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Effects of platelet-rich plasma on angiogenesis and osteogenesis-associated factors in rabbits with avascular necrosis of the femoral head

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Abstract. – OBJECTIVE: Platelet-rich plasma (PRP) contains various growth factors and cytokines that can enhance the recovery of the damaged tissues. The present study aimed to examine the effects of PRP on the recovery of avascular necrosis of the femoral head (ANFH), and to provide novel insights into the clinical treatment of this disease.

MATERIALS AND METHODS: A total of 24 New Zealand white rabbits were randomly divided into the normal control group, ANFH model and PRP-treated groups (n =1 2 each). Blood samples were extracted from the auricular vein at 4, 8 and 12 weeks after establishing the model to determine the hemorheological indexes, as well as the content of serum osteocalcin bone Gla-protein (BGP) and vascular endothelial growth factor (VEGF). In addition, femoral head tissue was collected, with part of it used for hematoxylin and eosin (HE) staining to observe the histological changes. The remaining was used to detect the mRNA expression levels of alkaline phosphatase (AKP), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), bone morphogenetic protein 2 (BMP-2) and platelet-derived growth factor B (PDGF-B) by reverse transcription-polymerase chain reaction.

RESULTS: Compared with the model group, PRP treatment significantly improved the hemorheological indexes, as well as significantly increased the contents of BGP and VEGF. In the PRP group, the expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B were significantly upregulated, while AKP expression was downregulated compared with the model group. Furthermore, PRP evidently improved the histological structure of the ANFH tissue.

CONCLUSIONS: PRP was able to improve the hemorheological indexes following femoral neck fracture, repair the local blood vessels, and promote the expression of osteoblast-associated and angiogenesis-associated factors, which suggested a high efficiency in repairing ANFH.

Key Words:
Platelet-rich plasma, Avascular necrosis of the femoral head, Angiogenesis, Osteogenesis.

Introduction

Hip fractures affected about 2 million people worldwide in 2000 because of osteoporosis, accidents, or other reasons, especially in the elderly population, and this number was predicted to have doubled or tripled by 2050[1,2]. Femoral neck fractures account for the majority of the hip fracture cases occurring in elderly patients after simple falls, causing serious economic problems for the patients and their families[3]. As a rare but severe complication of femoral neck fractures, avascular necrosis of the femoral head (ANFH) involves the death of the femoral head bone tissue due to the insufficient blood supply of the femoral head following fractures[4,5]. Blood in the femoral head periphery is mainly supplied by different vessel groups, including the lateral epiphyseal and the inferior metaphysical vessels[6,7]. Damage of these two groups of vessels significantly decreases the amount of blood transported to the

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femoral head, which in turn leads to ANFH[8]. The occurrence of ANFH consequently increases the difficulties in treating a femoral neck fracture[9,10]. Corresponding treatment strategies aiming to promote the vascular and bone growth have been developed to treat ANFH. Among these, internal fixation has been proven to be an effective strategy; however, its application is challenged by the low regenerative ability of the bone, as well as by safety problems[11]. Therefore, developing effective and safe treatments for femoral neck fracture are crucial.

Platelet-rich plasma (PRP), a blood product with a higher platelet density compared with the physiological whole blood, has been demonstrated to be a powerful tool for tissue healing[12,13]. PRP contains various growth factors, including fibroblast, insulin-like, transforming and platelet-derived growth factors, resulting in its ability to stimulate the formation of new tissue following tissue damage and to inhibit bacterial infection[14]. It has been observed that the activation of platelets releases multiple functional factors to coordinate the interaction between cells, as well as between cells and the extracellular matrix[15]. Previous studies[16] have also suggested that PRP can achieve an osteogenic function by increasing the proliferation rate and differentiation rate of human mesenchymal stem cells. In addition, PRP is able to promote the differentiation of human adipose-derived stem cells[17]. In view of the important biological functions of PRP and the pathogenesis of ANFH, it is suggested that PRP may also be used to improve the recovery of patients with ANFH.

In the present study, the potential application of PRP in the treatment of ANFH was investigated in a rabbit femoral neck fracture model. The treatment efficacy was evaluated by determining the hemorheological indexes, as well as the content of serum osteocalcin (BGP) and vascular endothelial growth factor (VEGF). In addition, the effects of PRP on the tissue histological structure, and the expression of osteoblast-associated and angiogenesis-associated factors were also analyzed.

Materials and Methods

Experimental Animals

In total, 24 adult New Zealand white rabbits (half male and half female, restriction on gender) with a weight of 2.6 ± 0.2 kg were purchased from the Animal Center of Binzhou Medical College (Yantai, China). The present study was approved by the Ethics Committee of Yantaishan Hospital. The basic instruments used in the surgical procedures were provided by Yantaishan Hospital.

