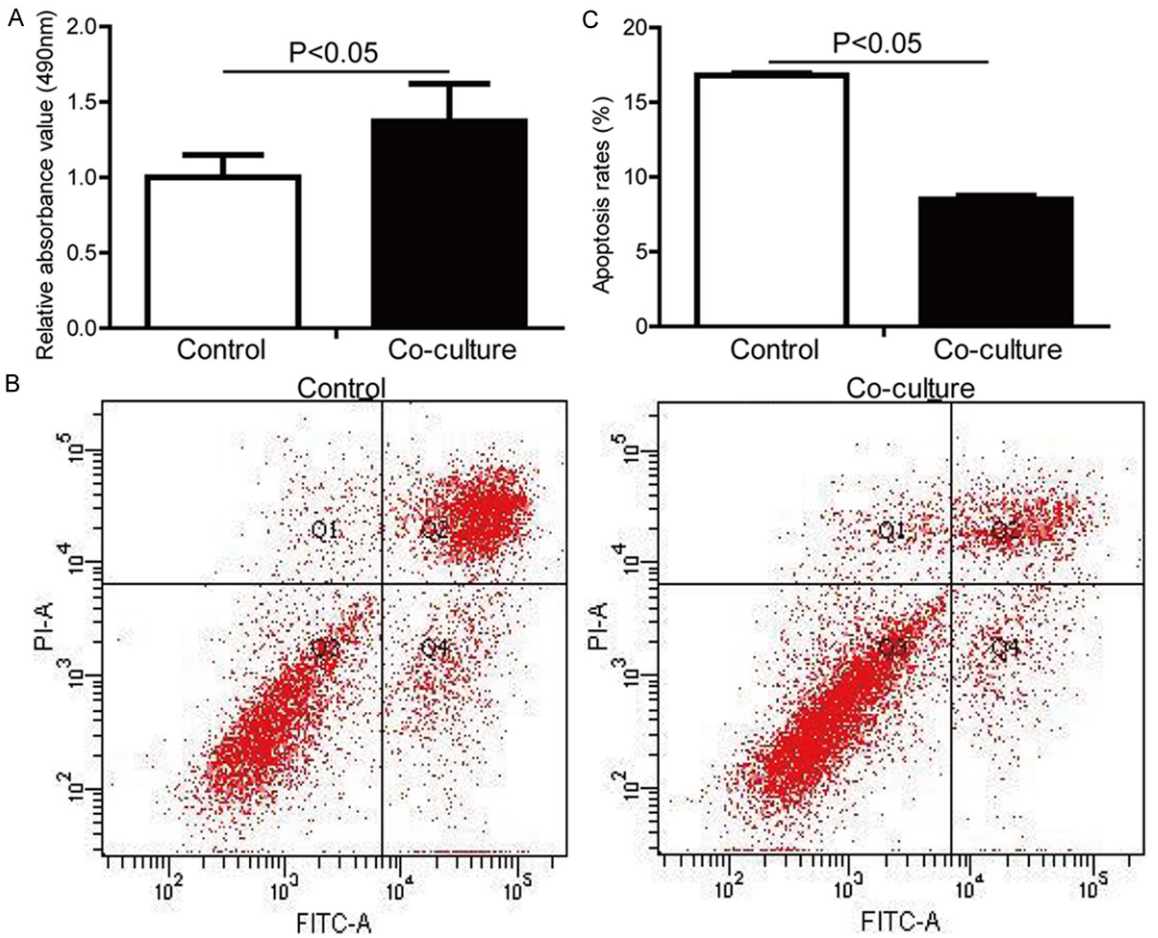

Figure 1. Primary BMSCs enhanced the activity and inhibited the apoptosis of primary OB in co-cultured system. Primary BMSCs and OB were co-cultured for 3 days in noncontact co-cultured system. A. The activity of OB was examined by MTT assay. B. The apoptosis of OB was measured by dual Annexin V-FITC and PI staining, following flow cytometry analysis. C. The result of flow cytometry analysis was quantified.

work was carried out under protocols approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Isolation and culture of cells

Primary BMSCs were isolated from the bone marrow of tibiae and femurs of rabbits as previously described [14]. Cells were maintained in growth media of DMEM (Gibco BRL Life Technologies) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin and streptomycin. Cells of passage 3 were used for the experiments.

