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The application of PRP combined with TCP in repairing avascular necrosis of the femoral head after femoral neck fracture in rabbit

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Abstract. – OBJECTIVE: In view of the high occurrence of avascular necrosis of the femoral head (ANFH) after femoral neck fracture and the difficulties in the treatment, our work aimed to explore the effects of platelet-rich plasma (PRP) combined with tri-calcium phosphate (TCP) on the repair of ANFH after femoral neck fracture and to provide reference for clinical treatment.

MATERIALS AND METHODS: Thirty New Zealand white rabbits were randomly divided into control group, TCP group, and PRP+TCP group. The rabbit ANFH model was established and femoral head tissues were collected. HE staining was used for histological observation. Image analysis and statistical analysis were used to calculate the New Bone Area fraction (NBA %). The levels of bone morphogenetic protein (BMP)-7, transforming growth factor (TGF)-β1, basic fibroblast growth factor (bFGF), interleukin (IL)-6 and tumor necrosis factor (TNF)-α in serum were detected by Enzyme-Linked ImmunoSorbent Assay (ELISA).

RESULTS: The new bone area of TCP group was significantly lower than that of PRP+TCP group ($p<0.05$). Compared with the control group, the levels of BMP-7, TGF-β1 and bFGF were significantly increased in both TCP and PRP+TCP groups ($p<0.05$), and the increase in PRP+TCP group was higher than that in TCP group. TCP and PRP+TCP can both significantly reduce the content of IL-6 and TNF-α ($p<0.05$); however, higher decrease was found in PRP+TCP group compared with the TCP group at 8 weeks after injection.

CONCLUSIONS: PRP combined with TCP, which can promote new bone formation and inhibit inflammatory response, showed higher efficiency in repairing ANFH than internal fixation alone.

Key Words

PRP, TCP, Femoral neck fracture, Avascular necrosis of the femoral head after femoral neck fracture, Repair.

Introduction

The incidence of hip fracture was about 1.6 million in 2000 worldwide, and this number was predicted to be increased up to 4.5-6.3 million by 2050[1,2]. Previous studies indicated that various populations were in poor health, suffering from it[3,4]. Femoral neck fractures, which are more common in women than men, account for 51.97% of all the cases of proximal femoral fracture cases[5]. After femoral neck fracture, the blood supply to the femoral head will be largely reduced, which in turn leads to the occurrence of avascular necrosis of the femoral head (ANFH). ANFH can bring negative effects on the recovery of hip joint function, so the difficulty in the treatment of femoral neck fracture will be increased[6-8]. Due to the complications of different degrees of necrosis, femoral neck fracture can seriously affect the life quality of old people or even become the main cause of death in old patients with serious conditions [9]. Previous studies have shown that the firmness of internal fixation and the degrees of comminuted fractures can affect the degrees of necrosis[10]. However, several researches found that the bone mineral density was not significantly affected by the firmness of internal fixation for femoral neck fractures[11]. Also, it has been proved that Singh index can only be used in determining the degree of necrosis but not in the prediction of fracture healing[12]. All those contradictory information increased the complexity of the treatment of femoral neck fracture. The degrees of ANFH following femoral neck fracture, are affected by the types of femoral neck fracture, fracture

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site, gender, age, as well as internal fixation[13]. Locations and materials of the internal fixation can usually determine the blood supply, which affects the development of ANFH[14]. Tri-calcium phosphate (TCP) scaffold has widely been used in the treatment of necrosis. In the treatment of steroid-associated osteonecrosis, TCP scaffold was found to be able to effectively prevent hip joint collapse[15]. The TCP artificial bone implantation was also found to be able to induce the formation of cartilage tissue and neovascularization-like tissue without inducing serious adverse reaction[16,17]. Platelet-rich plasma (PRP) is a product from blood with higher density of platelets compared with the physiological whole blood[18]. PRP contains various growth factors, including fibroblast growth factors, transforming growth factors, insulin-like growth factors, and platelet derived growth factors, which confer PRP the ability to stimulate the formation of new bone and vascular tissue[19]. In view of the important functions of TCP scaffold and PRP in the formation of new bone and vascular tissue, we suggest that the application of TCP combined with PRP will bring greater clinical value than the application of TCP alone. In order to investigate the efficacy of TCP combined with PRP in the treatment of ANFH, a rabbit ANFH model was established and the effects of TCP combined with PRP on the tissue histological conditions, bone formation-related factors, and inflammation related factors, were investigated.