PRP Preparation

Prior to surgery, the rabbits were treated with intravenous anesthesia using 3% pentobarbital sodium (30 mg/kg; Shanghai Haling Biological Technology Co., Ltd., Shanghai, China). Next, a 5 -ml blood sample was extracted from the central ear artery using a 10 ml syringe containing 1 ml sodium citrate serving as an anticoagulant (Sichuan Nangeer Biology Medical Co., Ltd., Chengdou, China). The blood sample was mixed with the anticoagulant and transferred to a 10 ml centrifuge tube (Corning Inc., Corning, NY, USA), followed by centrifugation at 1500 × g for 10 min at 4°C. Next, the sample was separated into two layers, namely the upper supernatant layer and the lower layer containing the red blood cells. The entire upper supernatant layer and red blood cells in the lower layer adjacent to 1-2 mm to the interface were collected, transferred to another 10 ml centrifuge tube and subjected to centrifugation at a speed of 1000 × g for 10 min at 4°C. PRP preparation was completed subsequent to removing the top 3/4 volume of the supernatant. The entire procedure of PRP preparation was performed under sterile conditions. Finally, PRP (0.8 ml) and coagulant (0.2 ml; 10 % $CaCl_2$ and 1,000 U thrombin) were transferred to a 2 -ml syringe containing a small amount of air, and then agitated for 10 s until PRP obtained a jelly-like consistency. PRP was obtained from each rabbit. Then, rabbits were treated with their own PRP.

Construction of Rabbit ANFH Model and Grouping

36 New Zealand white rabbits were randomly divided into the normal control, model and PRP-treated groups, 12 rabbits in each group. The rabbit ANFH model was established as described previously[18]. Briefly, following anesthesia, the rabbits were subjected to surgical treatment under sterile conditions. After separating the femur, a thin bone knife was used to cut off the neck of the femur to establish femoral neck fracture. Subsequent to performing a femoral neck fracture, a 'tunnel' with a length of 1 cm was drilled into the femur. PRP was placed in this 'tunnel' of rabbits in PRP group, while physiological saline was

used for rabbits in the model group. The fracture was fixed with two 0.8-mm Kirschner wires. Following surgery, a daily intramuscular injection of penicillin (400,000 U; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was performed for 5 days to prevent infection.

Detection of Hemorheological Indexes

Blood samples were extracted from the auricular vein of the rabbits at 4, 8 and 12 weeks after the model was established. The blood viscosity, plasma viscosity, hematocrit and erythrocyte aggregation index were measured using LBY-N6B hemorheology analysis instrument (Leader Precision Instrument Co., Ltd., Beijing, China).

Enzyme-Linked Immunosorbent Assay (ELISA) to Detect the Levels of BGP and VEGF in the Serum

Blood samples were extracted from the auricular vein of the rabbits at 4, 8 and 12 weeks after the model was established. Corresponding ELISA kits (VEGF ELISA Kit Cat. No. ABIN775887, VEGF ELISA Kit Cat. No. ABIN1116312, antibodies-online, Aachen, Germany) were used to detect the serum content of BGP and VEGF according to the manufacturer's protocol.

Histological Observation

A total of 4 rabbits were selected from each group at 4, 8, and 12 weeks after surgery for histological examination. The rabbits were sacrificed through cervical dislocation. Femoral head samples were collected and fixed in 10% neutral formaldehyde solution (Tianjin Kunhua Chemical Co., Ltd., Tianjin, China) for 72 h at room temperature. Subsequently to fixation (72 h at room temperature), the samples were rinsed with water for 1 h, and decalcification was performed using EDTA solution. The EDTA solution was replaced every 2 days for 5 weeks. Next, the specimens were dehydrated using a graded series of ethanol concentrations (60, 70 and 80%) at room temperature for 4 h each. The specimens were further dehydrated by butanol for 8 h. Following embedding in paraffin, the samples were sliced into 5-μM sections with an RM2235 model paraffin slicer (Leica Microsystems GmbH, Wetzlar, Germany). Subsequently, hematoxylin and eosin (HE) staining (Sigma-Aldrich, St. Louis, MO, USA) was performed and neutral resin was used to seal the slides. The pathological alterations of the femoral head tissues were observed under a microscope (Olympus Corp., Tokyo, Japan).

