


Bone Regeneration and Angiogenesis by Co-transplantation of Angiotensin II-Pretreated Mesenchymal Stem Cells and Endothelial Cells in Early Steroid-Induced Osteonecrosis of the Femoral Head

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Abstract

Mesenchymal stem cells (MSCs) have been shown to exert a positive impact on osteonecrosis of the femoral head (ONFH) in preclinical experiments and clinical trials. After the femoral head suffers avascular necrosis, the transplanted MSCs undergo a great deal of stress-induced apoptosis and senescence in this microenvironment. So, survival and differentiation of MSCs in osteonecrotic areas are especially important in ONFH. Although MSCs and endothelial cells (ECs) co-culture enhancing proliferation and osteogenic differentiation of MSCs and form more mature vasculature *in vivo*, it remains unknown whether the co-culture cells are able to repair ONFH. In this study, we explored the roles and mechanisms of co-transplantation of angiotensin II (Ang II)-MSCs and ECs in repairing early ONFH. *In vitro*, when MSCs and ECs were co-cultured in a ratio of 5:1, both types of cells managed to proliferate and induce both osteogenesis and angiogenesis. Then, we established a rabbit model of steroid-induced ONFH and co-transplantation of Ang II-MSCs and ECs through the tunnel of core decompression. Four weeks later, histological and Western blot analyses revealed that ONFH treated with Ang II-MSCs and ECs may promote ossification and revascularization by increasing the expression of collagen type I, runt-related transcription factor 2, osteocalcin, and vascular endothelial growth factor in the femoral head. Our data suggest that co-transplantation of Ang II-MSCs and ECs was able to rescue the early steroid-induced ONFH via promoting osteogenesis and angiogenesis, which may be regarded as a novel therapy for the treatment of ONFH in a clinical setting.

Keywords

osteonecrosis of the femoral head, bone marrow mesenchymal stem cells, endothelial cells, angiotensin II, bone regeneration, co-transplantation, angiogenesis

Introduction

Osteonecrosis of the femoral head (ONFH) is a devastating and incurable disease that occurs in young and middle-aged adults^{1,2}. Several risk factors, including corticosteroid use, alcohol addiction, sickle cell anemia, lipid storage, smoking, and trauma (femoral neck fracture, hip dislocation, etc), are associated with ONFH. As the incidence of corticosteroid-associated ONFH after severe acute respiratory syndrome (SARS) was reported to be 24%, the incidence of this condition might increase rapidly after the COVID-19 pandemic^{3,4}. Left untreated, ONFH largely advances to femoral head collapse and finally necessitates total hip arthroplasty^{5–7}. For the younger generation, total hip arthroplasty is not ideal due to its potential for dislocation, infection, activity limitation, and later revisions^{8–10}. Once the articular surface of the femur collapses, treatment is extremely challenging and usually

ineffective. Therefore, searching for effective therapies to ONFH at the early stage is essential in the field of

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orthopedics. Effective treatment of early ONFH is still a difficult and urgent problem.

ONFH is a progressive disease of bone necrosis, characterized by severe shortage of blood supply and intraosseous pressure^{11,12}. Accumulating evidence indicates that bone marrow mesenchymal stem cells (BMSCs) are predominant precursor cells for bone regeneration and remodeling^{13–15}. Autologous BMSC transplantation is a promising candidate for the treatment of avascular necrosis of the femoral head. BMSCs, which are modified or enhanced by gene transfection technology, have become a new and popular treatment plan for the treatment of ONFH but carry the risk of insertional mutagenesis and tumor formation^{16,17}. To enhance the safety of this approach, considerable research works need to be carried out in this area. Generally, after the femoral head suffers avascular necrosis, the transplanted BMSCs undergo a great deal of stress-induced apoptosis and senescence in this microenvironment, which limits their efficacy^{18,19}. Survival and stemness of BMSCs in the bone necrotic area are key to transplantation effectiveness^{20,21}. Compared with endothelial cell (EC)-alone group, BMSC-EC implants formed more mature vasculature that inosculates with the systemic circulation^{22,23}. Large data suggest strongly that ECs regulate the MSC activities by enhancing the proliferation and osteogenic differentiation of MSCs^{24–27}. Nevertheless, the effects of co-cultured cells on osteonecrosis therapy have been rarely studied.

The renin-angiotensin system (RAS) is known as a circulating endocrine system that has powerful effects on blood pressure and sodium homeostasis. It has been revealed that the local tissue RAS plays a pivotal role in bone metabolism that is independent of the systemic RAS^{28,29}. Angiotensin II (Ang II) was able to increase the expression of vascular endothelial growth factor (VEGF) in MSCs³⁰. Moreover, the pretreatment of MSCs with Ang II significantly reduced the MSC apoptosis and improved the outcome of MSC-based therapy for myocardial infarction via the mechanisms of enhancing the paracrine growth factors and MSC survival, angiogenesis, and gap junction formation³¹.

Although BMSCs and ECs co-culture enhancing proliferation and osteogenic differentiation of MSCs and form more mature vasculature *in vivo*, it remains unknown whether the co-culture cells are able to repair ONFH. In addition, Ang II pretreatment enhanced MSC survival and angiogenesis. Therefore, in this study, we preliminarily explored the roles and mechanisms of Ang II-pretreated MSC and EC co-transplantation in repairing early steroid-induced ONFH.

Materials and Methods

Animals

Animal care and procedures were performed in accordance with the Laboratory Animal Care Guidelines approved by

Medical Ethics Committee of Xi'an Jiaotong University. We extracted BMSCs from newborn New Zealand white rabbits and built ONFH models using 50 adult male New Zealand white rabbits (2.5–33 kg). All rabbits were provided by the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. Efforts were made to minimize suffering of animals.

Isolation and Culture of BMSCs

BMSCs from rabbits were isolated and cultured as previously described³². Briefly, BMSCs were obtained from the long bone shafts of the newborn rabbits after the muscles and tissues were trimmed. Bone marrow was flushed and centrifuged on a 1.073 g/ml Percoll density gradient (Pharmacia, New York, NY, USA). Subsequently, the cells were washed with phosphate-buffered saline (PBS), seeded into 25 cm² cell culture flasks, and cultivated in L-DMEM (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 20 mg penicillin-streptomycin/ml (Gibco) in a humidified 5% CO₂ atmosphere at 37°C. The MSCs were then incubated at 37°C for 3 days before the first change of medium. When the cells had pooled at 80%–90% of the bottom of the flask, we digested the cells with trypsin/EDTA (0.25% trypsin and 0.02% EDTA) (Gibco). Three to five passage-cells were used for the experiments.

Identification and Labeling of BMSCs

Following culturing for three passages *in vitro*, the cells were detached and incubated with anti-CD44 (1:300; ab157107) and CD90 (1:300; ab225) at 4°C overnight. Cells were then observed under an inverted fluorescence microscope following immunofluorescence staining at a magnification of 200×. To further characterize BMSCs, specific cell surface molecules, CD44 (ab25224) and CD90 (ab226), were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). All antibodies were purchased from Abcam (Cambridge, UK).

Isolation and Culture of ECs

Aorta endothelial cells (AECs) were isolated from the same rabbits that produced MSCs. ECs were isolated from inside the lumen of abdominal aorta of rabbit as described previously³³. Briefly, the artery was cut into 8–10 small pieces and opened longitudinally. These pieces were placed with the intima side down into 6-mm dishes in M199 [20% FBS, 100 U/ml penicillin-G, 100 µg/ml streptomycin, 2 mM L-Glutamine, 100 µg/ml heparin, 100 µg/ml endothelial cell growth supplement (ECGS)]. Cultures were maintained at 37°C in 5% CO₂ and were identified as ECs from their typical cobblestone appearance about 1 week later. Three to five passage-cells were used for the experiments.