Materials and Methods

Experimental Animals

Thirty adult New Zealand white rabbits with weight of 2.6 ± 0.2 kg and without restriction on gender were provided by the Animal Center of Binzhou Medical College. The basic equipment required for surgery was provided by our hospital. This work was approved by the Ethics Committee of our institute.

PRP Preparation

The rabbits were subjected to intravenous anesthesia using 3% pentobarbital sodium (Shanghai Haling Biological Technology Co. Ltd., Shanghai, China) with a dose of 30 mg/kg. A 10 mL syringe preloaded with 1 mL of compound sodium citrate anticoagulant (Sichuan Nangeer Biology Medical Co., Ltd., Sichuan, China) was used to extract 5 mL blood from the rabbit central ear artery. The blood sample was mixed and transferred to a 10 mL centrifuge tube (Corning, NY, USA), followed by centrifugation (4°C, 3700 r/min) for 10 min. After that, the liquid sample was separated into two layers-the upper supernatant layer and the lower red blood cells layer. The supernatant layer and the red blood cells within the area 1-2 mm to the interface were transferred to another 10 mL centrifuge tube, followed by centrifugation (3000 r/min) for 10 min. The top 3/4 supernatant was removed and the remaining liquid was mixed to make PRP. All the operations of PRP preparation were performed under sterile conditions. PRP (0.8 mL), coagulant (0.2 mL) (10% $CaCl_2$, 1000 U thrombin), and a little air, were inhaled with a 2 mL syringe and mixed; 10 s later, PRP became jelly.

Construction of Rabbit Femoral Neck Fracture Model and Animal Grouping

Under sterile conditions, the rabbits were anesthetized and the incision was made on the lateral of the left hip to separate the muscle layer by layer. The femoral head was exposed and dislocated. An ultra-thin bone knife was used to cut off the femoral neck, and a tunnel with a length of about 2 cm was drilled on the cross-section. Thirty New Zealand white rabbits were randomly divided into 3 groups (n=10): (1) Control group: the tunnel was not subjected to any treatment; (2) TCP group: β-TCP stent (Shanghai Bio-Lu Biomaterials Co., Ltd., Shanghai, China) was implanted into the tunnel; (3) PRP + TCP group: PRP was injected into the tunnel with a dose of 0.4 mL/kg and β-TCP were implanted into the tunnel.

Histological Observation

Five rabbits were selected from each group at each time point of 4w and 8w after surgery. The rabbits were sacrificed by air injection. The femoral head specimens were collected and fixed in 10% neutral formaldehyde solution (Tianjin Kunhua Chemicals Co., Ltd., Tianjin, China) for 72 h and rinsed with water for 1 h. EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) was used for decalcification. EDTA solution was replaced every 2 days for 5 weeks. After that, the specimens were dehydrated with different concentrations of ethanol (60%, 70% and 80%, 4 h each) and butanol for 8 h. After dehydration, the specimens were embedded with paraffin (EG1160 embedding machine, Leica, Wetzlar, Germany) and sliced into slices with a thickness of 5 μM (RM2235 model paraffin slicer, Leica, Wetzlar, Germany). The paraffin slices were transferred to glass slides. Dewaxing was performed by treating twice with xylene, 30 min each time. The hydration was performed by treating with different concentrations of ethanol (100%, 95%, 90%, 85%, 80% and 75%, 5 min for each), and then paraffin slices were immersed in water for 5-10 min. The slides were immersed in hematoxylin solution for 5-10 min, and the floating reagent was removed by washing with water, followed by treatment with 0.1% hydrochloric acid for 5 s and water for 10 min. The slides were immersed in eosin solution for 8 s, and the floating reagent was removed by washing with water, followed by dehydration with different concentrations of ethanol (75%, 85%, 90%, 95%, 100% and 100%, 5 min each). The dehydrated sample was treated twice with xylene, 15 min each time. Neutral gum was used to seal the slides. The tissue was observed under a microscope (×200) (Nikon, Tokyo, Japan).