Detection of Gene Expression Levels in Femoral Head Tissue By Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression levels of alkaline phosphatase (AKP), basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), bone morphogenetic protein 2 (BMP-2) and platelet-derived growth factor B (PDGF-B) in the tissues were examined by RT-PCR. Briefly, total RNA was extracted from the femoral head tissue using a TRIzol kit (Thermo Fisher Scientific, Waltham, MA, USA). The purity and integrity of the total RNA were detected (A260/A280 between 1.8 and 2.0 indicated the quality of the RNA), and then an RT kit (TaKaRa, Dalian, China) was used to reverse transcribe the RNA into cDNA. Next, 1 μl cDNA and SYBR Green Master kit (TaKaRa, Dalian, China) were used to prepare the PCR reaction system. The primer sequences used for PCR were as follows: AKP,5'-TGAGCGACACGGACAAGA-3' (forward) and 5'-GAGTGTTGTTGATGGTCCGG-3' (reverse); bFGF, 5'-GGTTTCTTCCTGCGTATCCAC-3' (forward) and 5'-TCCTTGACCGGTAAGTATTGT-3' (reverse); TGF-β1, 5'-CGGCAGCTGTACATTGACTT-3' (forward) and 5'-AGCGCACGATCATGTTGGAC-3' (reverse); BMP-2, 5'-GCGGTGGACTGCACAGGGAC-3' (forward) and 5'-CTACCCTTCCCCGTGGGGGA-3' (reverse); PDGF-B, 5'-CATGACAAGACGGCACTGAAG-3' (forward); 5'-GACATCAAGAAGGTGGTGAAG-3' (reverse); β-actin, 5'-TGGCTCTAACAGTCCGCCTAG-3' (forward) and 5'-GCCTACAGGTGCAGCGTGA-3' (reverse). All the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The reaction was performed at 37°C for 15 min, 98°C for 5 min; 40 cycles at 92°C for 30 s, 56°C for 30 s and 65°C for 1 min. Subsequently to the PCR reaction, the product was subjected to 2% agarose gel horizontal electrophoresis. Following staining with ethidium bromide, a gel imaging system was used to observe the results and capture images of the gel. ImageJ software 1.48 u (National Institutes of Health, Bethesda, MD, USA) was used to calculate the relative gray value of each band against the band of β-actin, serving as the endogenous control. After normalization, the expression levels of AKP, bFGF, TGF-β1, BMP-2 and PDGF-B mRNAs of different groups were compared. $2^{-\Delta\Delta Cq}$ method was also used to quantify relative gene expression data[19].

Statistical Analysis

SPSS version 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used to analyze the monitoring data. All the data are expressed as mean ± standard deviation. One-way ANOVA followed by LSD test was used to analyze the data, and $p < 0.05$ was considered to indicate differences that were statistically significant.

Results

PRP Treatment Significantly Improves the Hemorheological Indexes

WBV was measured at the shear rates of 5 s^{-1} and 200 s^{-1}. As shown in Figure 1, the blood viscosity, plasma viscosity, hematocrit (HCT) and erythrocyte aggregation index (EAI) were significantly increased after ANFH injury, and according to our results, these parameters were then notably reduced following PRP treatment compared with the model group at 4, 8 and 12 weeks after surgery ($p < 0.05$). For example, the values for blood viscosity index at 4, 8 and 12 weeks after surgery in the model group measured at the shear rates of 5 s^{-1} were 10.98 ± 0.56, 11.42 ± 0.63 and 11.39 ± 0.61 mpa*s, respectively. However, the corresponding values of the blood viscosity index at these time points after surgery in the PRP group were 9.62 ± 0.43, 8.86 ± 0.37 and 8.42 ± 0.34 mpa*s, respectively. The similar trends were also detected in other blood viscosity, plasma viscosity, HCT, and EAI (Figure 1). Thus, significant differences were detected in the blood viscosity between the two groups at each time point. These data suggested that PRP was able to significantly improve the hemorheological indexes subsequent to femoral neck fracture in the rabbits.