Identification and Labeling of AECs

AECs were incubated with CD31 (bs-0468R; Bioss, Beijing, China) and vWF (bs-0586R; Bioss) primary antibody overnight at 4°C followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4°C and observed by fluorescence microscope (Leica Microsystems GmbH, Mannheim, Germany). Furthermore, angiogenesis assay was performed to test the ability of the isolated endothelial cells to form angiogenic tubes on Matrigel (356234; Corning, NY, USA). Formed tubes were observed by microscope (Leica Microsystems GmbH).

Co-culturing of BMSCs and AECs

BMSCs and AECs were mixed in 9:1, 5:1, 7:3, and 1:1 ratio prior to seeding. About 6×10^4 cells were transferred to six-well plates and then medium was added into each well. Only BMSCs and only AECs containing wells were included as controls. As the media for the co-culture groups had to support both cell types, growth media of BMSCs and AECs were mixed in 1:1 ratio. In the differentiation studies, supplements required for the differentiation of BMSCs, such as ascorbic acid (Sigma, St. Louis, MO, USA) (50 μ M), dexamethasone (Sigma) (100 nM), and β -glycerophosphate (Sigma) (10 mM), were added to each respective medium. The cells were co-cultured for 72 h. Finally, the expression of osteocalcin (OCN) and collagen type 1 (Col-I) was assayed by Western blot. Whole-cell extracts were prepared using lysis buffer (Beyotime, Shanghai, China) containing the protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany).

Calcium Accumulation Assay

Alizarin red sulfate staining was used to measure the formation of mineralized extracellular matrix *in vitro*. Once the ratio of BMSCs and AECs was determined, we treated the co-cultured cells with different concentrations of Ang II. After incubation in osteogenic differentiation medium for 28 days, cells were fixed with 4% paraformaldehyde solution and stained with a 0.5% Alizarin red solution for 30 min. Cells were washed with PBS to remove nonspecific staining and pictures were taken.

Tube Formation Assay

Matrigel-induced tube formation using AECs and BMSCs was performed to assess the angiogenic activity of BMSCs. Briefly, Matrigel (356234; Corning) was added to 96-well plates in a volume of 50 μ l per well. Plates were held at 37°C for 30 min to form a gel layer. After gel formation, first, 2.5×10^4 cells of AECs were applied to each well, along with the addition of 100 μ l of supernatant from different

treatment conditions including control (fresh DMEM), BMSC supernatant, and Ang II-pretreated (0.01 M or 0.1 M) BMSC supernatant. The plates were then incubated at 37 °C for 3 h with 5% CO₂. The number of tubes was counted under microscopy (Leica Microsystems GmbH). At least three wells were viewed, and the number of tubes/well was counted and averaged.

Rabbit ONFH Model and Cell Transplantation

Experimental animals. We used a rabbit model of steroid-induced ONFH that has been well established before³⁴. All rabbits were provided by the Experimental Animal Center of Xi'an Jiaotong University College of Medicine. Animal care and procedures were performed in accordance with the Laboratory Animal Care Guidelines approved by the Medical Ethics Committee of Xi'an Jiaotong University.

Establishment of a rabbit model of ONFH. A total of 50 male New Zealand white rabbits (age, 24 weeks; weight, 2.5–3.3 kg) were bred and maintained under a 12-h light/dark cycle with free access to food and water. Room temperature and relative humidity were set at 25°C \pm 3°C and 60% \pm 15%, respectively. All rabbits were injected with 10 μ g/kg body weight of *Escherichia coli* endotoxin [lipopolysaccharide (LPS)] (Sigma) through an ear vein. After 24 h, three injections of 40 mg/kg body weight of methylprednisolone acetate (MPSL; Pfizer Pharmaceutical, Hangzhou, China) intramuscularly into the right gluteus medius muscle at a time interval of 24 h were administered. After injecting the hormone, 200,000 U of penicillin was administered intramuscularly into the buttock of each animal.

Six animals were dead because of shock in the day of injecting LPS and three animals were dead within 3 days. Two weeks later, two rabbits were dead. Seven weeks later, magnetic resonance imaging (MRI) was conducted on two rabbits and killed to examine histopathologically for the diagnosis of ONFH. The remaining 37 rabbits were used for further experiment.

Surgery and cell transplantation. After ONFH was confirmed histopathologically, the 37 model rabbits were randomly assigned to five groups: ONFH group (ONFH, without treatment, $n = 6$), Core decompression group (core decompression, $n = 7$), BMSC group (1×10^7 BMSCs per rabbit, $n = 8$), BMSC + AEC group (9×10^6 BMSCs + 1.8×10^6 AECs per rabbit, $n = 8$), and Ang II-BMSC + AEC group (0.01 μ M Ang II + 9×10^6 BMSCs + 1.8×10^6 AECs per rabbit, $n = 8$). The BMSCs were pretreated with Ang II (0.01 μ M) for 72 h before being co-transplanted with AECs through the tunnel of core decompression. The core decompression combined with cell transplantation is performed under C-arm fluoroscopy of an X-ray machine for the treatment of early ONFH. Rabbits were administered their respective treatments in right sides of the femoral head.

To prevent infection, all rabbits were intramuscularly injected with one preoperative dose and two postoperative doses of gentamicin (4 mg/kg).

Histological and Immunohistochemical Staining

After 4 weeks of treatment, the rabbits were anesthetized using intravenous administration with 3% sodium pentobarbital (30 mg/kg) in an ear vein and the animals were subsequently sacrificed via air embolism. The right femoral head was divided into two parts along the coronal plane of the central hole, one placed in liquid nitrogen cryopreservation and another fixed with 10% formaldehyde solution (Sangon Biotech Co., Ltd., Shanghai, China) at 4°C for 48 h. Then the femoral head was decalcified with EDTA decalcification solution (AR1071; Boster Biological Technology, Ltd., Wuhan, China) for 2 months and embedded in paraffin. Sections of 5 μ m thickness were made and stored in thermostat of 37°C. Specimen sections were stained with hematoxylin and eosin (HE) and Masson staining.

For immunohistochemical (IHC) staining, sections were deparaffinized, antigen retrieved, blocked, and incubated with primary antibodies of OCN (1:400; GTX13418; GeneTex, Irvine, CA, USA) and vascular endothelial growth factor (VEGF) (1:250; BS1313R; Bioss, Beijing, China) and relevant secondary antibodies. Then sections were stained with DAB (3,3'-diaminobenzidine) and counterstained with hematoxylin. At last, each piece of the sections was observed by light microscope (Olympus Corporation, Tokyo, Japan).

Extraction of Total Protein from Bone Tissue

The femoral head was washed with PBS and crushed with a hammer tissue pulverizer and then the fragments were quickly transferred into a mortar containing liquid nitrogen. The powdered bone tissue was collected and extracted in lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics). After cleavage on ice for 30 min, we centrifuged it at 12,000 \times g for 15 min at 4°C and harvested the supernatant for Western blot analysis.

