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Original Article

Bone marrow-derived mesenchymal stem cell composited with platelet rich plasma improved repair of annulus fibrosus rupture

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Abstract: *Purpose:* This study was aimed to evaluate the effects of bone marrow-derived mesenchymal stem cells (BMSCs) composited with platelet rich plasma (PRP) on annulus fibrosus rupture repair. *Methods:* BMSCs were isolated from Ximeng goats (6 months, weighting 23-25 kg) and identified by detecting the expressions of CD29, CD34 and CD44 via immunohistochemistry. Then the proliferation of BMSCs was assessed using cell counting and MTT assay after cultured in medium containing 0, 0.1%, 0.2% and 0.3% PRP for 2 d, 4 d, 6 d and 8 d, respectively. In addition, the damaged intervertebral disc annulus fibrosus rupture model was built up with control, model and PRP-BMSCs groups. H & E staining, Masson tricolor gelatin staining, AB-PAS staining, and immunohistochemistry for type II collagen were performed to assess the histological changes of annulus tissue in different groups at 3, 6 and 12 weeks after surgery. *Results:* BMSCs were successfully isolated and identified with positive CD29 and CD34, but negative CD44. Cell proliferations of BMSCs were significantly increased with the PRP proportion increasing ($P < 0.05$). Decreasing bone and cartilage cells, maturated trabecular bone, increasing collagen fibers, as well as unclear cell and tissue structures were found in model group as compared with control group. Pathological conditions were notably improved in PRP-BMSCs group than those in model group ($P < 0.05$). Meanwhile, type II collagen was significantly increased in PRP-BMSCs group ($P < 0.05$). *Conclusion:* A combination therapy of BMSCs and PRP could effectively repair the damaged annulus tissue in goat model.

Keywords: Bone marrow-derived mesenchymal stem cells, PRP, annulus, type II collagen, lumbar disc herniation

Introduction

Lumbar disc herniation (LDH) is a common disease causing a series of problems, including neurologic deficits, perennial back and leg pain, physical deterioration, as well as socioeconomic problems due to the substantial treatment and healthcare costs [1]. The clinical outcomes of LDH after conservative treatment are poor, which is only 4-6% clinically significant [2, 3]. LDH is mainly caused by the lumbar nucleus pulposus, which oppresses the peripheral nerve tissue and causes a series of symptoms [4]. Thus, it is an effective treatment method for LDH to remove nerve nucleus by the way of minimally invasive surgery [5, 6]. However, the recurrence rate of LDH is quite high and the incisions in annulus and posterior longitudinal ligament during the primary discectomy are considered as the predisposing factors for the recurrence [7, 8]. Therefore, in order to reduce the recurrence rate of LDH surgery, a proper strategy to regenerate the damaged annulus fibrosus (AF) is critical and urgent.

Stem cells are a unique type of cells which are benefit for the regeneration of functional tissues, owing to their specialized capacity for self-renewal and the ability to differentiate into different tissues [9]. Bone marrow-derived mesenchymal stem cell (BMSCs) has been reported to simulate the intervertebral disc-like assembly and has the potential to regenerate AF [10]. Platelet-rich plasma (PRP) contains various growth factors, including transforming growth factor-β (TGF-β), platelet-derived growth

factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (ILGF), which are benefit for speeding up wound and bone healing [11]. PRP promotes the proliferation of bone precursor cells in various kinds of animals, such as goats and rabbits [12, 13]. Meanwhile, PRP has been demonstrated to promote the proliferation and differentiation into osteoblasts of BMSCs as well as promote the synthesis of collagen [14]. Nowadays, several studies have reported the combination therapies of stem cells and PRP. Autologous mesenchymal stem cells (AMSCs) in combination with PRP have been used for the regeneration of periodontal with a good healing [15]. A combined treatment of BMSCs and PRP produces a positive effect on the functional recovery of spinal cord injured rats via enhancing the remyelination of axonal [16]. However, there are still rare studies about the combined function of BMSCs and PRP on annulus fibrosus rupture repair.

The hypothesis of this present study was that BMSCs combined with PRP was benefit for annulus fibrosus rupture repair. BMSCs of goats were isolated, cultured and identified, and PRP was also isolated. A goat model was built up with damaged intervertebral disc annulus to investigate the combined function of BMSCs and PRP on annulus fibrosus rupture repair.

Material and methods

Animals

Skeletally mature female purebred Ximeng goats (6 months, weighting 23-25 kg) were used in this study, which were feed in the Laboratory Animal Center of Inner-Mongolian Agricultural University under pathogen-free conditions. All experiments were performed with the approval of the Experimental Animal Committee of the Inner-Mongolian Agricultural University.

