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Enhancement of tendon-bone healing after rotator cuff injuries using combined therapy with mesenchymal stem cells and platelet rich plasma

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Abstract. – OBJECTIVE: The injuries of rotator cuff will cause the shoulder dysfunctions. Due to limited self-regeneration abilities of the tendon-bone part, rotator cuff injuries remain a clinical challenge. Previous studies have proposed many strategies for treating this disease. In this work, we aimed to combine different strategies to achieve better beneficial effects on tendon-bone repair.

MATERIALS AND METHODS: We isolated mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP) and tested the effects of PRP on the gene expression, cell death resistance, and osteogenic differentiation of MSCs. Then, we utilized multiple strategies to treat rotator cuff injuries. We evaluated the expression of genes that related to tissue repair, bone formation, and tendon regeneration. We also tested the biomechanical property of repair tissues.

RESULTS: We found that the *in-vitro* co-culture with PRP endowed MSCs with enhanced production of growth factors, better osteogenic differentiation ability, and stronger ability to resist cell death. Next, we applied MSCs, PRP, and MSCs-PRP combined therapies in rat rotator cuff injury model to compare their therapeutic effects *in vivo*. Through biomechanical testing, we found that the combined therapy was most efficient to promote tissue regeneration and enhance the biomechanical property of the newly generated bone.

CONCLUSIONS: The combined treatment induced strongest signals related to angiogenesis, bone formation, and tendon generation in-situ. We demonstrated that the combination of MSCs and PRP synergistically promotes tendon-bone healing and holds great promise for the treatment of rotator cuff injuries.

Key Words

Mesenchymal stem cells, Platelet rich plasma, Rotator cuff injuries, Angiogenesis.

Introduction

The rotator cuff has critical functions in maintaining the normal properties of the shoulder, whose dysfunction are associated with dysfunctions and even disabilities in shoulder movements[1,2]. Nowadays, lots of strategies (such as stem cell therapies, growth factor therapies, and implantations of biological materials) have been proposed to hold the potential to treat rotator cuff injuries, and the targets of these therapies are mainly focused on tendon-bone healing process[2-4]. Previous studies[5-10] have revealed that many factors can suppress the recovery of tendon-bone tissues, including local inflammation, shortage of angiogenesis, dysregulation of collagen fiber among tendon-bone interface, limitation of physiological fibrocartilaginous transition restoration, bone injuries, and cartilage damages. Since the process of tendon-bone repair requires the synchronous regeneration of multiple different tissues including cartilage, fibrous cartilage, tendon and bone, the therapeutic effects of single treatment are often not stable[5,11-14]. To overcome such obstacles, it is necessary to develop combined therapies for tendon-bone healing, and these therapies will certainly improve the therapeutic effects on rotator cuff injuries. Moreover, several studies have attemped to apply combined therapies to treat the diseases and achieved a synergistic effect. The main purpose of our investigation was to compare the therapeutic effects of mesenchymal stem cells (MSCs), platelet-rich plasma (PRP) and combined therapy with both MSCs and PRP on treating tendon-bone injuries after rotator cuff injuries.

Mesenchymal stem cells are stem cells existing in almost all tissues[15,16]; they can undergo differentiation into neural cells, adipocytes, chondrocytes, endothelial cells, and vascular cells *in vivo* or *in vitro*[15-17]. Depending on their self-renewal property, pluripotency, and capability of growth factors secretion, these cells have been used to treat various kinds of tissue injuries and showed great potential to promote tissue regeneration[15,17]. Scholars[18-20] revealed that the application of MSCs from different tissues can enhance tendon-bone repair. In one