Western Blot

Protein was quantified by the BCA protein assay kit (Pierce, Rockford, IL, USA). Equivalent amounts of protein were applied to 10% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% bovine serum albumin (BSA)/TBST and incubated overnight at 4°C with the following primary antibodies: anti-OCN (1:1000; ab13420; Abcam, Cambridge, UK), Col-I (1:800; AB6308; Abcam), anti-runt-related transcription factor 2 (Runx2) (1:1000; AV36678; Sigma-Aldrich, MO, USA), anti-VEGF (1:800; BA04707;

Boster Biological Technology, Ltd., Wuhan, China), and anti-GAPDH (1:1000; AB8245; Abcam). The membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature, and protein expression was detected using an enhanced chemiluminescence Western blot detection reagent (Pierce). We took photographs using a gel imaging system (Geldoc, Quantity one software; BioRad, Hercules, CA, USA) and performed quantitative analysis using Image J software.

Statistical Analysis

Results were expressed as means \pm SEM. Differences between groups were determined by one-way analysis of variance (ANOVA) with the SPSS 20.0 program (SPSS Inc., Chicago, IL, USA). Differences were considered significant if $P < 0.05$.

Results

Culture and Identification of Primary BMSCs

BMSCs were cultured and taken under invert phase contrast microscopy after subcultures, followed by immunostaining identification. At 3 days in culture, BMSCs showed a spindle-shaped and fibroblast-like appearance without more distinct morphological differences (Fig. 1A, B). Also, immunocytochemical staining demonstrated that they were positive for CD44 and CD90 which are characteristic markers for BMSCs (Fig. 1C, D). Flow cytometry was also used to determine the purity of BMSCs. The positive rates of CD44 and CD90 were 99.95% and 99.27%, respectively (Fig. 1F, G).

Culture and Identification of Primary AECs

Primary AECs had polygonal morphology before confluency and showed characteristic cobblestone-like morphology when they reached confluency (Fig. 2A, B). Immunofluorescence assay displayed that they were positive for CD31 (Fig. 2C, E) and vWF (Fig. 2F, H). The following tube formation assay also ascertain that the cells were AECs.

The Ratios of BMSCs and AECs

To determine the better ratios of BMSCs and AECs for osteogenesis, we assessed the expression of OCN and Col-I. BMSCs and AECs were mixed in 9:1, 5:1, 7:3, and 1:1 ratio prior to seeding in differentiation medium (+Diff) for 3 days. As shown in Fig. 3B, the ratios of BMSCs and AECs at 9:1 or 5:1 caused an apparent increase in OCN production compared with BMSC group. And, results from Fig. 3C displayed that the BMSCs exhibited a very low basal protein expression of Col-I. However, Col-I expression was significantly increased compared with the BMSC group during

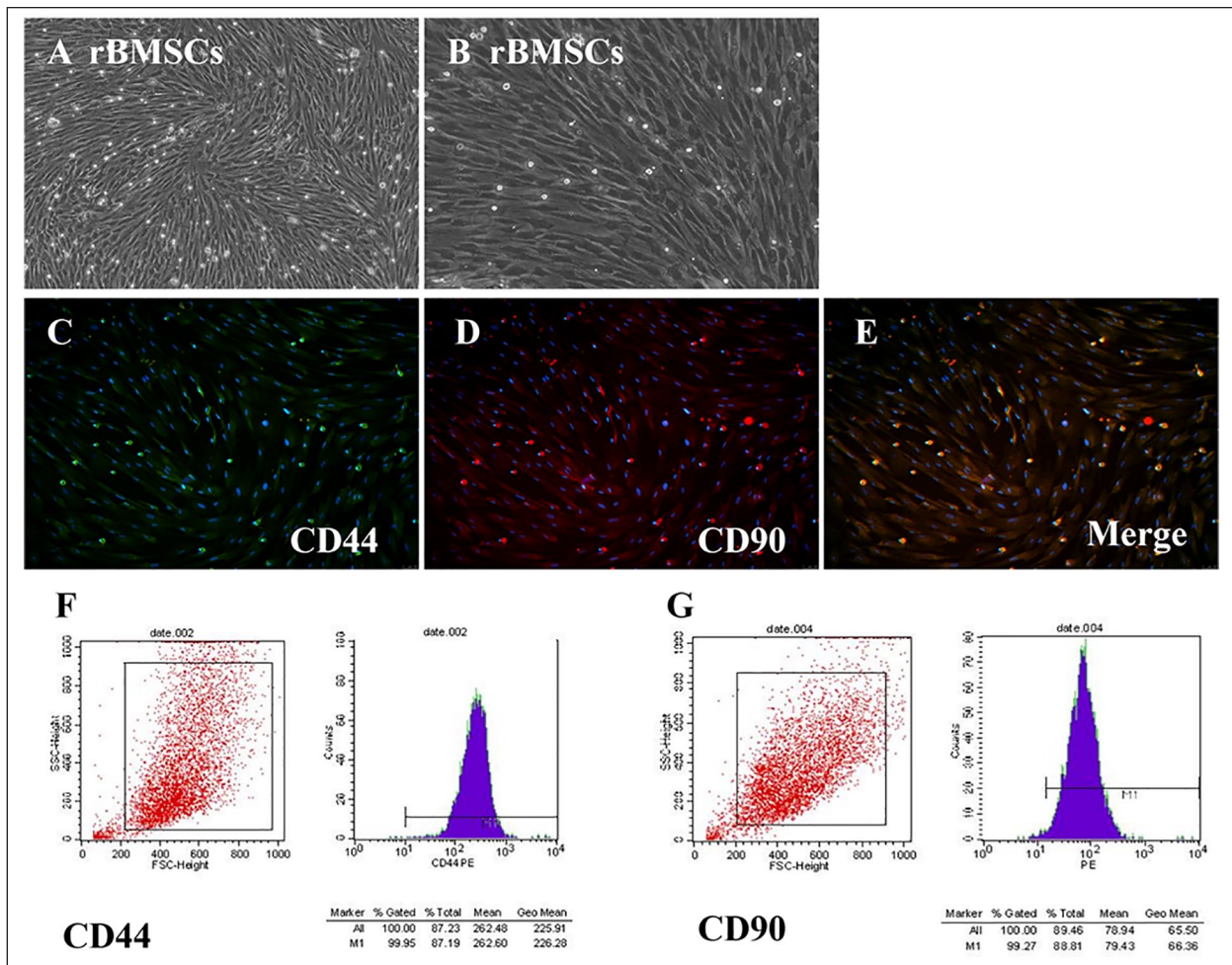


Figure 1. Identification of primary cultured rabbit BMSCs. The passage 3 BMSCs were spindle-shaped, arranged neatly, and fibroblast-like: (A) (100 \times) and (B) (200 \times). (C–E) Photomicrographs of dual-immunofluorescence labeling of cultured BMSCs for CD44 (green) and CD90 (red). Flow cytometry histogram of CD44-labeled (F) and CD90-labeled (G) BMSCs (obtained by FACSCalibur). Fractions of positive gated cells were indicated on histograms. BMSCs: bone marrow mesenchymal stem cells.

co-culture of BMSCs and AECs. The expression of OCN and Col-I was reached the peak at the ratios of 5:1 (Fig. 3B, C).

Effect of Ang II on the Proliferation of BMSCs and Osteogenic Differentiation of Co-cultured Cells

In this study, CCK-8 assay was used to detect the proliferation of BMSCs. The result indicated that Ang II (0.001, 0.01, 0.1, 1, 10 μ M) could promote the proliferation of BMSCs at 72 h (Fig. 3D). However, the difference is not significant. Then, BMSCs and AECs were mixed in the ratio of 5:1 in differentiation medium, following treatment of the cells with Ang II (0.01, 0.1, 1 μ M). After 4 weeks, a large number of calcium nodules were formed, and Alizarin red was positive, especially on the Ang II-stimulated co-cultured cells (Fig. 3E). So, we demonstrate that Ang II may promote the osteogenic potential of co-cultured cells *in vitro*.