Isolation and identification of goat BMSCs

Bone marrow was aspirated from the iliac crest of the 6-month-old dairy goats. The goats were anaesthetized with Sumianxin II by intramuscular injection and laid on the operating table as lateral position. After shearing, the skin was disinfected with 2% povidone-iodine. Inner walls of bone needle and 20 ml syringe were infiltrated with 0.1 ml 3000 u/ml heparin to prevent the occurrence of clotting. Total 4 ml bone marrow fluid was extracted and then transferred into a 15 ml centrifuge tube and resuspended with 5 ml L-DMEM. Then 5 ml dilution was loaded onto 5 ml Percoll solution (density, 1.073 kg/L; Gibco, USA) and separated by centrifugation at 1500 rpm for 30 min at room temperature. The milky white supernatant was transferred to a new centrifuge tube with 5 ml L-DMEM, followed by centrifugation at 1500 rpm for 15 min and the supernatant was discarded. After mixed with 1 ml L-DMEM supplemented with 20% fetal bovine serum (FBS), 120 μg/ml streptomycin and 120 μg/ml penicillin, the cells were seeded in 25 cm^2 flasks and cultured at a 37°C incubator containing 5% CO_2. The cells were washed with phosphate buffer saline (PBS) to remove the unattached cells and digested with 0.25% trypsin containing 0.02% EDTA for passaging when the cell density reached to 85%-90%. Morphological characteristics, growth and proliferation of the cells were daily observed under an inverted microscope. The passage 3 cells were seeded in 24-well plates at the density of 1×10^4/mL to conduct the identification of BMSCs. Firstly, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 (in PBS), and blocked with 5% bovine serum albumin (BSA) for 2 h. Cells were then incubated with primary antibodies, CD29, CD34 and CD44 (1:100; Santa Cruz, USA), at 4°C overnight. After washed with PBS for three times, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:200; Santa Cruz) in the dark for 45 min, followed by nuclear staining with DAPI (Sigma, MO, USA) in the dark for 5 min. Finally, an inverted fluorescence microscope (Olympus, Tokyo, Japan) was utilized to observe the staining with 10 random fields for each sample.

Extraction of platelet-rich plasma (PRP)

Under sterile conditions, 45 ml goat neck venous blood was extracted using 50 ml syringe which added with 5 ml sodium citrate before. The mixed liquid was centrifuged at 4000 rpm for 10 min, and the supernatant was centrifuged again at the same condition. Then the supernatant was collected as PRP. The prepared PRP mixture (0.8 ml) was activated by adding 0.2 ml anticoagulant consisting of 10%

calcium chloride and 1,000 units/u thrombin, and incubated for 10 sec to obtain PRP gel [11, 17]. The prepared PRP gel was then added to serum-free DMEM to prepare PRP gel culture medium as the final concentration of 0, 0.1%, 0.2% and 0.3%, respectively.

Cell counting and MTT assay

Primary cells were seeded into 24-well plate at the density of 1.0×10^4 cell/well. Three holes in each group were randomly selected to count the number of cells every other day to obtain growth curves. Meanwhile, MTT assay was also performed to assess the proliferation of BMSCs cultured in medium with different PRP proportions. Briefly, the cells were seeded in 96 well/plates at the density of 2×10^3 in 100 μl L-DMEM medium containing 10% FBS, and serum-free L-DMEM was exchanged after 4 h. After incubated for 24 h, the cells were treated with 100 μl medium containing 0, 0.1%, 0.2% and 0.3% PRP, respectively. The cell proliferation was examined at 2 d, 4 d, 6 d and 8 d by reacting with 20 μl 50 g/L MTT for 4 h. Finally, the medium was removed and replaced with 200 μl dimethyl sulfoxide (DMSO) to be thawed for 10 min, and absorbance (A) was measured at 450 nm using a Microplate reader (Thermolab System, Franklin, MA).

Damaged intervertebral disc annulus animal model

A total of 18 skeletally mature female purebred Ximeng goats were divided into three groups with 6 goats per group, namely control group, model group and PRP-BMSCs group. Meanwhile, 30 intervertebral discs were used for annulus injury per group and 5 intervertebral discs were operated in each goat. The surgical sites were posited from thoracic vertebra 1 (Th1) to lumbar spine 5 (L5). The goats in model group and PRP-BMSCs group were anaesthetized with Sumianxin II by intramuscular injection and laid on the operating table as a lateral position. After shaving and disinfection, a 10-15 cm longitudinal cut was made next to the spinous process. Periosteum and soft tissue were stripped using periosteal stripping, and then a 1 × 1 cm gap was made in the annulus with a knife. For the PRP-BMSCs group, 1 × 1 cm gelatin sponge (GS) scaffolds (Gelfoam; Upjohn, Kalamazoo, MI, USA) containing 0.3 ml BMSCs suspension (5.5×10^7 cells/mL) was implanted into the hole and PRP gel was laid on the surface. Finally, the wound was sutured using 4 sutures for fascia and 8 sutures for skin (Ethicon, Johnson & Johnson, USA). Annulus tissues in different groups were taken out and observed at 3, 6 and 12 weeks after surgery, and 10 injured annulus tissues were extracted from 2 goats under anesthesia state for a time point detecting per group.