Computational Image Analysis

The area of new bone was calculated by Image-Pro Plus 6.0 (GraphPad Software, La Jolla, CA, USA). The average value of the new bone area was used to calculate the new bone area (NBA%) using the following formula: NBA% = new bone area/total defect area × 100%. The efficiencies of the treatment methods were compared.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

The levels of bone morphogenetic protein-7 (BMP-7), transforming growth factor (TGF-β1) and basic fibroblast growth factor (bFGF) in serum were measured by ELISA kit (Dingguo Biotechnology Co., Ltd., Chongqing, Sichuan, China). The levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in serum were also measured by ELISA kit (Dingguo Biotechnology Co., Ltd., Chongqing, Sichuan China).

Statistical Analysis

Statistical analysis was performed using SPSS19.0 (SPSS Inc., Chicago, IL, USA) statistical software. The measurement data were expressed as mean ± standard deviation. *t*-test was used for the comparison between groups. $p<0.05$ was considered to be statistically significant.

Results

Histological Observation

Circular defects appeared around bone marrow cells at 4 W and 8 W after surgery (Figure 1A). Four weeks after surgery, fibroblasts were found in TCP group within the tunnel, but circular defects were also found around bone marrow cells in TCP group; fibroplasia was detected in PRP+TCP group within the tunnel, a few bone cells were also found in the artificial bone tunnel. Also, the circular defects were replaced by fibroblasts (Figure 1A). Eight weeks after surgery, bone cells and new bone tissues can be seen in the PRP+TCP group within the artificial bone tunnel, the new bone trabecular plate was layer- or woven-like, and the number of fibroblasts were increased; only sparse tissues (mainly fibrous tissue) can be found in TCP group within the artificial tunnel bone (Figure 1A). The new bone areas of each group were all increased to different degrees along with the time (Figure 1B). Four weeks after TCP injection, the NBA% was increased from 6.12% to 23.21% ($p=0.00$). Four weeks after the injection of PRP combined TCP4, the NBA% was increased from 6.12% to 39.31% (Figure 1B). Compared with TCP group, injection of PRP combined with TCP can significantly increase the new bone area. Eight weeks after injection, the NBA% of PRP+TCP was 53.23%, while the NBA% of TCP was 37.09% (Figure 1B), significant difference was found between those 2 groups ($p=0.00$), indicating that PRP combined with TCP accelerates bone defect repair.

The Changes of the Levels of Serum BMP-7, TGF–β1 and bFGF Protein

At 4 weeks after surgery, the concentrations of BMP-7 in serum of TCP group and PRP+TCP group were significantly higher than that in control group ($p<0.05$); the concentration of BMP-7 in serum of the PRP+TCP group was 235.21 pg/ml, which was significantly higher than that in serum of the TCP group (184.04 pg/ml) ($p=0.04$). At 8 weeks after surgery, the concentrations of BMP-7 in serum of TCP group and PRP+TCP group were also significantly higher than that in control group ($p<0.05$), while no significant different was found between TCP group and PRP+TCP group (Figure 2A). The changes of the concentration of TGF-β1 in serum of each group showed similar pattern with BMP-7 (Figure 2B). The concentration of bFGF in serum of PRP+TCP group was significantly higher than