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case[21], ultrasound-guided injection of umbilical cord blood-derived MSCs are found to support the regeneration of the full-thickness rotator cuff tendon without surgical repair, and the newly regenerated tissues were predominantly composed of type I collagens. Yin et al[22] showed that, with the pretreatment of TGF-β and connective tissue growth factor, MSCs can maintain a condition with highly tenogenic differentiation; these differentiated MSCs are found to promote the tendon repair efficiently. Moreover, the therapeutic effects of MSCs in treating tendon-bone injuries can be enhanced by gene modifications or the treatment by other factors[23,24]. Compared to normal MSCs, those genetically modified with bFGF and BMP2 can stronger augment new bone formation with the better mechanical property, thus contributing to the tendon-bone repair after anterior cruciate ligament (ACL) reconstruction[23]. In another case, MSCs can express higher levels of genes related to tenogenic differentiation upon BMP12 pretreatment, such as Scx and Tnmd[25,26]. These BMP-12-treated MSCs are found to better improve the tendon-bone healing with increased cellular alignment/organization and cell elongation[25,26].

Platelet-rich plasma (PRP) is a kind of concentrated platelets in a small volume of plasma[27-29]. The potential therapeutic effects of PRP are largely dependent on the production and secretion of various growth factors by platelets, which could mobilize and recruit immune cells, endothelial cells and stem cells to regulate inflammation, angiogenesis, and matrix synthesis[2,30]. Considering the great demand for growth factors during tendon-bone repair, PRP is postulated to hold promise for the treatment of rotator cuff injuries[31,32]. However, the preclinical studies using PRP to treat tendon-bone injuries are inconsistent. Thus, further investigations can improve the effects of PRP-related strategies. Furthermore, whether PRP and their secretome can regulate the properties of MSCs is largely unknown, and the answer to this question will provide further information regarding the potential application of PRP in stem-cell-mediated therapies.

Here, our work showed that PRP could enhance the properties of MSCs including growth factors secretion, osteogenic differentiation ability, and cell death resistance. Furthermore, we compared the therapeutic effects of MSCs, PRP, and combined therapy with MSCs and PRP on treating tendon-bone injuries and found that the application of combined therapy with MSCs and PRP could promote the expression of beneficial genes and achieved better effects on promoting tendon-bone repair after rotator cuff injuries in the rat rotator cuff injury model.

Materials and Methods

Cell Isolation and Culture

The 2-month-old Sprague-Dawley (SD) rats were maintained in Zhejiang Chinese Medical University Laboratory Animal Research Center and euthanized for the isolation of the bone marrow cells from thighbones and shinbones by using 10 mL syringe containing low glucose Dulbecco's Modified Eagle's Medium (DMEM) medium [10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin] (Gibco, Rockville, MD, USA). Mesenchymal stem cells were then isolated through percoll (1.073 g/mL) mediated density gradient centrifugation at 2500 rpm/min for 20 minutes. The cells pellet was resuspended in low glucose DMEM medium and cultured in standard incubators (37°C, 5% CO_2). MSCs within 3-8 passages will be used for the experiments.

Platelet Rich Plasma Preparation and the Co-culture with MSCs

Autologous rat platelet rich plasma (PRP) was produced as previous reported. Briefly, whole blood was obtained through the marginal auricular vein by using an 18-gauge catheter. Then, the blood was kept in a sterile centrifuge tube that containing 1.5 mL of sodium citrate. The tube was centrifuged at 1500×g for 10 min to isolate the plasma from the red blood cells. The isolated plasma was then centrifuged at 2500×g at 4°C for 20 min to deplete the blood cells and cell debris further, and the precipitated platelets were collected.

The prepared PRP was added to treat MSCs, and after the 48 hours, a part of the treated MSCs were collected and lysed to extract the RNA for the further analysis of the gene expression of MSCs; the other part of MSCs were treated under starvation or H_2O_2, and then, apoptosis analysis through flow cytometry was performed.

Rotator Cuff Injury Model of Rat

The 2-month-old male Sprague-Dawley (SD) rats were maintained in Zhejiang Chinese Medical University Laboratory Animal Research Center. All experimental operations were approved by the Experimental Animals Committee. The experimental procedure included the detachment

and the supraspinatus tendon repair as previously reported. In brief, the deltoid was split and the supraspinatus was detached from its footprint. Then, a 0.5 mm incision was made in the bone from the anterior of the greater tuberosity to its posterior side. The supraspinatus tendon was then detached from its insertion in the greater tuberosity. The rats were randomly enrolled into different treated groups. The model group without insertion was used as the negative control group. Sutures of the tendon were then performed through the bone tunnels by Mason-Allen technique. Animals were sacrificed after 8 weeks, and the tissues were collected for the biomechanical testing.