Hematoxylin and eosin (H & E) staining and scoring

For these three groups, tissue samples were collected from the annulus to underwent H & E staining [18]. These specimens were stored in 10% formaldehyde solution (PBS, pH 7.2) for fixation for 24 h, and decalcified using 10% EDTA and microwave for a month. The specimens were dehydrated by graded series of alcohol, embedded in paraffin and cut into 4-7 μm thickness sections. After stained with H & E, the paraffin-embedded sections were observed under inverted microscope (Olympus). Furthermore, the sections were also assessed according to the modified scoring principles of Mankin with a maximum score of 14 points, which is recommended for semiquantitative histological assessment [19]. The Mankin's score was modified and divided into four stages depended on the degeneration, namely 0-1 points for normal tissues; 2-5 points for mild degenerative tissues; 6-9 points for moderate degenerative tissues and 10-14 points for severe degenerative tissues.

Trichrome-masson staining

Trichrome-masson staining [20] was performed to obverse the fibrosis. After dewaxed and hydrated, the sections were successively stained with 100 μl Masson staining solution for 5-10 min, followed by 100 μl phosphomolybdic acid solution for 3-5 min and 100 μl aniline blue staining solution for 5 min. Then the sections were added with 100 μl differentiation solution for 1 min, followed by dehydrated with graded series of alcohol. After mounted with neutral gum, the sections were observed under inverted microscope (Olympus) and scored in accordance with the areal density of collagen fibers.

AB-PAS staining

AB-PAS staining [21] was carried out to obverse the changes of cartilage morphology for the different groups. Briefly, each section was imme-

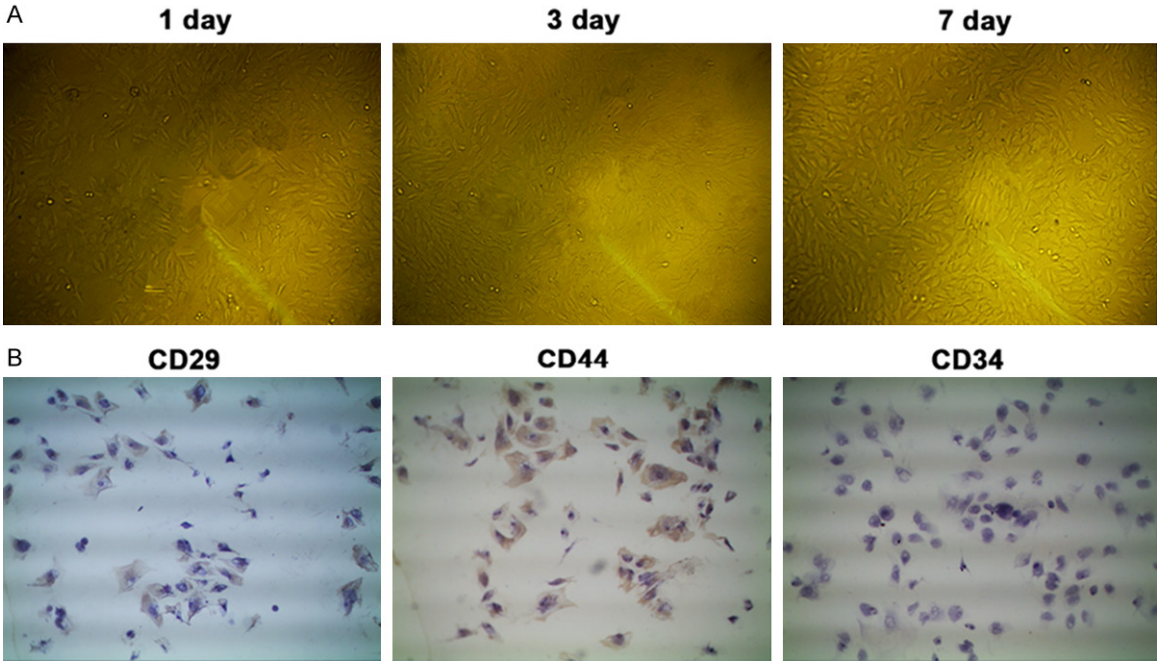

Figure 1. Morphology and identification of bone marrow mesenchymal stem cell (BMSCs). A: The morphologies of passage 3 BMSCs are observed on 1 day, 3 day and 7 day; B: Immunocytochemistry showed that the passage 3 BMSCs were positive for CD29 and CD34, while negative for CD44 (100 ×).

rsed in alcian blue solution for 40 min, followed by 3% acetic acid for 4 min and Schiff's reagent for 10-20 min to promote oxidation. After counterstained with hematoxylin, the sections were observed under inverted microscope (Olympus).