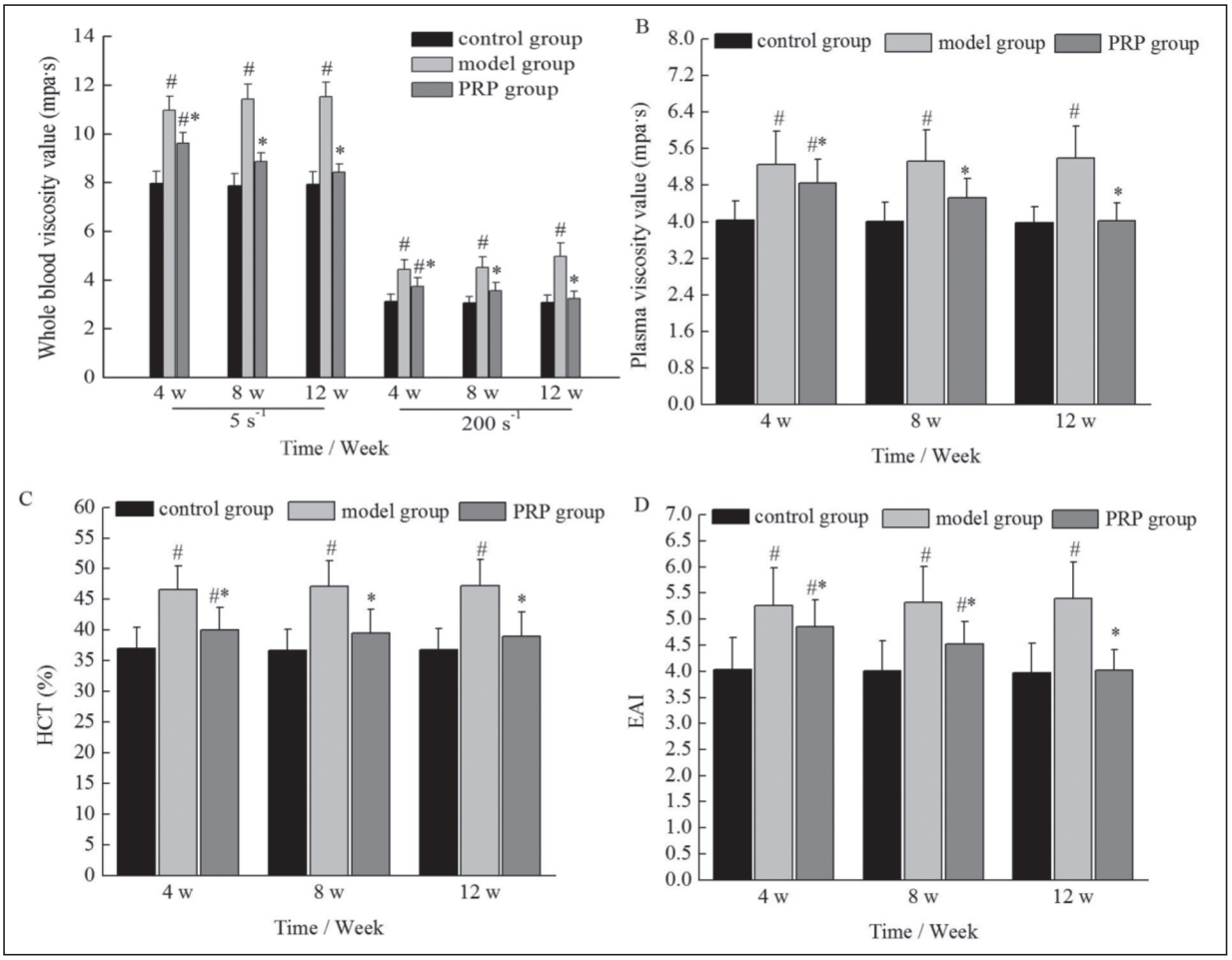

Figure 1. Effects of PRP on the hemorheological indexes of rabbits with avascular necrosis of the femoral head. ***(A)*** Blood viscosity (5 s-1 and 200 s-1), ***(B)*** plasma viscosity, ***(C)*** HCT and ***(D)*** erythrocyte aggregation index were examined. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$ *vs.* model group. PRP, platelet-rich plasma; HCT, hematocrit; RBC, red blood cell.

PRP Significantly Increases the Content of Serum BGP and VEGF in Rabbits with ANFH

As shown in Figure 2, compare with control group, the levels of serum BGP and VEGF in model group and PRP group were decreased, which were significantly elevated in the PRP group as compared with those in the model group at 4, 8 and 12 weeks after surgery. At 4 weeks after surgery, the levels of BGP and VEGF in the PRP group were 329.86 ± 18.62 and 323.75 ± 16.76 ng/l, respectively, which were significantly higher when compared with those in the model group (302.26 ± 11.37 and 284.79 ± 11.96 ng/l, respectively; $p < 0.05$; Figure 2). Furthermore, at 8 weeks after surgery, the levels of BGP and VEGF in the serum of the PRP group were 359.72 ± 22.74 and 342.62 ± 17.35 ng/l, respectively, while the levels in the model group were 297.58 ± 21.46 and 289.86 ± 13.25 ng/l, respectively; thus, statistically significant differences were identified between the two groups ($p < 0.05$; Figure 2). Compared with the levels at 8 weeks, the BGP and VEGF concentrations exhibited a decrease in the PRP group at 12 weeks, but remained significantly higher in comparison with those in the model group ($p < 0.05$). These data suggested that the content of serum BGP and VEGF in the rabbits with ANFH was increased by PRP treatment.