Ang II Pretreatment BMSC Supernatant Enhanced AEC Tube Formation

To evaluate the influence of Ang II pretreatment on the angiogenic activity of BMSCs, tube formation assay was performed using AECs. As shown in Fig. 4B–D, the number of tube formation was significantly increased at 1 μ M after 3 h. Compared with AECs treated with BMSC media, those incubated with Ang II-pretreated (0.01 μ M) BMSC media formed hollow, tube-like structures apparently within 3 h (Fig. 4E–G). After 6 h, we found that the number of tube formations decreased significantly.

MRI and Histopathological Examination to Confirm ONFH

To observe co-transplantation cell osteogenesis *in vivo*, we used LPS combined with MPSL to establish an early

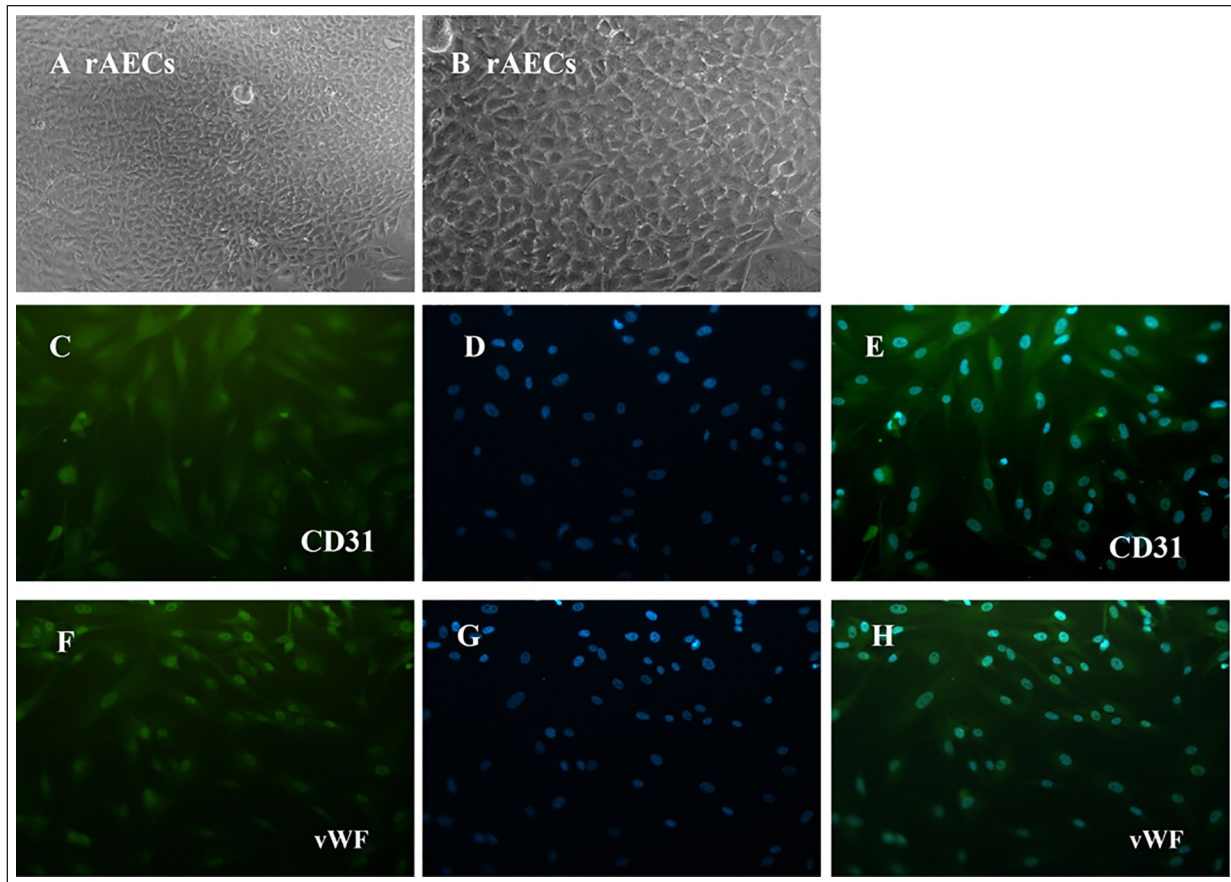


Figure 2. Identification of primary cultured rabbit aorta endothelial cells (AECs). The attached cells display spindle-shaped and cobblestone-like appearances: (A) (100 \times) and (B) (200 \times). Immunofluorescence for CD31 (green) expression in plasmas of AECs (C, E) (200 \times) and vWF (green) expression in both plasmas and nucleus of AECs (F, H) (200 \times), and counterstained with DAPI (blue) (D, G) (200 \times).

steroid-induced ONFH model in rabbits. At week 7, there was no clear abnormality observed in the normal group femoral heads (Fig. 5A). In T2-weighted imaging, MRI revealed mixed signals of different heights in the femoral head which was not smooth (Fig. 5B).

Furthermore, HE staining showed that in the normal group, the trabecular bone was complete and arranged, the bone marrow was rich in hematopoietic cells, and osteoblasts were observed on the surface of the trabecular bone (Fig. 5C). However, the medullary cavity was filled with a large amount of adipose tissue, the trabecular bone had become thinner, and plenty of empty lacuna were observed in the ONFH group (Fig. 5D). These results confirmed the success of early steroid-induced ONFH model.

Ang II–Pretreated Co-transplantation Repaired Early Steroid-Induced ONFH

Transplantation of BMSCs into femoral heads after core decompression surgery was carried out, which is a routine clinical treatment for early-stage ONFH patients. After surgery,

we removed one rabbit each from the co-transplantation group and the Ang II–pretreated co-transplantation group, respectively, by wound infection. At 4 weeks after surgery, the right femoral head was harvested for histological, immunohistochemistry, and Western blot study.

Outcomes of histological detection in treatment groups. Histological evaluation of bone repair was also performed by HE staining (Fig. 6A) and Masson staining (Fig. 6B). As the figures presented, the core decompression and BMSC groups displayed more empty lacunae than the other groups. The BMSC/AEC and Ang II–BMSC/AEC groups showed reduced numbers of empty lacuna (Fig. 6A), and Ang II–pretreated group seemed to be better. Besides, there were less necrotic bone and collagenous tissue in the BMSC group and BMSC/AEC group than the core decompression group (Fig. 6B). The proportion of collagenous tissue was further reduced in the Ang II–BMSC/AEC group at 4 weeks after the transplantation (Fig. 6B). As one of the main causes of ONFH is insufficient blood supply, angiogenesis in the femoral head was evaluated next by IHC staining. The result