Immunohistochemistry

The sections were utilized to make a comparison between protein expression and morphology by immunohistochemistry assay. After dewaxed and hydrated, the sections were blocked with non-immune serum for 10 min, and then incubated with primary antibodies against Collagen II (Abcam, Cambridge, UK) for 1 h, followed by biotin-labeled second antibody (Vector Laboratories, Burlingame, California, USA) for 10 min. After washed with PBS, the sections were incubated with horseradish peroxidase-labeled streptavidin for 30 min. Finally, the sections were stained with 3,3'-diaminobenzidine (DBA) and re-stained with hematoxylin. Random 10 fields were taken for each section to be observed with a microscope (Olympus, Japan) and 100 cells per field. The scores were divided into four stages depending on the depth of color: 0 point for negative cells, 1 point for yellowish, 2 point for yellow, and 3 point for brown. Meanwhile, another scoring was performed according to the percentage of positive cells: 1 point for positive cells < 5%, 2 point for 5%-25% positive cells, 3 point for 26%-50% positive cells, 4 point for 51%-75% positive cells, and 5 point for positive cells > 75%. The product of these two scores was considered as the final criteria.

Statistical analysis

All the scoring procedures were performed by three independent researchers who were blind to the experiment grouping design. Inter-examiner agreement was assessed using weighted coefficient Kappa and a high Kappa score higher than 0.8 was considered as a high inter-observer agreement. All experiment data were shown as mean ± SD and analyzed using SPSS software (version 19.0; SPSS Inc., Chicago, IL). The significance differences among groups were performed by one-way analysis of variance (ANOVA) followed by LSD multiple comparisons. $P < 0.05$ was considered as statistically significant difference.

Results

Growth, morphology and identification of BMSCs

Primary cells were mostly adhered after cultured for 24 h with spindle or spherical. The

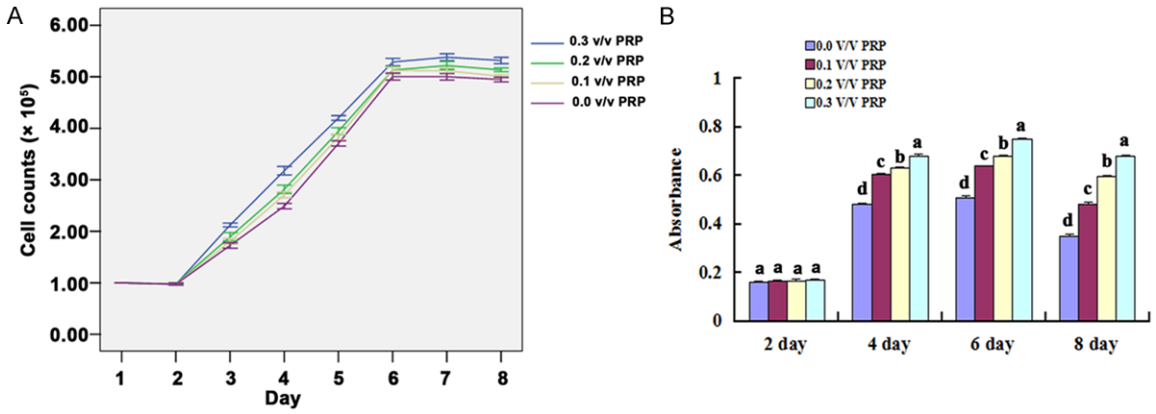

Figure 2. Platelet rich plasma (PRP) promoted the proliferation of bone marrow mesenchymal stem cell (BMSCs). A: The growth curves of BMSCs with 0, 0.1, 0.2, 0.3 v/v PRP treatment; B: MTT assay was also performed to assess BMSCs proliferation after treated with 0, 0.1, 0.2, 0.3 v/v PRP. Columns, mean (n=3); bars, SD; the significance differences among groups were performed by one-way analysis of variance (ANOVA) followed by LSD multiple comparisons and different lowercase letters (a-c) are used to indicate statistically significant differences ($P < 0.05$).