PRP Improves The Histopathological Structure of the Femoral Head of Rabbits with ANFH

As shown in Figure 3, in the normal control group, the bone trabeculae was thick and solid, the structure was continuous, the hematopoietic tissue in the marrow cavity was evenly distributed, osteoblasts with normal morphology were rich in the medullary cavity, while the ratio of hematopoietic cells to adipocytes was normal during the whole experiment. In the model group, HE staining of femoral head tissues indicated that the bone trabeculae of the rabbits in the model group were sparsely distributed and disordered with increased distance between them since week 4. The sample of week 8 showed that hematopoietic tissue in the marrow cavity was almost replaced by adipocytes, and osteoblasts decreased sharply. At week 12, we still observed sparsely distributed bone trabeculae, congestion and necrotic materials were observed in the marrow cavity. In the PRP group, sample from week 4 showed thin bone trabeculae, which were sparsely distributed. There was a small number of adipocytes: at week 8, the bone trabeculae were sparsely distributed, hematopoietic cells in the medullary cavity were decreased, the number of hematopoietic cells increased and adipocytes decreased. At week 12, the bone trabeculae were still sparsely distributed, there was a small number of red blood cells and necrotic material found in the medullary

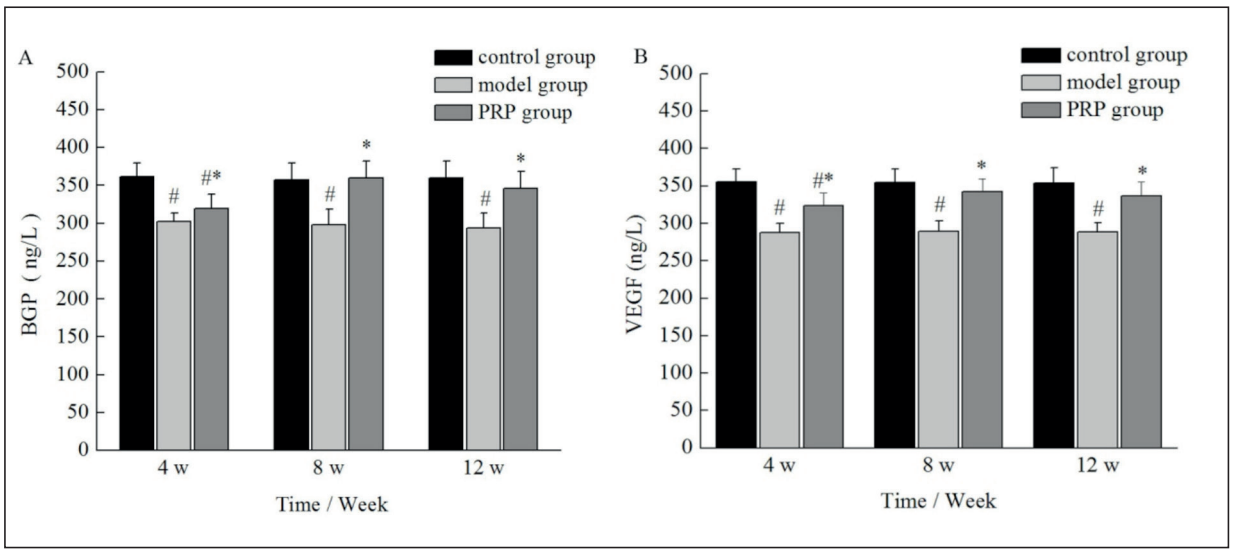

Figure 2. Effects of PRP on the content of serum. ***(A)*** BGP and ***(B)*** VEGF of rabbits with avascular necrosis of the femoral head. $^{\#}p < 0.05$ *vs*. control group;$^{*}p < 0.05$ *vs*. model group. PRP, platelet-rich plasma; BGP, serum osteocalcin; VEGF, vascular endothelial growth factor.