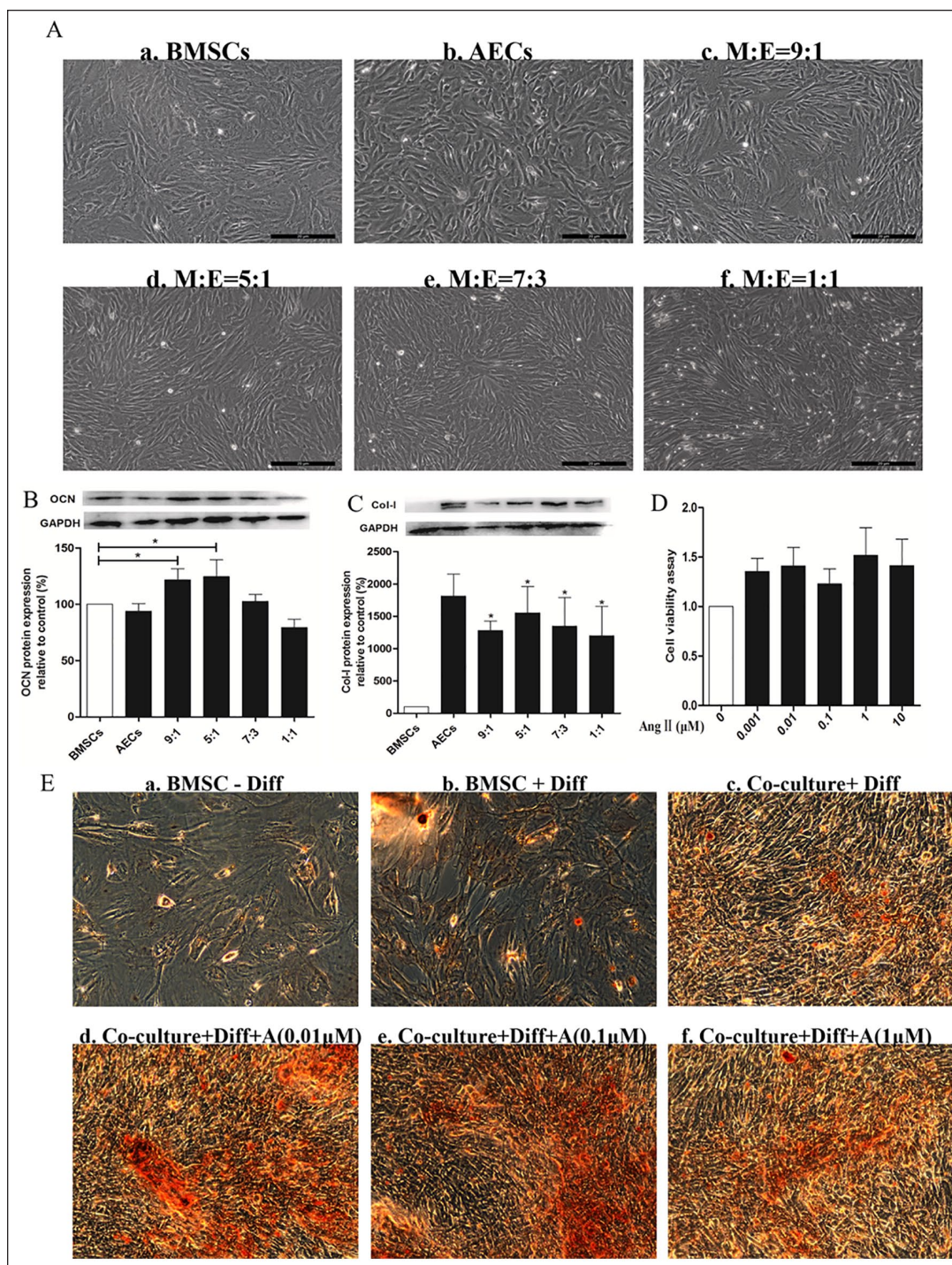


Figure 3. Effect of different ratios of BMSCs/AECs on osteogenic and Ang II-induced proliferation and osteogenesis of BMSCs. BMSCs and AECs were mixed in ratios of 9:1, 5:1, 7:3, and 1:1 in differentiation medium for 72 h. (A) Micrographs of co-cultured cells in different ratios. Scale bar: 20 μm. (B) Protein expression of OCN; (C) protein expression of Col-I. BMSCs were incubated with different concentrations of Ang II (0.001, 0.01, 0.1, 1, 10 μM) for 72 h. BMSCs without any treatment were taken as control. Cell viability was measured by CCK8 assay (D). Moreover, the Alizarin red staining of co-cultured BMSCs and AECs (5:1) incubated in osteogenic medium with Ang II (0.01, 0.1, 1 μM) stimulation showed the induced role of Ang II on extracellular mineralization. After 4 weeks, a large number of calcium nodules were formed, Alizarin red was positive, especially on the Ang II-stimulated co-cultured cells (E). Results are expressed as means ± SEM (n = 3). *P < 0.05 vs BMSCs. AECs: aorta endothelial cells; BMSCs: bone marrow mesenchymal stem cells; OCN: osteocalcin.