The Analysis of the Gene Expression in MSCs and in Tissues

MSCs were collected after different treatments for RNA extraction. The specimens were collected after the euthanization of the animals. The samples were weighed and immediately stored in liquid nitrogen. The pestles were precold in order to grind the tissue while maintaining them in a low temperature. Then, TRIzol (Invitrogen, Carlsbad, CA, USA) was added to lyse the sample and extract the RNA for the RT-PCR experiments. The primers used for the RT-PCR were: β-actin, forward: 5'-TTCCAGCCTTCCTTCTTGGG-3', reverse: 5'-TGTTGGCATAGAGGTCTTTACGG-3'; Osterix forward: 5'-ATGGCGTCCTCTCTGCTTG -3', reverse: 5'-TGAAAGGTCAGCGTATGGCTT-3'; Runx2 forward: 5'-CCACGGCCCTCCCTGAACTCT-3', reverse: 5'-ACTGGCGGGGTGTAGGTAAAGGTG-3'; Osteocalcin forward: 5'-GCCCTGAGTCTGACAAAGGTA-3', reverse: 5'-GGTGATGGCCAAGACTAAGG-3'; VEGF forward: 5'-GTACCTCCACCATGCCAAGT-3', reverse: 5'-TCACATCTGCAAGTACGTTCG-3'; PDGF-A forward: 5'-CCCCTGCCCATTCGGAGGAAGAG-3', reverse: 5'-TTGGCCACCTTGACGCTGCGGTG-3'; EGF forward: 5'-TTCTCACAAGGAAAGAGCATCTC-3', reverse: 5'-GTCCTGTCCCGTTAAGGAAAAC-3'; FGF-2 forward: 5'-GCGACCCACACGTCAAACTA-3', reverse: 5'-TCCCTTGATAGACACAACTCCTC-3'.

Statistical Analysis

All experiments in our study were performed for at least three times. The results are shown as the mean ± SEM and analyzed by unpaired two-tailed Student's *t*-test. The differences were considered significant when $p<0.05$.

Results

Isolation of Mesenchymal Stem Cells and the Effects of Platelet Rich Plasma on These Cells

To investigate whether platelet-rich plasma (PRP) and mesenchymal stem cells (MSCs) could achieve synergistic effects in treating rotator cuff injuries, we first isolated MSCs from rat bone marrow. We observed that these cells could be cultured for at least 10 passages in incubators supplemented with 5% CO_2 at 37°C. The MSCs cultured *in vitro* at 3 passages or 5 passages were collected for the test of the morphological property, and we found that the morphology of MSCs at 3 passage or 5 passage were similar and both are fibrotic (Figure 1). Then, we isolated PRP and tested the effects of PRP on the gene expression of MSCs. We treated MSCs with PRP in a transwell system for 48 hours and then collected the cells. We first analyzed the effects of PRP on the anti-apoptotic abilities of MSCs. We found that both the hydrogen peroxide and the starvation treatment could efficiently induce the apoptosis of MSCs, and the PRP treatment largely promoted the anti-apoptotic ability of MSCs and decreased the percentage of apoptotic cells (Figure 2). These results indicated that PRP may enhance the survival of MSCs *in vivo*. Next, we tested the expression of several growth factors, including VEGF, PDGF, EGF, FGF-2, and BMP-2 after the extraction of mRNA form these samples. We found that MSCs indeed expressed these genes. Notably, PRP could significantly enhance the expression of these genes (Figure 3). Such observation suggested that, other than directly benefiting the tissue repair during rotator cuff injuries, PRP could promote tissue repair through enhancing the functionality of injected MSCs. Of note, we found that MSCs with PRP treatment showed a faster and more efficient osteogenic differentiation *in vitro* (Figure 4A). Gene expression analysis showed that PRP treatment promotes the mRNA expression of Osterix, Runx2 (runt-related transcription factor 2), and OCN (osteocalcin) in MSCs during osteogenic differentiation (Figure 4B).