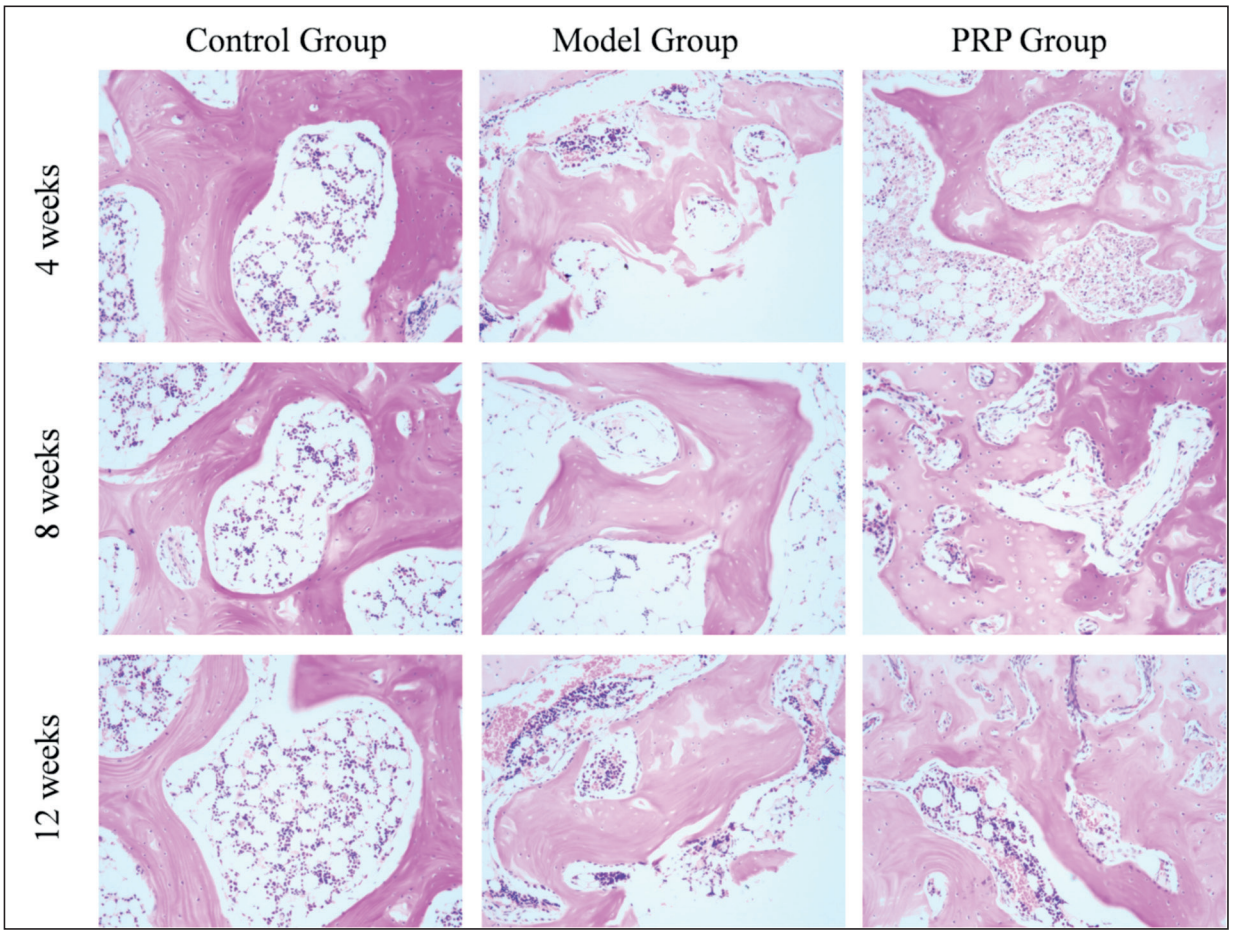

Figure 3. Effects of PRP on the histopathological structures of the femoral head tissues of rabbits with avascular necrosis of the femoral head examined by hematoxylin and eosin (HE) staining (magnification: x 200). PRP, platelet-rich plasma.

cavity, and the ratio of hematopoietic cells to adipocytes was almost normal. These data indicated that PRP treatment promoted the bone tissue repair in rabbits with ANFH.

PRP Upregulates the Expression Levels of TGF-β1, bFGF, BMP-2 and PDGF-B mRNA, and Downregulates the Expression of AKP mRNA in Rabbits with ANFH

In animals with ANFH, the expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B mRNA were decreased and AKP mRNA expression was elevated according to our RT-PCR analysis, indicating a disturbance TGF-β/BMP signaling pathway. Then, the RT-PCR results revealed that the expression level of AKP mRNA was significantly decreased by 8% compared with the model group at 4 weeks after surgery ($p < 0.05$; Figure 4 A). By contrast, the expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B mRNA in the PRP group were significantly increased by 9, 16, 12, and 8%, respectively, as compared with the model group at the same time point ($p < 0.05$; Figure 4 B-E). In addition, at 8 weeks after surgery, the expression of AKP mRNA in the PRP group was significantly decreased by 17% ($p < 0.05$; Figure 4 A), whereas the expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B were significantly increased by 21, 31, 26, and 19%, respectively ($p < 0.05$; Figure 4 B-E). Similar expression patterns for these factors in the two groups were detected at 12 weeks after surgery. These data suggested that PRP promoted the expression of osteoblast-associated and angiogenesis-associated factors in rabbits with ANFH.