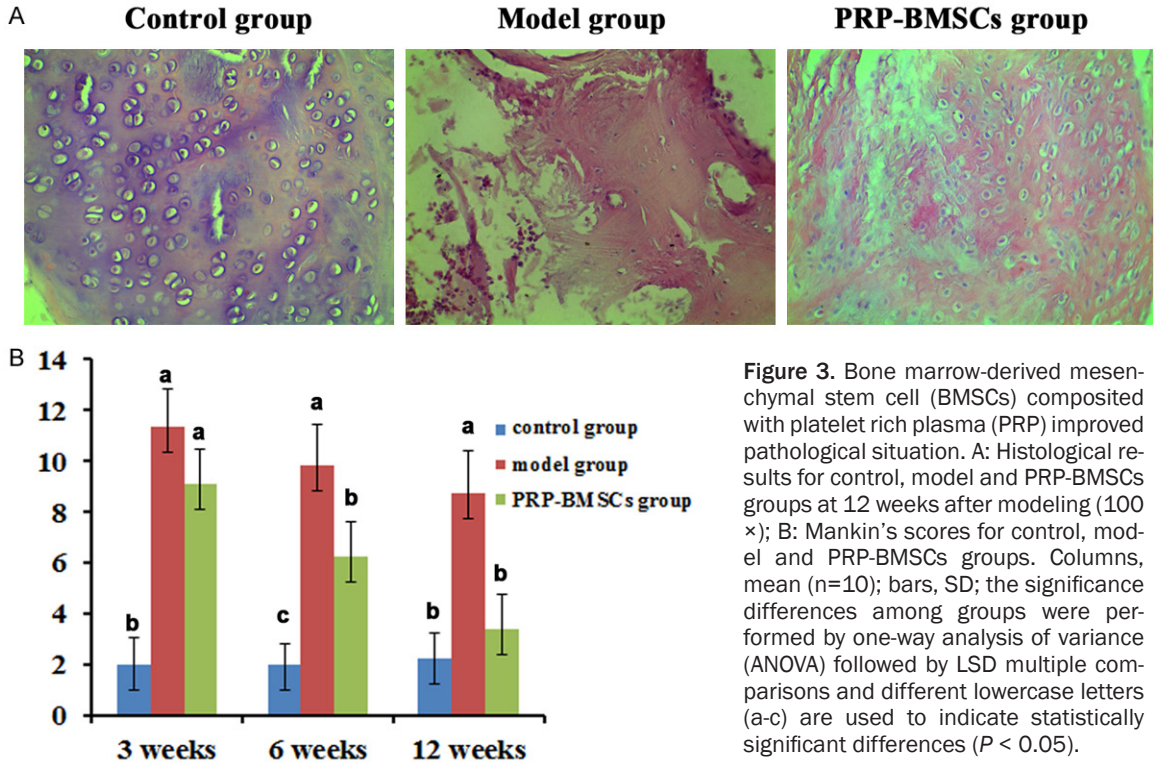

Figure 3. Bone marrow-derived mesenchymal stem cell (BMSCs) composited with platelet rich plasma (PRP) improved pathological situation. A: Histological results for control, model and PRP-BMSCs groups at 12 weeks after modeling (100 ×); B: Mankin's scores for control, model and PRP-BMSCs groups. Columns, mean (n=10); bars, SD; the significance differences among groups were performed by one-way analysis of variance (ANOVA) followed by LSD multiple comparisons and different lowercase letters (a-c) are used to indicate statistically significant differences ($P < 0.05$).

morphologies of the passage 3 cells are shown in **Figure 1A**. Form day 1 to day 7, the cells kept getting better with increasing adherent cells, narrow gaps and clear cell morphology. Immunocytochemistry showed that the passage 3 BMSCs were positive for CD29 and CD34, while negative for CD44, which proved these cells were indeed goat BMSCs, instead of hematopoietic cells or other cells (**Figure 1B**).

PRP promoted the proliferation of BMSCs

As it was shown in **Figure 2A**, the cell numbers on 2-6 d were increasing in all groups. The proliferation of BMSCs was higher after treated with PRP, and it was highest in the 0.3 v/v PRP group (2-8 d, $P < 0.05$). Consistent with these results, on significant differences of A were found among these four groups on day 2 ($P > 0.05$, **Figure 2B**), while the promoting function