Primary OB was isolated from the rabbits according to the previous method [15, 16]. The femoral head fragments were treated with trypsin and digested with type II collagenase. Cells were collected and resuspended in growth media. Cells of passage 3 were used for the experiments.

Co-culture of BMSCs with OB

Noncontact co-culture was achieved by BMSCs (5×10^4/well) seeded onto the upper chamber of the transwell plate (Corning), while the lower chamber was filled with OB (5×10^4/well). Cells were suspended in growth media.

Determination of cell activity and apoptosis

BMSCs and OB were co-cultured for 3 days. Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) assay (1×10^4 cells/well). Absorbance was determined on a spectrophotometric microplate reader (Bio-Rad, Hercules, CA).

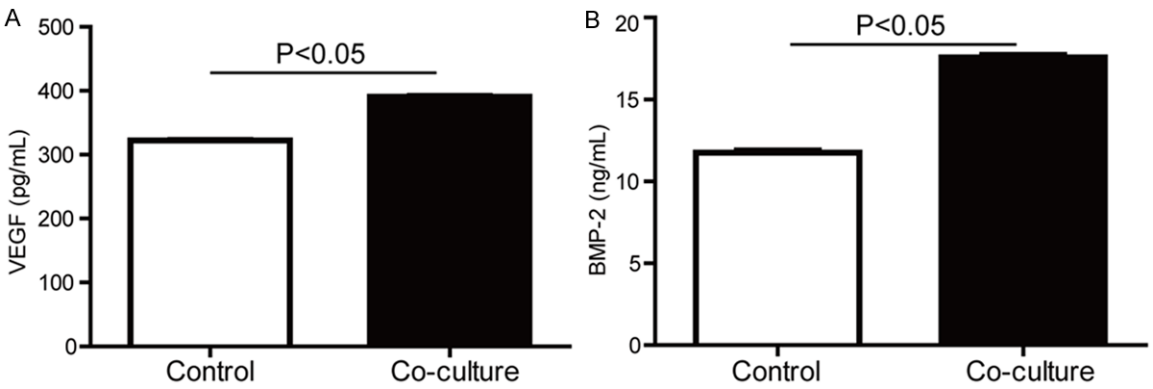

Figure 2. The concentrations of VEGF and BMP-2 in the medium of OB co-cultured with BMSCs were significantly increased. Primary BMSCs and OB were co-cultured for 3 days, and the supernatant was collected. (A) VEGF and (B) BMP-2 in the co-culture medium were quantified by ELISA.