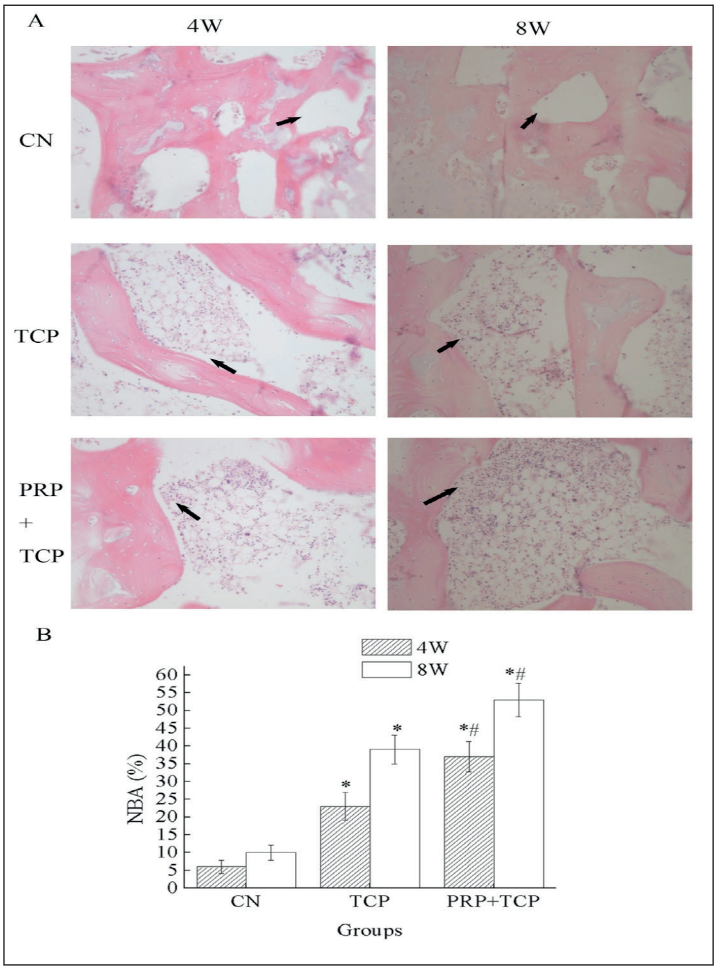

Figure 1. Histological observation after injection with TCP or PRP combined with TCP. **A**, Results of HE (×200), the arrows point the new-formed bone tissues. **B**, NBA% of each group; *compared with control group at the same time point $p<0.05$; #compared with TCP group at the same time point $p<0.05$.

that in control group and TCP group ($p<0.05$) at both 4 weeks and 8 weeks after surgery (Figure 2C). The results showed that the application of PRP combined with TCP in the treatment was more effective than the application of TCP alone.

The Changes of the Levels of Inflammation Related Factors After Injection of TCP or PRP/TCP

TCP and PRP+TCP can both significantly reduce the content of IL-6 and TNF-α in serum (Figure 3). No significant difference was found in levels of serum IL-6 and TNF-α between TCP and PRP+TCP groups at 4 weeks after injection ($p>0.05$). Eight weeks after injection, the levels of IL-6 and TNF-α in serum of PRP+TCP group were decreased to 9.07 pg/ml and 7.98 pg/ml respectively, which were both significantly lower than those of TCP group ($p<0.05$). Our data suggested that PRP combined with TCP can significantly inhibit inflammation.

Discussion

Femoral neck fracture can not only seriously affect old people but also significantly reduce the life quality of the young and middle-aged patients with illness and inactivity[20]. Various vulnerable groups suffered from it[21,22]. Femoral neck fracture causes significant health care as well as economic problems to the patients and their families[23]. Therefore, safe and effective treatment methods of femoral neck fracture are always needed. Internal fixation has been proved to be as effective way to treat femoral neck fracture in many different cases. However, the safety and efficacy of internal fixation are largely determined by the materials and positions. Previous studies have shown that inappropriate positions of internal fixation can damage the surrounding tissues including lateral epiphyseal artery, which in turn increases the injury[24]. Zlowodzki et al[25] reported that some materials used in internal fixation including three-