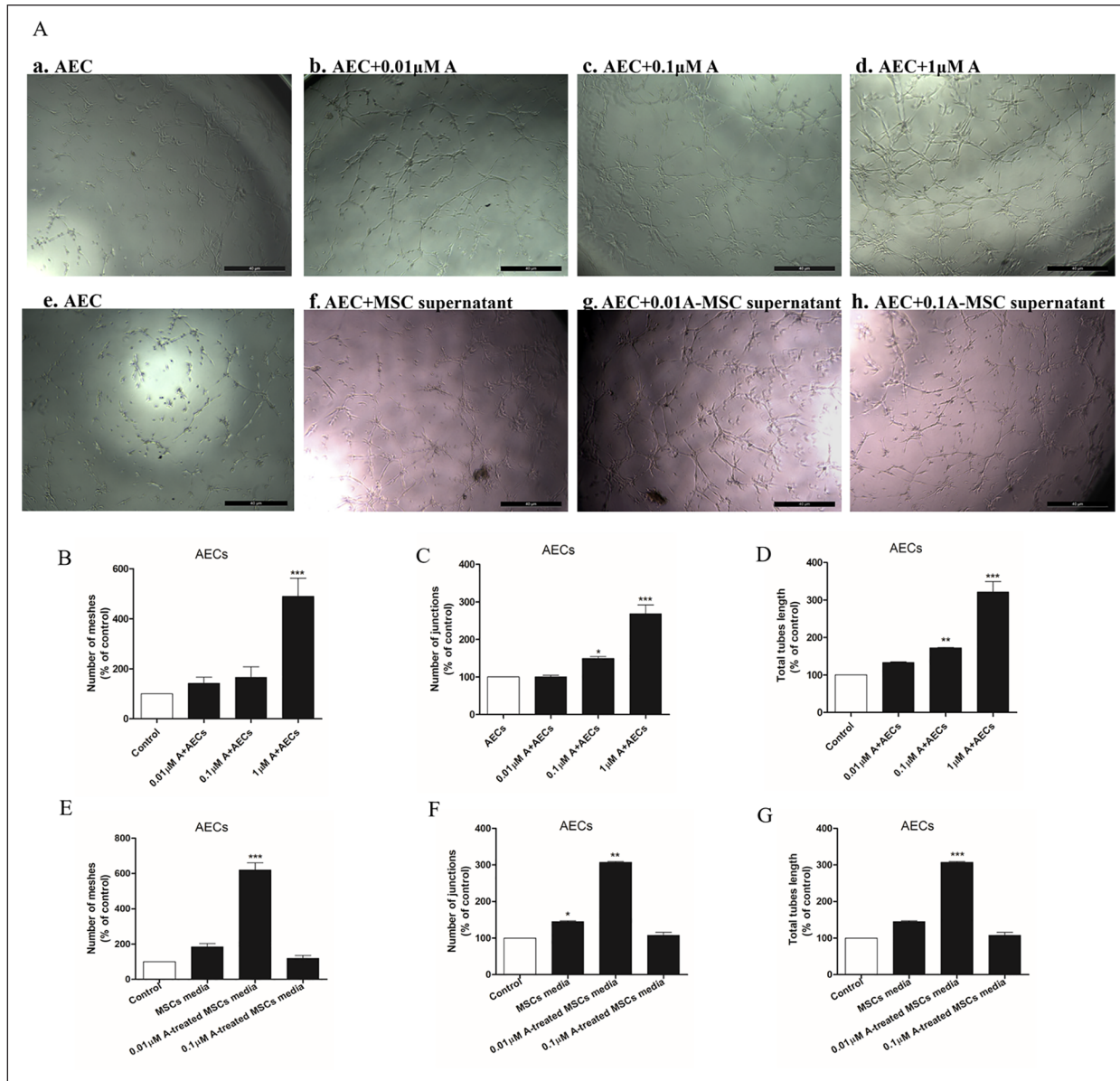


Figure 4. Tube formation assay *in vitro*. [A(a–d)]: Representative pictures of tube formation of AECs. Cells were cultured in Matrigel and treated with Ang II (0.01, 0.1, 1 μ M) for 3 h. (B) Quantitative analysis of the number of capillary-like rings from a to d. (C) Quantitative analysis of the number of junctions from a to d. (D) Quantitative analysis of total tube length from a to d. [A(e–h)]: Representative photographs of tube formation of AECs. Cells were cultured in Matrigel and treated with Ang II–pretreated (0.01 M or 0.1 M) BMSC supernatant for 3 h. (E) Statistical analysis of capillary-like rings in AECs from e to h. (F) Statistical analysis of the number of junctions in AECs from e to h. (G) Statistical analysis of total tube length in AECs from e to h. At least five wells were viewed, and experiments were repeated three times. All values were expressed as means \pm SEM, * P < 0.05 vs AECs, ** P < 0.01 vs AECs, *** P < 0.001 vs AECs. AECs: aorta endothelial cells; BMSCs: bone marrow mesenchymal stem cells; MSC: mesenchymal stem cells.

revealed that VEGF was higher positively expressed in Ang II–pretreated BMSC and AEC group than the other treatment groups (Fig. 6C).

Outcomes of Western blot. Western blot was used to further study the effect of co-transplantation of Ang II–pretreated BMSCs and AECs on rabbits with early steroid-induced ONFH. Total protein was extracted from the femoral head

tissue, and levels of osteogenic markers such as Runx2, OCN, and Col-I and angiogenic marker VEGF were detected by Western blot. The results from Fig. 7 displayed that the expressions of Runx2, OCN, Col-I, and VEGF in the cell treatment groups were significantly increased in comparison with the ONFH group. It was noteworthy that the expression was the highest in the Ang II–pretreated BMSC and AEC group.

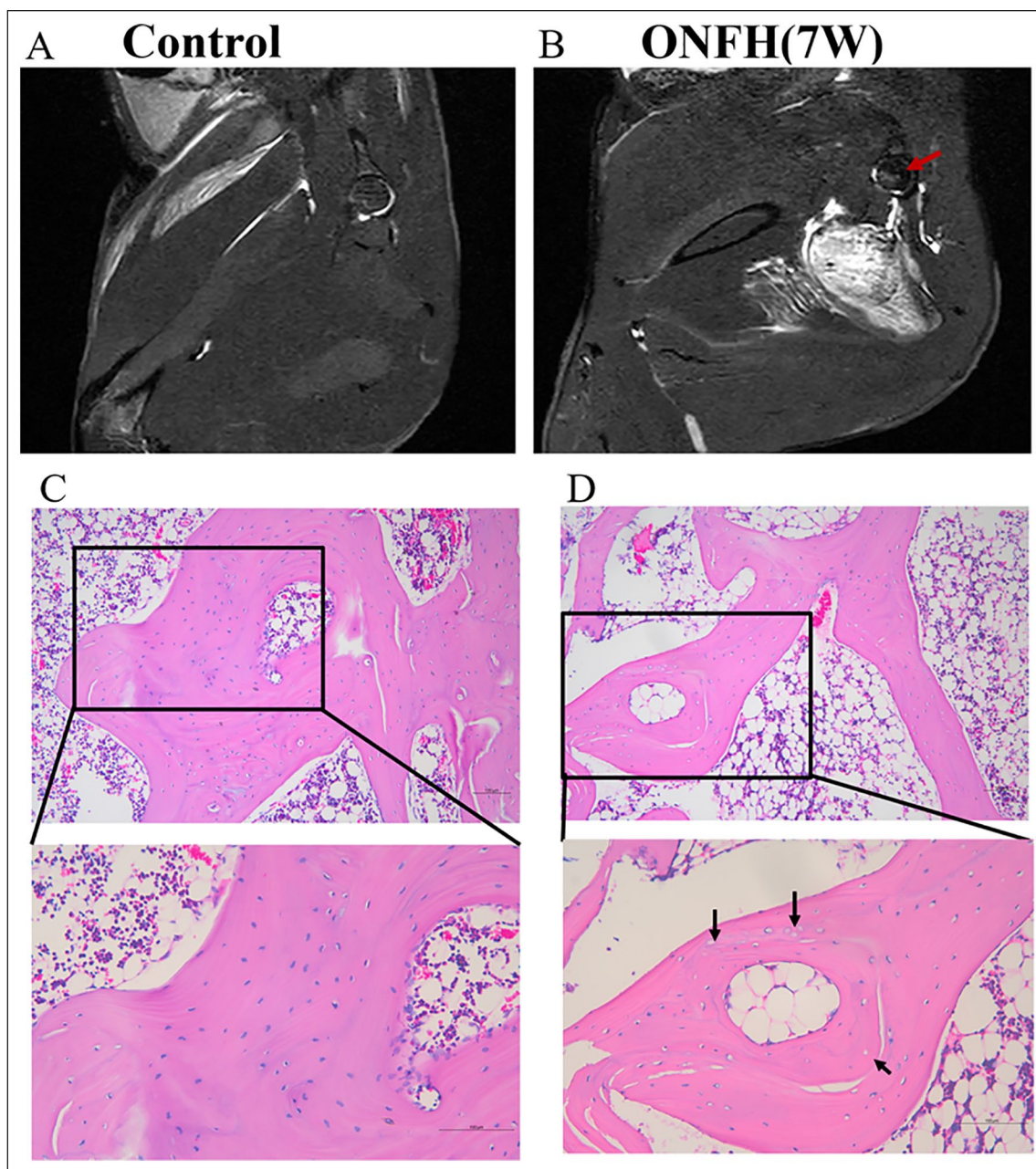


Figure 5. Confirm ONFH by MRI and histopathological examination in a rabbit model. Lipopolysaccharide and methylprednisolone were used to build the model; saline was used as control. MRI scans were performed on rabbits in the (A) normal and (B) model group and representative images are presented. In T2-weighted imaging, MRI revealed mixed signals of different heights in the femoral head which was not smooth. Hematoxylin and eosin (HE) staining was performed on the femoral head from the (C) normal group and the (D) model group at 7 weeks after treatment. The black arrows showed the empty lacuna in the necrotic region. Scale bar = 100 μ m. MRI: magnetic resonance imaging; ONFH: osteonecrosis of the femoral head.