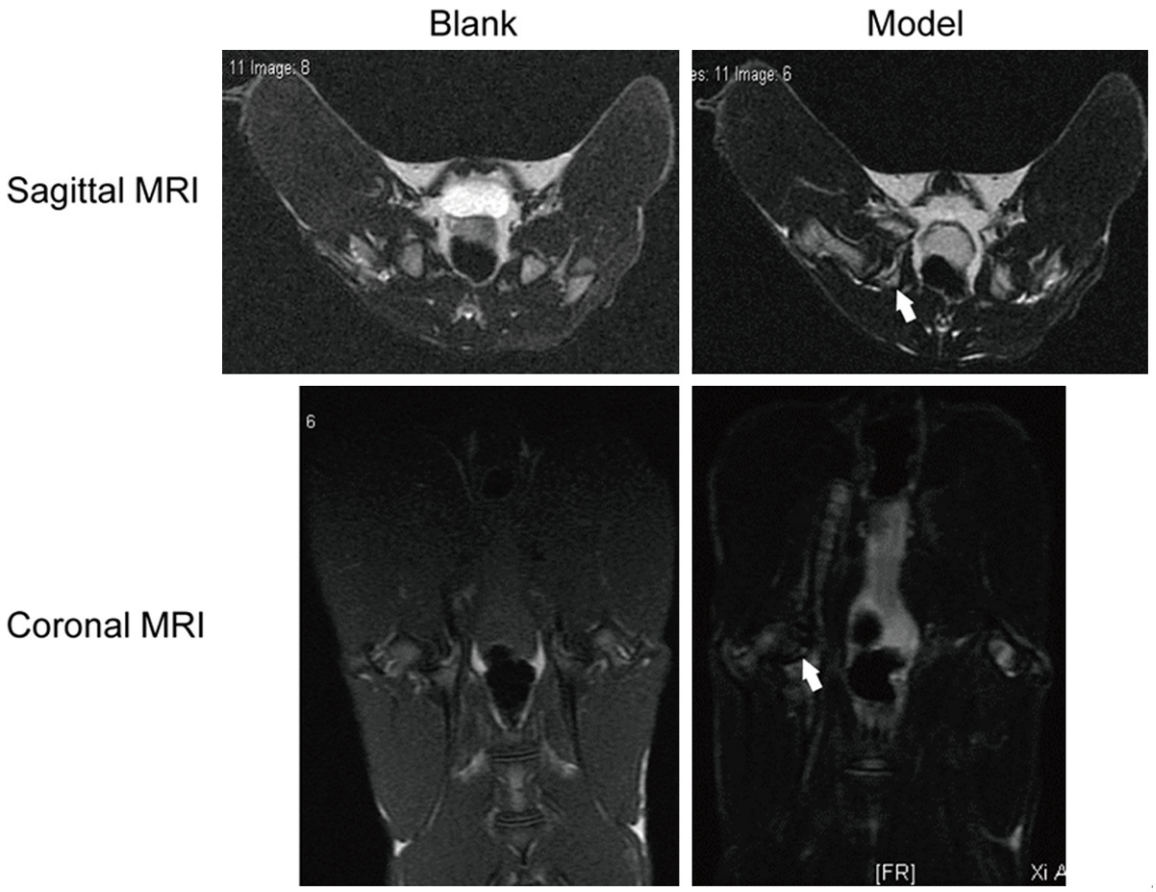

Figure 3. Development of ONFH models in rabbit. Rabbits were given 10 μg/kg Escherichia coli endotoxin LPS and 20 mg/kg methylprednisolone gluteal to induce ONFH model. Sagittal and Coronal MRI with T2-weighted fat suppression sequences showed the necrotic areas of femoral head. Black arrows depicted inhomogeneous low signal area in the ONFH model group.

Cell apoptosis were measured by flow cytometry. Treated OB were collected and resuspended in binding buffer containing Annexin V-fluoroisothiocyanate (FITC) and propidium iodide (PI) according to the manufacturer's recommendations. The number of stained cells was quantified using a FACS can flow cytometer, and analyzed by FlowJo software.