Effects of Different Strategies on Gene Expression and Pathologies of Rotator Cuff Injuries

To compare the therapeutic effects of single treatments and combined strategies on rotator cuff injuries, we induced rat supraspinatus repair

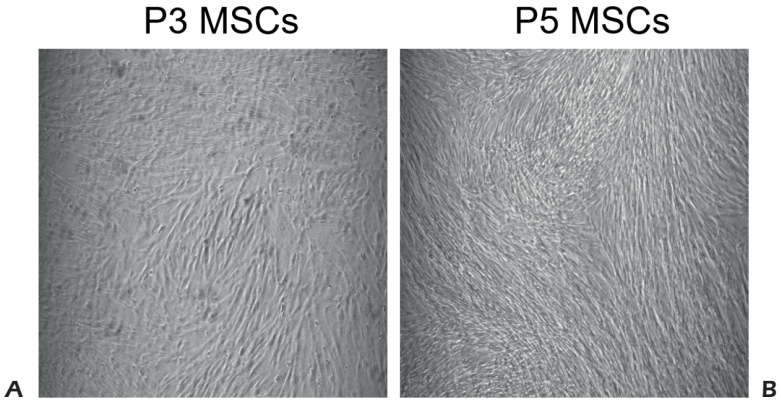

Figure 1. The morphologies of MSCs at passage 3 and passage 5 (Magnification: 10×).

model and then applied different treatments (PRP alone, MSCs alone, PRP+MSCs) just after surgery. The rats without any treatment were used as positive controls. We collected samples for gene expression detection and protein expression analysis at 4 weeks after the induction of rotator cuff injury model in rat. We measured the expression of genes that related to tendon-bone healing and found that, compared to positive control group and single treatment groups (PRP