Discussion

ONFH is a catastrophic disease of the femoral head that often causes disability in younger patients in their 30s and 40s³⁵. It is a heavy burden for the patients' families and the society financially because early diagnosis is difficult and conservative treatment is invalid³⁶. Further research into

potential novel treatments of early ONFH is necessary. Currently, many studies of the ONFH mechanism are focusing on the balance of osteogenesis, bone resorption, and angiogenesis³⁷⁻³⁹. At the same time, the influenced osteogenic differentiation, proliferation, and migration of MSCs are considered as the top factors leading to ONFH^{40,41}. MSC transplantation has been used to treat early steroid-induced

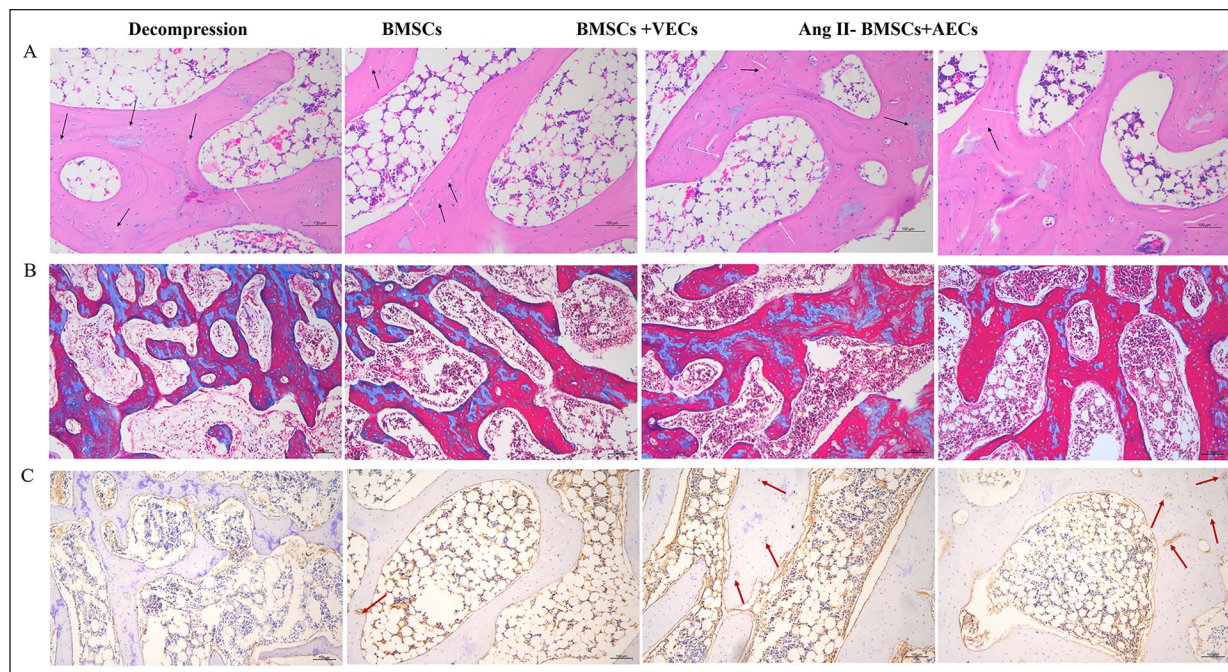


Figure 6. Evaluate bone reparation and angiogenesis of the femoral head in ONFH rabbits. (A) HE staining of the empty lacuna in the necrotic region of the femoral head. Black arrows showed the empty lacuna in the necrotic region. White arrows indicate osteoblasts along the trabeculae. (B) Masson staining of collagenous tissue in the femoral head. The proportion of collagenous tissue was reduced in the Ang II-BMSC + AEC group at 4 weeks after transplantation. (C) The VEGF expression in the femoral head was detected with immunohistochemical staining. Representative images are presented. Red arrows showed the vessels. Scale bar = 100 μ m. Decompression: core decompression only; BMSCs: only transplantation BMSCs; BMSCs/AECs: co-transplantation of BMSCs and AECs; Ang II- BMSCs + AECs: co-transplantation of Ang II-pretreated BMSCs and AECs. AEC: aorta endothelial cells; BMSCs: bone marrow mesenchymal stem cells; ONFH: osteonecrosis of the femoral head; VEGF: vascular endothelial growth factor.

ONFH. A recent study revealed that there was no noted case of related complications such as malignancy, bone overgrowth on the trochanter, and infection in the BMSC therapy for ONFH patients⁴². However, the regenerative capacity of transplanted cells is impaired by the hostile environment of necrotic tissue *in vivo*, limiting their clinical efficacy¹⁷. More and more studies have shown that survival and stemness of MSCs in the bone necrotic area are key to transplantation effectiveness^{43–45}. ECs function not only to form the microvasculature that delivers nutrients to developing bone but also to enhance osteogenic differentiation of MSCs *in vitro* and *in vivo*^{24–27}. In addition, Ang II is not only a good trigger for pretreating MSCs to enhance the paracrine release of growth factors (such as VEGF), but is also a promising drug for use as a trigger for preconditioning MSCs *in vitro*^{30,31}.

In this study, we demonstrate that co-culturing of BMSCs and AECs with a ratio of 9:1 or 5:1 for 3 days significantly increased the protein expression of osteogenic markers OCN and Col-I, which reached the peak at the ratio of 5:1. This result is in line with other research⁴⁶. Both types of cells managed to proliferate without leading to the domination of one cell type over the other. To improve the proliferation and osteogenic capacity of the cells for their use in cell-based therapy for ONFH, Ang II was added in the differentiation

medium. The results further demonstrate that Ang II has potent regulative effects on osteogenic differentiation throughout the culture time. In clinical practice, MRI is the most accurate imaging method available and is particularly useful to diagnose ONFH at its early stage⁴⁷. In the present study, MRI scans revealed an edema and high-level signal in the right femoral head. These results are consistent with the image characteristics of ONFH^{48,49}. Furthermore, HE staining also revealed that the rabbit model of ONFH was successfully established in the present study. The *in vivo* experiment showed that Ang II-pretreated BMSC and AEC group had more reduced numbers of empty lacuna and proportion of collagenous tissue by HE and Masson staining. Meanwhile, the expression of Runx2, OCN, and Col-I in the cell treatment groups was significantly increased, and the expression was the highest in the Ang II-pretreated BMSC and AEC group. These indicate that Ang II can be used as a pro-osteogenic agent for stem-cell-based therapy of ONFH. Given these findings, we speculate that pretreatment of BMSCs with Ang II improved the outcome of BMSC-based therapy for ONFH via the mechanisms of enhancing the paracrine production of Runx2, OCN, and Col-I.

Although the pathogenesis of ONFH has not yet been fully elucidated, it is generally accepted that the final