Discussion

As a disease which rarely recovers spontaneously, the development of ANFH is induced and promoted by various etiologic factors caus-

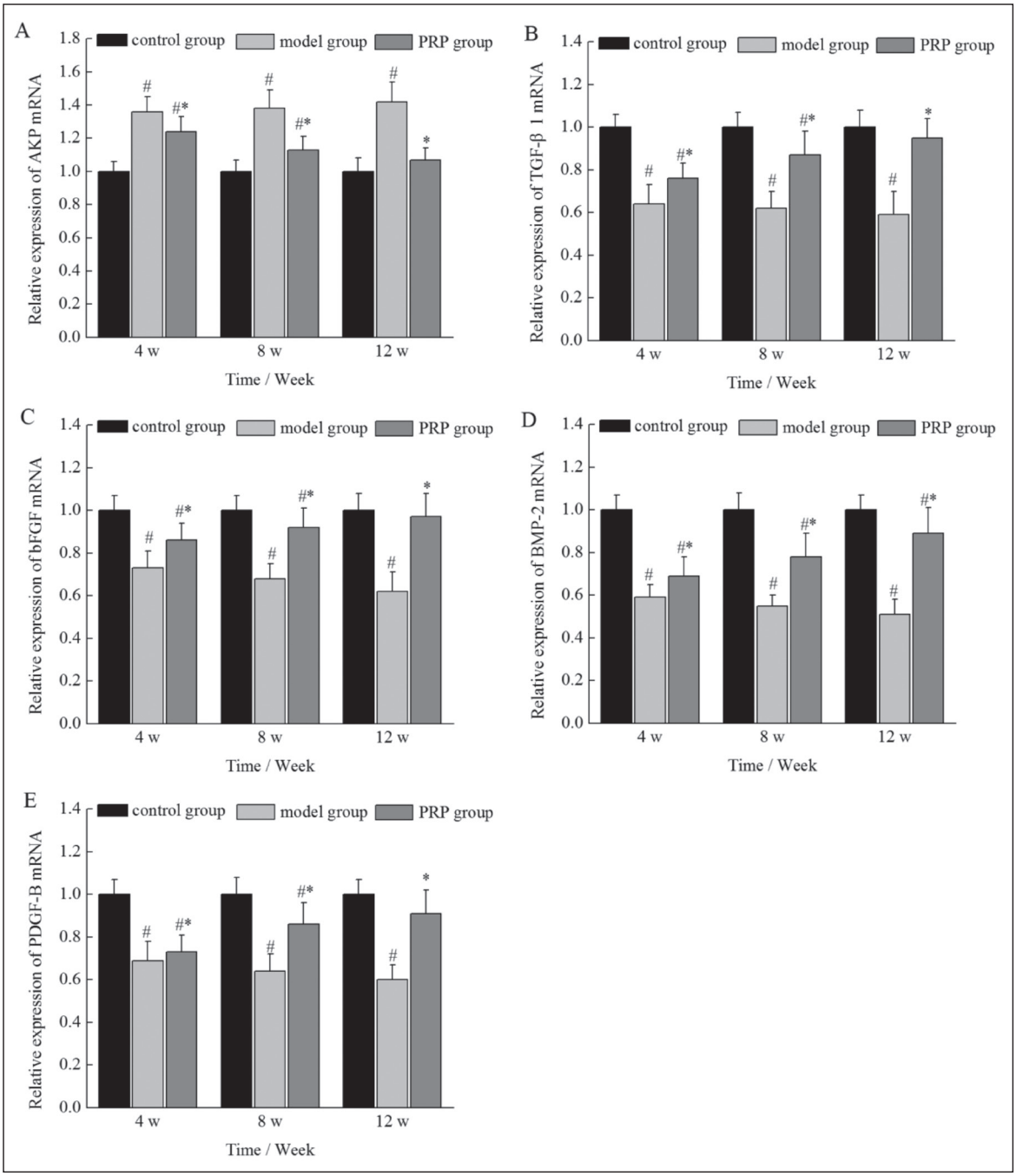

Figure 4. PRP upregulated the expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B mRNA, whereas it downregulated the expression of AKP mRNA in rabbits with avascular necrosis of the femoral head. The relative mRNA expression levels of ***(A)*** AKP, ***(B)*** TGF-β1, ***(C)*** bFGF, ***(D)*** BMP-2 and ***(E)*** PDGF-B were detected by reverse transcription-polymerase chain reaction. $^{\#}p < 0.05$ *vs*. control group; $^{*}p < 0.05$ *vs*. model group. PRP, platelet-rich plasma; AKP, alkaline phosphatase; TGF-β1, transforming growth factor β1; bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic protein 2; PDGF-B, platelet-derived growth factor B.