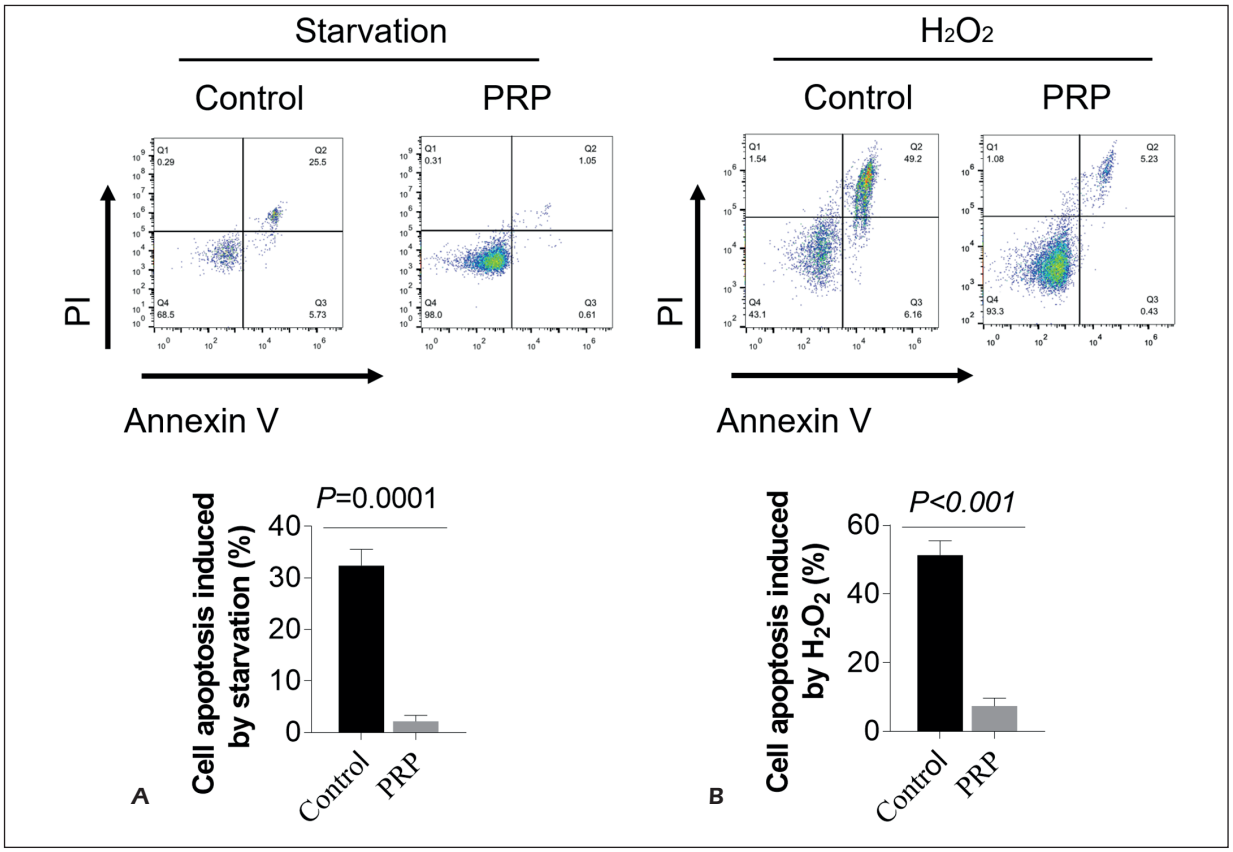

Figure 2. PRP enhanced cell death resistance of MSCs. MSCs were treated with or without PRP in a transwell system; then, the cells were collected for further experiments. **A**, Some of MSCs were then treated with starvation stress for 48 hours, and the apoptosis of MSCs are tested. **B**, Some of MSCs were treated with H_2O_2 for 1 hour, and the apoptosis of these cells were analyzed by flow cytometry.