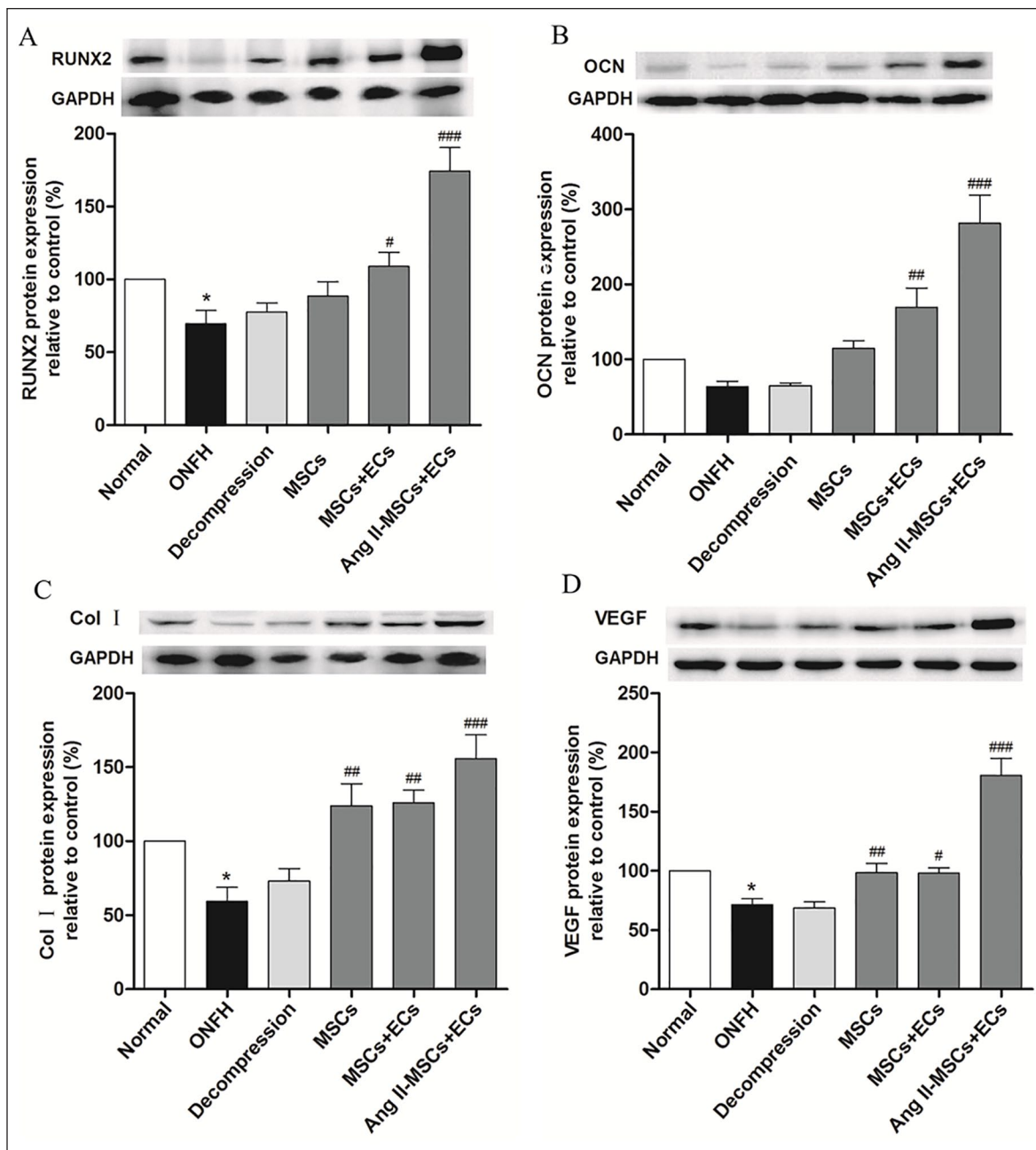


Figure 7. Ang II-pretreated BMSCs and AECs to promote the repair of early steroid-induced ONFH. The BMSCs were pretreated with Ang II for 72 h before being co-transplanted with AECs through the tunnel of core decompression under C-arm fluoroscopy for the treatment of early ONFH. Western blot analysis of Runx2 (A), OCN (B), Col-I (C), and VEGF (D) expression in femoral head osteonecrosis ($n = 6$). Results are expressed as means \pm SEM ($n = 3$). * $P < 0.05$ vs Normal, # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs ONFH. AECs: aorta endothelial cells; BMSCs: bone marrow mesenchymal stem cells; ECs: endothelial cells; MSC: mesenchymal stem cells; OCN: osteocalcin; ONFH: osteonecrosis of the femoral head; VEGF: vascular endothelial growth factor.

common pathway is the decrease in the vascular supply to the femoral head, resulting in the necrosis of osteocytes and marrow and destruction of the bone architecture. More and more existing clinical strategies have paid attention to vascular problems. However, no good therapeutic effect has been achieved so far as the extreme hypoxia of the ONFH tissue is

not conducive to angiogenesis⁵⁰. Osteogenesis and angiogenesis are tightly coupled during bone repair and remodeling. To evaluate the influence of Ang II pretreatment on the angiogenic activity of BMSCs, tube formation assay was performed in this study. The results of the present experiment exhibited that AECs treated with Ang II-pretreated BMSC

media formed more tube-like structures. This demonstrates that the paracrine factors of BMSCs contribute to angiogenesis. Similar result was obtained from another study in which human umbilical vein endothelial cells cultured with Ang II–pretreated MSC media increased tube formation⁵¹. As a key mediator of angiogenesis, VEGF has direct and indirect effects on bone formation^{52–54}. Moreover, VEGF can help to promote the construction of vascular network, to improve the local supply of oxygen and nutrients, and to increase the recruitment and survival of MSCs⁵⁴. Therefore, we also explored the expression of VEGF in an MSC-based therapy for ONFH model. Our further *in vivo* experiments confirmed that VEGF expression in the Ang II–pretreated co-transplantation group was significantly increased in comparison with the ONFH group. Similarly, Liu et al. showed that pretreatment of MSCs with Ang II improved the outcome of MSC-based therapy for myocardial infarction via the mechanisms of enhancing the paracrine production of VEGF, angiogenesis, and gap junction formation³¹. In addition, the pretreatment of human umbilical cord MSCs with Ang II improved the outcome of MSC-based therapy for severe acute pancreatitis via enhancing angiogenesis and ameliorating endothelial cell dysfunction⁵¹. According to our findings and the previous study^{31,51}, we suggest that Ang II–pretreated MSCs exhibited better therapeutic effects in ONFH through enhancing angiogenesis, such as VEGF paracrine.

Combined cell implantation has been widely applied in tissue engineering in recent years⁵⁵. It is generally known that MSCs can be isolated from many different tissues, including bone marrow, adipose tissue, and dental pulp, while the routinely wasted umbilical cord seems an inexpensive source that hosts MSCs and umbilical vein ECs, both obtainable non-invasively. More and more studies are focusing on the attempts to overcome cell transplantation's shortcomings, such as immunocompatibility, stability, heterogeneity, differentiation, and migratory capacity. Therefore, in clinical practice, it is possible to combine transplantation of human umbilical cord MSCs and umbilical vein ECs for the treatment of early ONFH. Although we designed a very safe and effective therapeutic schedule which could contribute to clinical applications, co-culturing with ECs was essential in the pretreatment of MSCs. However, the mechanism underlying the synergy between pretreatment and co-culturing has not been investigated. For future studies, we will explore the mechanism research in this filed.

Conclusions

In this study, we preliminarily confirmed that co-transplantation of Ang II–pretreated BMSCs and AECs exerted effects on repairing the early steroid-induced ONFH through improving bone regeneration and angiogenesis. Compared with core decompression alone in the treatment of ONFH, the

combined Ang II–pretreated MSC and EC implantation probably has better pain relief and clinical outcomes and can delay the collapse of the femoral head more effectively.

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Authors' Contributions

JZ was involved in financial support, conception and design, execution of experiments, collection and analysis of data, manuscript writing, and final approval of the manuscript. WH and HZ were involved in design and execution of animal experiments, discussion and analysis of results, and revision and approval of the manuscript. RZ was involved in design experiments, collection and analysis of data, and revision and approval of the manuscript. HY was involved in discussion of results, supervision of research work, and revision and approval of the manuscript.

Ethical Approval

This study was approved by the Medical Ethics Committee of Xi'an Jiaotong University (Xi'an, China).

Statement of Human and Animal Rights

All animal experiment protocols in this study were conducted in accordance with the Medical Ethics Committee for Laboratory Animal Care Guidelines, the Experimental Animal Center of Xi'an Jiaotong University College of Medicine.

Statement of Informed Consent

There are no human subjects in this article, and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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