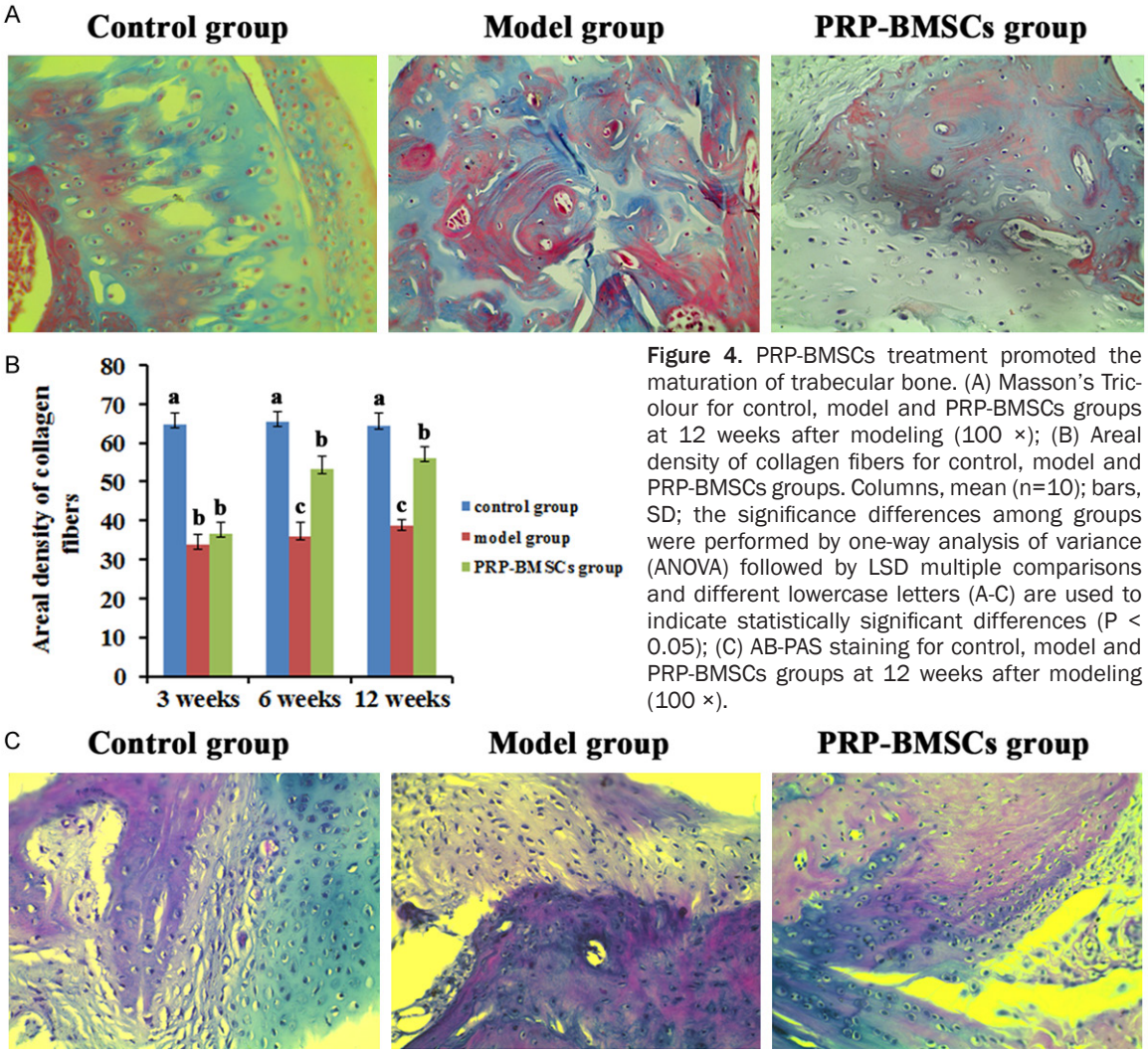

Figure 4. PRP-BMSCs treatment promoted the maturation of trabecular bone. (A) Masson's Tricolour for control, model and PRP-BMSCs groups at 12 weeks after modeling (100 ×); (B) Areal density of collagen fibers for control, model and PRP-BMSCs groups. Columns, mean (n=10); bars, SD; the significance differences among groups were performed by one-way analysis of variance (ANOVA) followed by LSD multiple comparisons and different lowercase letters (A-C) are used to indicate statistically significant differences ($P < 0.05$); (C) AB-PAS staining for control, model and PRP-BMSCs groups at 12 weeks after modeling (100 ×).

of PRP on the growth and proliferation of BMSCs were obviously detected on day 4-day 8 ($P < 0.05$, **Figure 2B**).

PRP-BMSCs significantly improved pathological situation

Histological results are shown in **Figure 3A** and a high inter-observer agreement was obtained with Kappa score of 0.89. In the control group, the cells were normal with closely arranged bone and cartilage cells, clear cell outline, and complete morphology. For the model group, it could be seen with a small amount of bone and cartilage cells, smaller cells, large nucleus, surrounded by a small amount of collagen and matrix, a small amount of fibroblasts, defect morphology, as well as unclear cell and tissue structure. The tissue morphology was notably improved in PRP-BMSCs group. The number of bone cells, cartilage cells and fibroblast cells were all notably increased, and the cells were with larger cell volume and clearer form in PRP-BMSCs group. At 3 weeks, the Mankin's scores in model and treatment groups were all higher than that in control group ($P < 0.05$, **Figure 3B**). However, the scores were significantly decreased at 12 weeks in PRP-BMSCs group, which were notably lower than that in model group ($P < 0.05$, **Figure 3B**) and similar to that in control group ($P > 0.05$, **Figure 3B**).