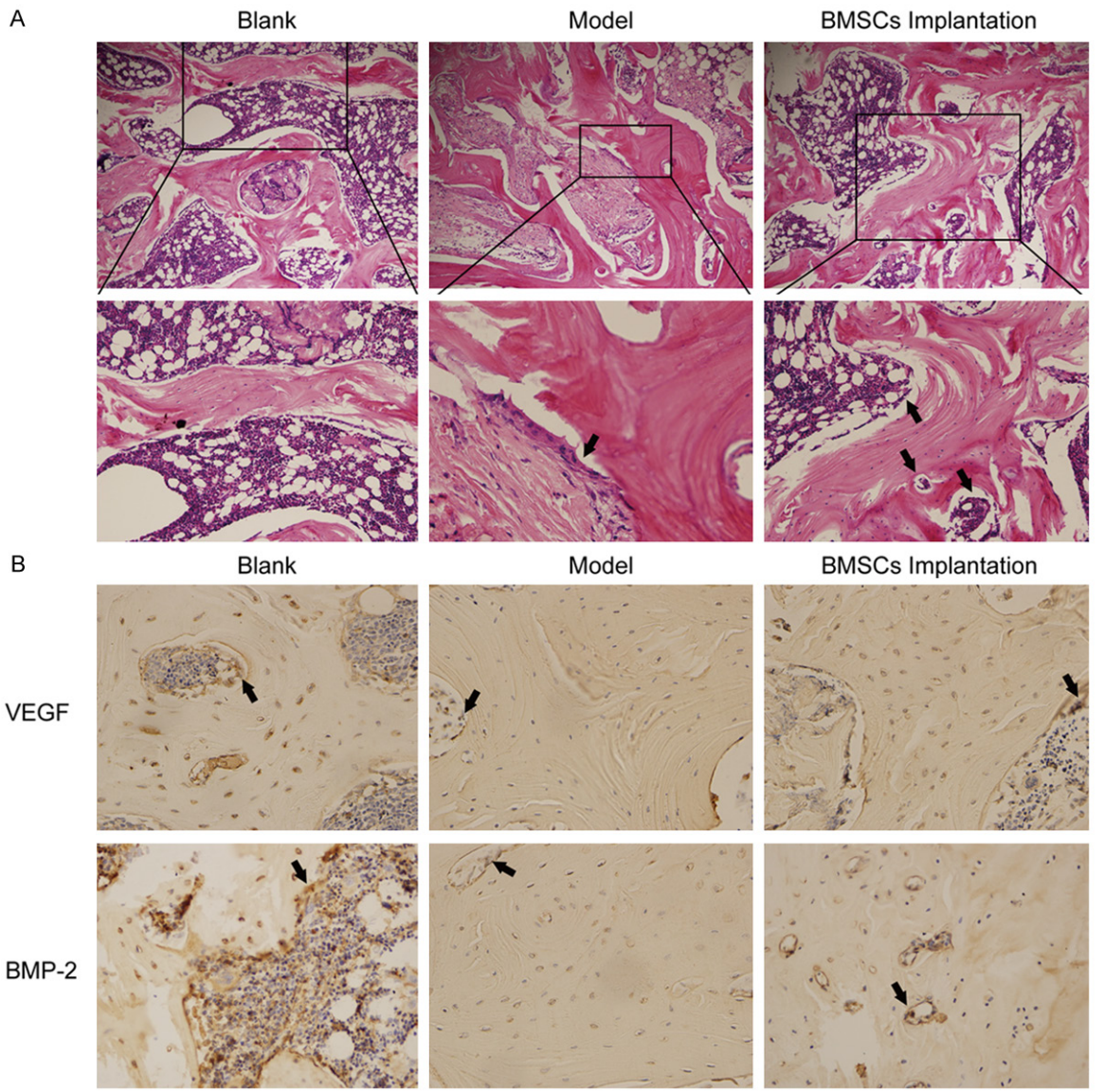

Figure 4. BMSCs implanted into the ONFH rabbits. BMSCs implanted into the ONFH rabbit for 4 weeks. The femoral head fragments were collected and sectioned. A. The femoral head tissues were analyzed by H&E staining. Black arrows depicted OB and capillary. B. VEGF and BMP-2 expressions were examined by immunohistochemistry.

Determination of VEGF and BMP-2 concentrations by ELISA

BMSCs and OB were co-cultured for 3 days. Culture supernatant was collected and centrifuged for 10 min (3,000 rpm). VEGF and BMP-2 concentrations were measured by ELISA (Cat. No. ABIN431314 and Cat. No. ABIN416053, Elabscience) according to the manufacturer's recommendations.

Development of the ONFH rabbit model

ONFH models were established as previously described [17]. Rabbits were given 10 μg/kg Escherichia coli endotoxin LPS by ear vein injection, following 20 mg/kg methylprednisolone gluteal by muscle injection for three consecutive days and were breeding under conventional condition. The animal models were verified by MRI after 6 weeks. For the implantation, BMSCs were adjusted to 10^6/ml. Autologous BMSCs (1×10^6) suspension in PBS was implanted to decompression channel, and 1 ml PBS was injected in the control group.

Immunohistochemical assay

BMSCs implanted into the ONFH rabbits after 4 weeks. Rabbits were sacrificed by vein injection

of air. The femoral head fragments were collected and sectioned. By using rabbit VEGF and BMP-2 primary antibody (BA0407 and BA0624, BOSTER, Wuhan) and anti-mouse HRP-conjugated secondary antibody, rabbit VEGF and BMP-2 was detected. The sections were visualized with motic digital slices scanning and application system.

Statistical analyses

All results were showed as mean ± SE. Statistical analysis was performed by using ANOVA, followed by Student's t-test. *P* value <0.05 was considered to be statistically significant.

Results

The effect of BMSCs on OB in vitro

BMSCs enhanced the activity and inhibited the apoptosis of OB in co-cultured system: To determine the effect of BMSCs on OB in vitro, primary BMSCs and OB were isolated and co-cultured by transwell plate. We found that the cell activity of OB with BMSCs co-culture was significantly higher than that of the control (OB without co-culture) ($P<0.05$) by MTT assay (**Figure 1A**). Furthermore, the results of flow cytometry analysis showed that the apoptotic rates of OB were markedly decreased in the co-cultured group compared with the control group ($P<0.05$) (**Figure 1B**), and the results were quantified (**Figure 1C**). These results indicated that BMSCs enhanced the activity and inhibited the apoptosis of OB in noncontact co-cultured system.