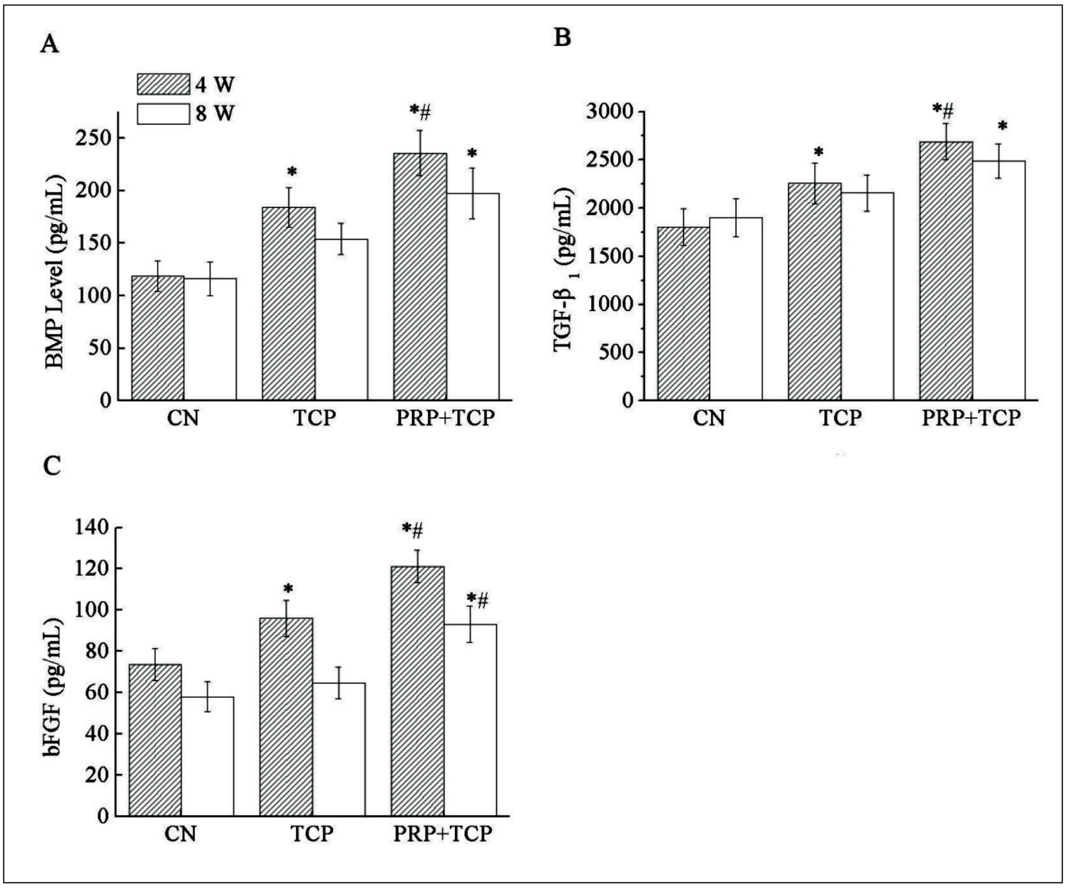

Figure 2. The concentrations of BMP-7, TGF-β1 and bFGF in serum of each group after injection of TCP or PRP/TCP; *compared with control group at the same time point $p<0.05$; #compared with TCP group at the same time point $p<0.05$.

wing screws can potentially separate the end of the fracture, so pains and secondary bone injury will be caused by the knocking. Later on, three-wing screws fixation was replaced by the multi-screw and multi-needle fixation as the latter one can maintain better blood supply in femoral head, so the multi-screw and multi-needle therapy has been widely applied in the clinical treatment of femoral neck fracture[26]. However, although the small needle used in this new generation therapy can significantly reduce the damage to articular capsule and blood circulation, it also can cause pain un-