The effects of PRP-BMSCs on the collagen fibers and chondrocytes

Collagen fibers, chondrocytes and trabecular bone were observed by Trichrome-masson staining at 12 weeks (**Figure 4A**). For control group, there were a large number of chondrocytes companying with large number of mature

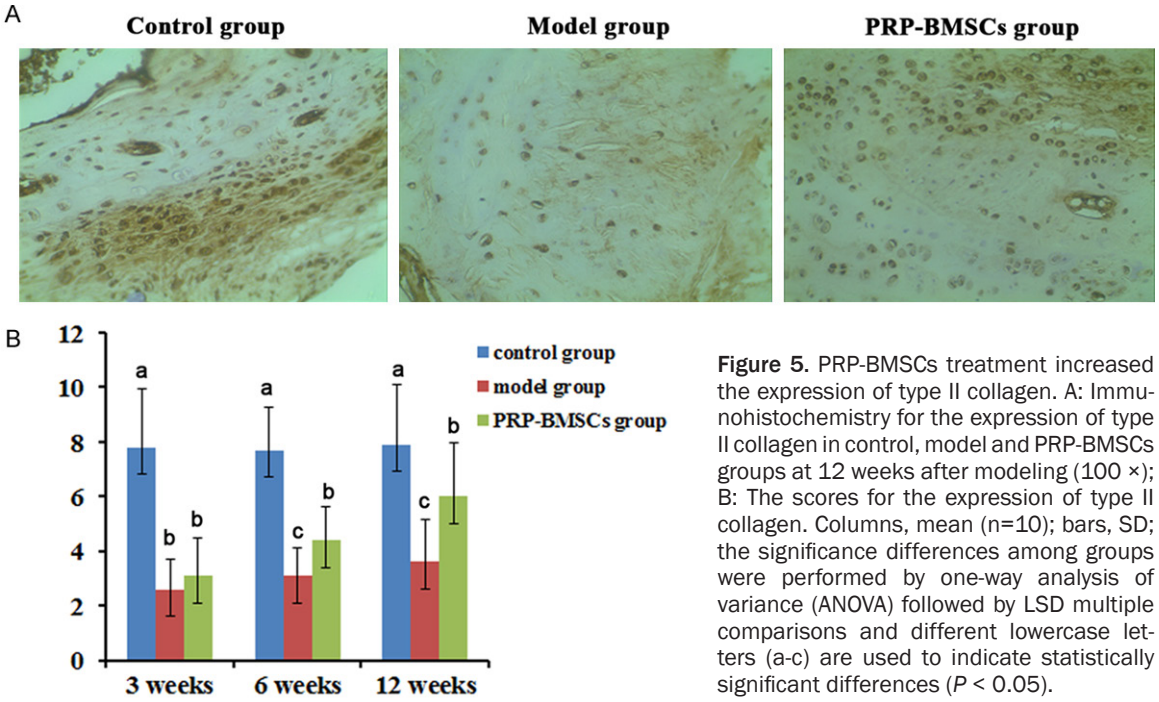

Figure 5. PRP-BMSCs treatment increased the expression of type II collagen. A: Immunohistochemistry for the expression of type II collagen in control, model and PRP-BMSCs groups at 12 weeks after modeling (100 ×); B: The scores for the expression of type II collagen. Columns, mean (n=10); bars, SD; the significance differences among groups were performed by one-way analysis of variance (ANOVA) followed by LSD multiple comparisons and different lowercase letters (a-c) are used to indicate statistically significant differences ($P < 0.05$).

trabecular bone (blue), whereas there was no immature trabecular bone (red). After intervertebral disc annulus damaged, chondrocytes, collagen fibers and cartilage matrix were obviously decreased, as well as a large number of immature trabecular bone were found. However, the situation of the damaged intervertebral disc annulus was remarkably improved by PRP-BMSCs treatment via increasing the mounts of chondrocytes, collagen fibers and cartilage matrix, and promoting the maturation of trabecular bone. Furthermore, the areal density of collagen fibers was significantly increased in PRP-BMSCs group, as compared with that in model group at 6 and 12 weeks after surgery ($P < 0.05$, **Figure 4B**). The results of AB-PAS staining showed that PRP-BMSCs treatment also could make boundaries between cartilage and bone plate more clear and promoting the maturation of cartilage matrix (**Figure 4C**).