The concentrations of VEGF and BMP-2 in the medium of OB co-cultured with BMSCs were significantly increased: To illustrate cytokines secreted by BMSCs in co-cultured system, we quantified VEGF and BMP-2 in the co-culture medium by ELISA. The results showed that the concentrations of VEGF (**Figure 2A**) and BMP-2 (**Figure 2B**) in the co-cultured medium were higher than that of the control (OB without co-culture).

The effect of BMSCs on OB in vivo

To further explore the effect of BMSCs on OB in vivo, we developed ONFH models in rabbit. Sagittal and Coronal MRI with T2-weighted fat suppression sequences showed that the blank group showed abnormal signal. However, femoral head of the ONFH rabbits exhibited inhomogeneous low signal area with visible articular fluid as black arrows depicted (**Figure 3**). MRI performance represented early avascular necrosis in ONFH rabbits, suggesting that the ONFH model was successfully developed.

Implantation of BMSCs increased OB and capillary in the ONFH rabbits: Next, BMSCs were implanted into the ONFH rabbits. The femoral head tissues were analyzed by H&E staining. We found that OB surrounding the trabecular bone and capillary were increased in BMSCs implantation group compared with the ONFH model group as black arrows depicted (**Figure 4A**). The ONFH model group developed necrosis of OB. Femoral trabecular was arranged regularly in the blank group.

VEGF and BMP-2 expressions in the necrotic areas of rabbits with BMSCs transplantation were increased: Similarly, VEGF and BMP-2 expressions were also examined by immunohistochemistry. We found that VEGF and BMP-2 expressions were increased in the BMSCs implantation group compared with the ONFH model group (**Figure 4B**). Expressions of VEGF and BMP-2 were positively stained in the blank group.

Discussion

ONFH is a common and severe disease with a high morbidity rate in the orthopedics field. Recent pioneer studies by Hernigou et al. [18] have demonstrated the efficacy of autologous BMSCs implantation into the femoral head during the early-stage of ONFH. Autologous BMSCs incurred neither an acute or chronic rejection response, nor a graft-versus-host disease. Thereby, autologous BMSCs implantation is a safe and effective treatment against early-stage ONFH. However, the underlying mechanisms remain unknown. Therefore, we aimed to investigate the effect of BMSCs on the early-stage ONFH.

Although the mechanisms of ONFH development remain elusive, the poor blood supply to the femoral head is mainly reason. Therefore, rebuilding and improving the blood supply has been proposed as an effective therapeutic measure for ONFH. Kinnaird et al. found that BMSCs could enhance new blood vessel growth both in vitro and in vivo models [19]. Here, we found that implantation of BMSCs increased

capillary in the ONFH rabbits. The results indicated that BMSCs treated the ONFH possibly through improving blood supply.

Bone formation is a coordinated process involving BMPs and VEGF, orchestrating these factors may greatly enhance this process [20, 21]. The combination of BMP-2 and VEGF induced significantly early bone formation, and VEGF might enhance BMP-2-induced bone formation [22]. Furthermore, local BMP-2 also affects the production and presence of VEGF [23]. In this study, we found that both VEGF and BMP-2 increased in the medium of co-culture. Importantly, the noncontact co-cultured system allows the passage of cells secreted molecules through the transwell hole, but does not allow direct cells contact, indicating that cytokines from BMSCs affected the proliferation and apoptosis of OB possibly by the paracrine way. Moreover, recent research also showed that BMSCs transplantation enhanced vascular regeneration mainly through a paracrine action of VEGF [24]. Therefore, our results supported that BMSCs may improve the cytokines of the microenvironment in the early stage ONFH.

In conclusion, BMSCs treated the early ONFH possibly through increasing OB and capillary, as well as VEGF and BMP-2 expressions in the femoral head. These results provided possible mechanisms for the treatment of early-stage ONFH with BMSCs transplantation.

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Disclosure of conflict of interest

None.

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