ing disorder of the local vascular supply. The insufficient blood supply will eventually lead to the death of femoral head bone tissue, significantly reducing the life quality of the patients[20]. The development of ANFH is usually accompanied by functional disorder of the hip joint, collapse of the femoral head, and damage of trabeculae[21]. Therefore, improving the local blood supply is the biggest challenge for the clinical treatment. To date, there are no effective and

safe strategies to treat ANFH at an early stage. The traditional bone marrow transplantation has been widely used as the standard method to improve the hip joint function in patients[22,23]. Song et al[24] have demonstrated that the transplantation of peripheral blood stem cells that were cultured with small intestine submucosal extracellular matrix in the treatment of ANFH achieved an improved outcome when compared with the traditional treatments. In addition, stem cell therapy is another promising treatment of ANFH[25]. In 2002, Hernigou and Beaujean[26] first described a technique for injecting mesenchymal stem cells combined with standard core decompression to introduce biologics into an area of necrosis. ANFH patients with relatively lower stem cell concentrations (measured by *in vitro* colony-forming assay) or a history of alcohol use or organ transplantation, demonstrated faster disease progression compared with patients with higher stem cell concentrations[25,26]. Stem cell therapy was typically performed by injecting stem cell into the necrosis area through a core decompression tract[25,26]. However, the application of this novel therapy is challenged by the limited stem cell resources.

Given its ability of inducing tissue formation, PRP has been widely used in the treatment of various diseases, including disability diseases. Compared with the placebo treatment, the application of PRP demonstrated a higher efficacy in the treatment of knee osteoarthritis[27]. Albanese et al[28] observed that PRP significantly improved the wound healing and promoted bone regeneration following oral and dental surgery. In addition, the anti-inflammatory function of PRP suggests that it can be safely used as a treatment subsequent to surgery[29]. In the present study, the results demonstrated that PRP treatment significantly improved the hemorheological indexes, decreasing the blood viscosity, plasma viscosity, hematocrit and erythrocyte aggregation in the rabbit model of ANFH. These observations indicated that PRP improved the disrupted blood supply. In addition, the histopathological characteristics of ANFH, including the damage of trabeculae, the decreased number of subchondral medullary hematopoietic cells and the increased number of adipocytes, were also observed in the rabbits, indicating that ANFH model was successfully established. Upon the application of PRP, the histopathological structure of the necrosis area was significantly improved.

BGP, which is also known as bone Gla-protein, is considered to be the most important non-collagen protein in the bone matrix[30]; thus, BGP is typically used as a biomarker of the bone formation[30]. It has been observed that the onset and development of ANFH is usually accompanied with a decrease in the level of BGP, which in turn leads to the disorder of bone metabolism and to an abnormal bone structure[31]. Furthermore, VEGF is an essential growth factor responsible for vasculogenesis and angiogenesis[32]. The decreased level of VEGF during ANFH further accelerates the disease progression[33]. In the present study, significantly higher levels of serum BGP and VEGF were observed in PRP-treated group compared with ANFH model group. These data indicated that PRP was able to improve the blood supply and promote new bone tissue formation in rabbits with ANFH.

The development of ANFH and new bone tissue formation are complex processes with a variety factors involved. The TGF-β/BMP signaling pathway has been demonstrated to be involved in bone formation[34]. Previous studies have also revealed that TGF-β1, bFGF and PDGF-B are able to increase the level of BMP receptor-IB, which is a type I receptor of activin receptor-like kinase 6, thus enhancing the BMP-2-induced osteogenic functions[35], and this pathway is known as the TGF-β/BMP signaling pathway. In addition, AKP is a hydrolase enzyme highly expressed in liver, kidney and bone tissues, and an abnormally high expression level of AKP usually indicates bone diseases[36]. In the current study, the effect of PRP on these factors was analyzed, and the results indicated higher expression levels of TGF-β1, bFGF, BMP-2 and PDGF-B mRNA and lower expression of AKP in the PRP group as compared with the model group. These data suggested that PRP may promote bone formation following ANFH through the TGF-β/BMP signaling pathway and by inhibiting the expression of AKP.

In the present study, the application of PRP improved the hemorheology of the rabbits with a femoral neck fracture. PRP was also able to promote the expression of osteoblast-associated and angiogenesis-associated factors, which in turn prevented ANFH following femoral neck fracture. Our results indicated that TGF-β/BMP pathway might play an important role in the PRP treatment in this disease. Although this study shows promises by using PRP to treat rabbits with a femoral neck fracture, it still needs further investigation before clinical application.

Conclusions

PRP was able to improve the hemorheological indexes following femoral neck fracture, repair the local blood vessels, and promote the expression of osteoblast-associated and angiogenesis-associated factors, which suggested a high efficiency in repairing ANFH.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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