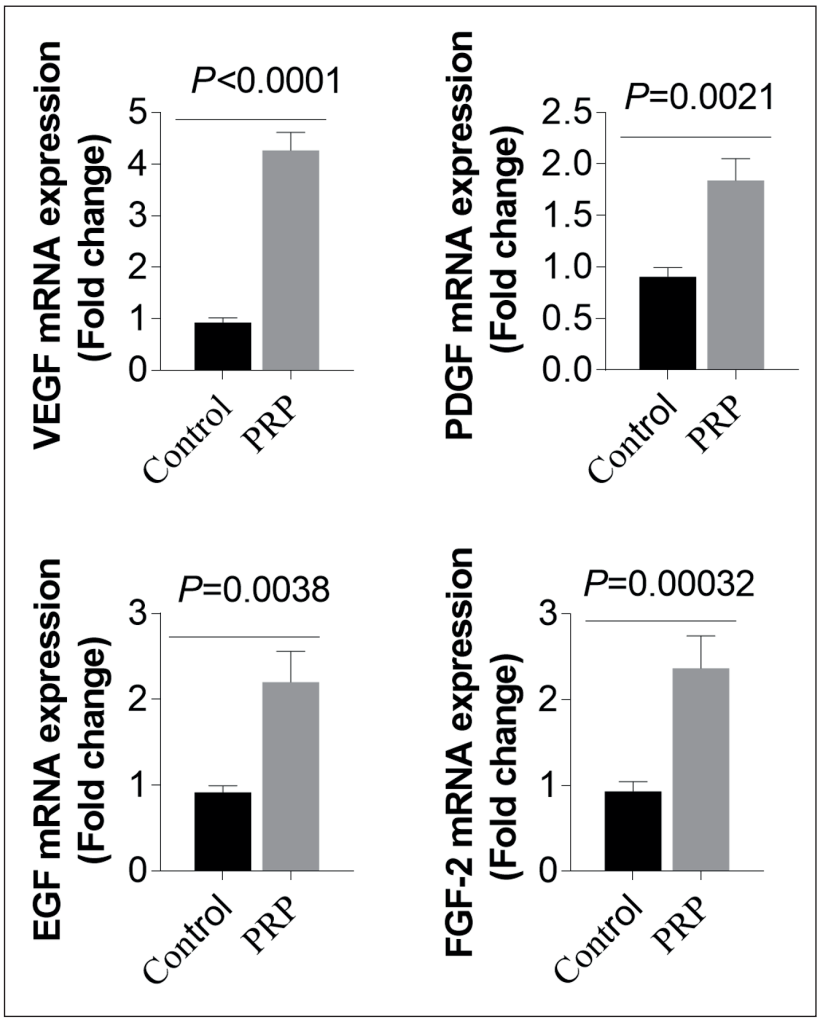

Figure 3. PRP enhanced the gene expression of MSCs. MSCs were treated with PRP in a transwell system for 48 hours, then the cells with or without PRP treatment were collected. These cells are used for the extract of mRNA. The mRNA expression levels of different genes (VEGF, PDGF, EGF, and FGF-2) were examined by RT-PCR.

alone or MSCs alone), combined therapies could enhance the expression of VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), EGF (epidermal growth factor), TGF-β (transforming growth factor-β), BMP-2 (bone morphogenetic protein 2), and BMP-7 (bone morphogenetic protein 7) (Figure 5). Since the functions of these genes were related to angiogenesis, bone formation, and tissue regeneration, their upregulation in damaged tissues should have beneficial effects on enhancing the recovery of rotator cuff injury. To test this suggestion, we next measured the expression of genes related to bone and tendon generation by RT-PCR and Western blot. We found that the levels of proteins that associated with tendon generation including Col I (collagen I), TNMD (tenomodulin), SCX (scleraxis), and P-ERK1/2 were significantly higher in combined therapy group (Figure 6A). Furthermore, we detected that combined therapy with PRP and MSCs significantly promoted the mRNA expression of genes related to osteogenic differentiation such as Osterix, Runx2, and OCN *in-situ,* compared to other groups (Figure 6B).

Combined Therapy with PRP and MSCs Promoted the Function of Newly Regenerated Bone

We also tested the functionality of the newly regenerated bone by a biomechanical test. We found that the maximum loads of the bones derived from combined therapy group were significantly higher than that of bones derived from single therapy groups (Figure 7A). Furthermore, the stiffness of bones was also higher in combined therapy group, compared to the positive control group and single treatment groups (Figure 7B). These data revealed that the application of PRP and MSCs at the same time could achieve a synergistic effect on promoting tendon-bone repair *in vivo.*