PRP-BMSCs treatment increased the expression of collagen II

The Kappa score for the immunohistochemistry was 0.91, which indicated a high inter-observer agreement. According to the immunohistochemistry results and scores (**Figure 5**), lower contents of type II collagen were found in model and PRP-BMSCs groups, as compared with that in control group ($P < 0.05$). No significant differences of scores were found between model and PRP-BMSCs groups at 3 weeks ($P > 0.05$). However, the scores for collagen II expression in PRP-BMSCs group were significantly increased and much higher than those in model group at 6 and 12 weeks ($P < 0.05$).

Discussion

Regeneration of damaged annulus is a critical process for reducing the recurrence rate of LDH in patients underwent minimally invasive surgery. During the past decades, transplantation of mesenchymal stem cells (MSCs) to the intervertebral disc has been considered as a therapeutic model for disc degeneration in variety of animal models. Sakai and his collogues demonstrated that the disc degeneration, disorientation of annular structure and cell depletion in the nucleus pulposus were notably prevented by MSCs injection in rabbits [22]. Overexpression of FasL in cells differentiated from the transplanted MSCs contributes to maintain the immune privilege of intervertebral discs in dogs [23]. Meanwhile, MSCs could differentiate toward disc-like cells and survive in the porcine disc for at least 6 months [24]. In this present study, we demonstrated that PRP promoted the proliferation of BMSCs and a combination of PRP with BMSCs had a synergistic effect on annulus fibrosus rupture repair in goats. As a

stimulating factor, PRP could promote the growth and proliferation of BMSCs (**Figure 2**), further accelerating BMSCs amplification, avoiding immune rejection response and providing the impetus for annulus repair.

Cartilage damage is common in LDH surgery and self-repair ability of cartilage is poor owing to its avascularity and the low mitotic ability of chondrocytes [25]. Increasing studies have revealed that BMSCs have the ability to differentiate into chondrocytes as a promising seed cell source for cartilage repair [26]. However, the amount of BMSCs mobilized by microfractures is very limited [27]. Li *et al.* reported that chondrogenic differentiation of BMSCs could be promoted by supplementing TGF-β1 into culture medium [28]. Additionally, E7 and rhTGF-β1 (CBrhTE) are identified as the most favorable for BMSCs survival and can promote the chondrogenic differentiation ability of BMSCs by synchronously improving the basic components required for cartilage tissue engineering [29]. PRP contains a number of growth factors, including TGF-β1 [30]. Mishra et al. has reported that PRP enhanced proliferation of stem cells and induced the differentiation of human MSCs into chondrogenic and osteogenic cells [31]. According to the H & E and AB-PAS staining results, increasing chondrocytes cells and cartilage matrix were found in PRP-BMSCs group. We certified that PRP could significantly promote the differentiation ability of BMSCs into cartilage cells, which not only by increasing the BMSCs amplification but also by the function of TGF-β1 on BMSCs. The differentiation of BMSCs into cartilage cells was necessary and critical for annulus fibrosus rupture repair.

Collagen is an important part of annulus, which can enhance the tensile strength of annulus cells and plays a pivotal role in the cell metastasis and growth [32]. Type II collagen owns the properties to transform into cartilage matrix for cartilage repair and high expression of type II collagen is associated with low levels of chondrocyte apoptosis [33]. PRP is reported to enhance cartilage repair, promote cell proliferation and collagen synthesis, and induce the formation of cartilage matrix riched with type II collagen and proteoglycans [33]. Combination of PRP and collagen matrix facilitates a physiological microenvironment, which might be help to maintain the chondrocyte homeostasis [34]. Human MSCs promote rat meniscal regeneration by increasing the expression of type II collagen after they are activated [35]. Compared to undifferentiated monolayer cells, upregulated expressions of type II collagen and aggrecan gene exist in MSCs [36]. Consistently, a significant increasing of type II collagen expression was found in PRP-BMSCs group when compared with that in model group. Meanwhile, a lot of mucopolysaccharides were seen among cartilage matrix according to the immunohistochemistry results, which are necessary for type II collagen to repair tissues. Therefore, we concluded that the differentiation of BMSCs into type II collagen was critical for annulus fibrosus rupture repair.

Conclusion

BMSCs were successfully isolated and identified from goat, which were with positive CD29 and CD34, while negative CD44. PRP could significantly increase cell proliferation of BMSCs. A combination therapy of PRP and BMSCs could successfully repaired the damaged annulus tissue by improving pathological situation, promoting the maturation of trabecular bone, increasing chondrogenic differentiation of BMSCs, and increasing the expression of collagen II. This experiment provided an animal experiment basis for decreasing the recurrence of LDH and provided a reliable foundation for further clinical trials.

Disclosure of conflict of interest

None.

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