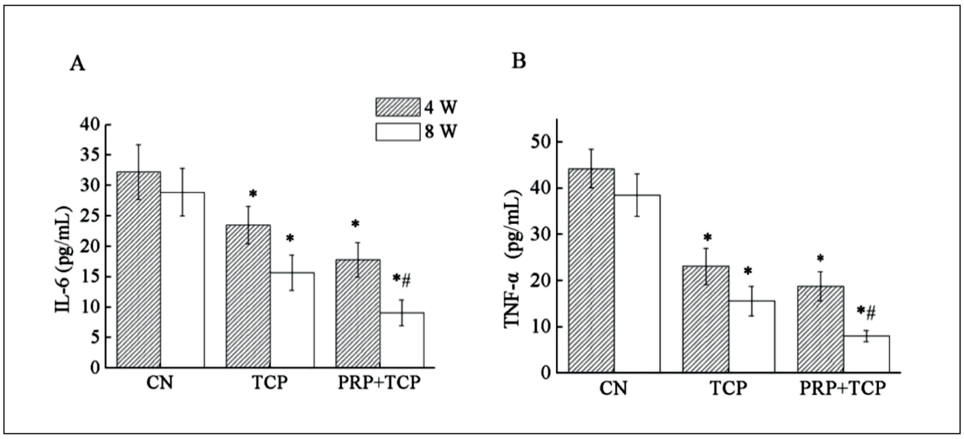

Figure 3. The concentrations of IL-6 and TNF-α in serum of each group after injection of TCP or PRP/TCP; *compared with control group at the same time point $p<0.05$; #compared with TCP group at the same time point $p<0.05$.

derneath the skin, which will significantly reduce the postoperative life quality of the patients. So, new treatments of femoral neck fracture are still needed. In recently years, a variety of biomaterials, which have been proved to be able to repair the damaged bone or even replace the damaged tissue without inducing significant adverse reaction, have been widely used in clinical practice[27]. The chemical and structural similarities shared by TCP and natural bone tissue confers TCP the ability to replace the inorganic part of the bone[28]. Hench et al[29] showed that hydroxyapatite (HA) and TCP can both induce the formation of new bone tissues, and the combination of HA and TCP has higher osteoconductive than TCP alone[30]. The TCP artificial bone implantation was also found to be able to stimulate the growth of cartilage tissue and neovascularization-like tissue[14,15]. Consistent with previous researches, our work also found TCP can significantly increase the new bone area (Figure 1). PRP, which contains multiple growth factors, has been widely used in clinic. Patel et al[31] reported that the efficacy of PRP is higher than that of placebo in the treatment of knee osteoarthritis. It was also found that the application of PRP can significantly enhance the bone regeneration[28]. In our paper, based on the results of histological observation, the application of PRP and TCP in the treatment of femoral neck fracture in rabbit model gave better outcomes than the application of TCP alone (Figure 1), indicating that PRP can accelerate the process of bone defect repair. TGF-βs, BMP-7, and bFGF play pivotal roles in the bone formation and bone homeostasis. TGF-βs and BMPs can act on the tetrameric receptor complex to trigger the signaling transduction of both Smad-dependent and -independent signaling pathways to regulate the formation of bone tissue[29]. As the basic fibroblast growth factor, bFGF was also found to be able to accelerate bone regeneration[30]. In the present research, TCP was found to be able to increase the concentrations of TGF-βs, BMP-7 and bFGF in serum of the rabbits with femoral neck fracture (Figure 2), indicating that TCP can accelerate the bone regeneration at molecular level. In addition, the application of TCP combined with PRP increased the concentrations of TGF-βs, BMP-7, and bFGF to higher levels compared with the application of TCP alone, suggesting that TCP combined with PRP were more effective in the treatment of femoral neck fracture than TCP alone. Inflammatory response is also a serious problem in the treatment of femoral neck fracture, and serious inflammatory response may lead to the failure of the surgery[31]. In our work, TCP alone and the combination of PRP and TCP can both significantly reduce the content of inflammatory factors including IL-6 and TNF-α in serum (Figure 3), but PRP combined with TCP can reduce the content of IL-6 and TNF-α to a lower level than TCP alone. All the data suggest that PRP combined with TCP can significantly reduce the inflammatory response after surgery, which will benefit the recovery.

Conclusions

PRP combined with TCP showed higher efficiency in repairing ANFH after femoral neck fracture than internal fixation alone or TCP. PRP combined with TCP provide a scaffold for growth of osteoblast, which in turn promotes osteoblast growth, and accelerates bone repair. PRP combined with TCP can also upregulate the expression of bone formation-related factors and downregulate the inflammation related factors. Our work provided a reference for clinical treatment of femoral neck fracture.

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Conflict of Interest

All authors declared they have no conflict of interest.

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