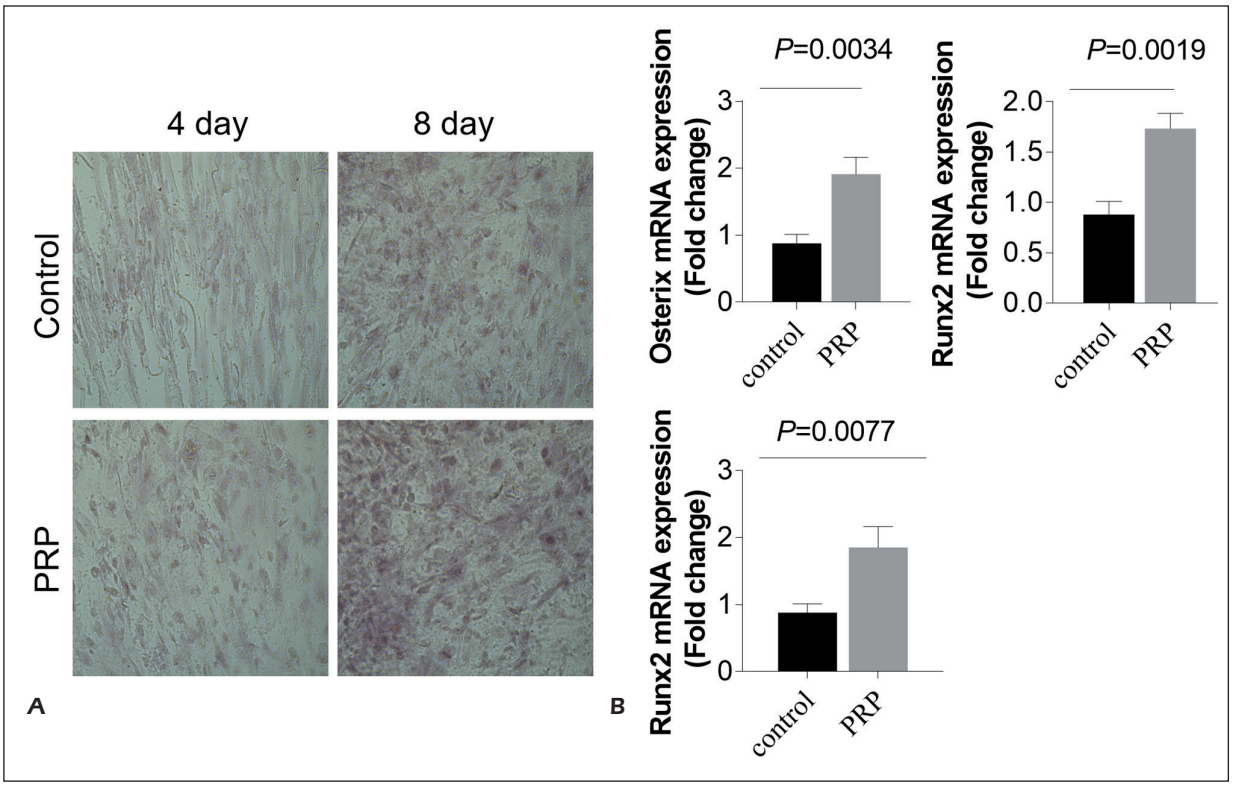

Figure 4. PRP promoted the ability of MSCs to undergo the osteogenic differentiation. MSCs with or without PRP treatment (48 hours) in the transwell system were collected and then cultured under osteogenic differentiation conditional medium. **A**, The efficiency of osteogenic differentiation in MSCs were determined by identifying the calcium deposition in these cells through Alizarin Red S staining at different times (Magnification: 40×). **B**, The gene expression of Osterix, Runx2, OCN, in MSCs were determined by RT-PCR after 10 days of osteogenic differentiation.

In summary, investigating the therapeutic effects of different strategies (PRP alone, MSCs alone, PRP+MSCs) on rotator cuff injuries, we found that the therapeutic effects of the combined therapy (PRP+MSCs) were much better in promoting tendon-bone healing in rats. These studies would provide more information in improving the efficiency of the clinical strategies in treating tendon-bone interface-related diseases.

Discussion

Tendon-bone interface is a critical structure to maintain the normal physiological function of joints and shoulders[11,33,34]. The rotator cuff tears can induce the dysfunction and disabilities of the shoulder, which remains a big clinic challenge. The efficiency of tendon-bone healing determines the outcome of rotator cuff recovery. Previous studies[2,11,12,31,32,35] have shown that strategies mediated by stem cells, growth factors or other biological materials can promote the recovery of tendon-bone injuries in rotator cuff. However, whether the combination of these therapies could achieve a synergistic effect is still largely unknown. Furthermore, which combination can reach a better efficacy is not determined. The purpose of the research is to compare the effects of MSCs, PRP, and a combined therapy with MSCs and PRP on promoting supraspinatus repair after rotator cuff injuries, thus confirm the potential of the combined therapy in promoting tendon-bone healing.

Investigations[2,36] about the function and structure of tendon-bone interface region have revealed that such interface is very complex and composed of multiple parts including tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone. The effective repair of each part requires many kinds of factors and can show interactive influences on the regeneration of other parts, thus determining the outcomes of rotator cuff[2]. To further improve the recovery of tendon-bone damage caused by surgical treatment

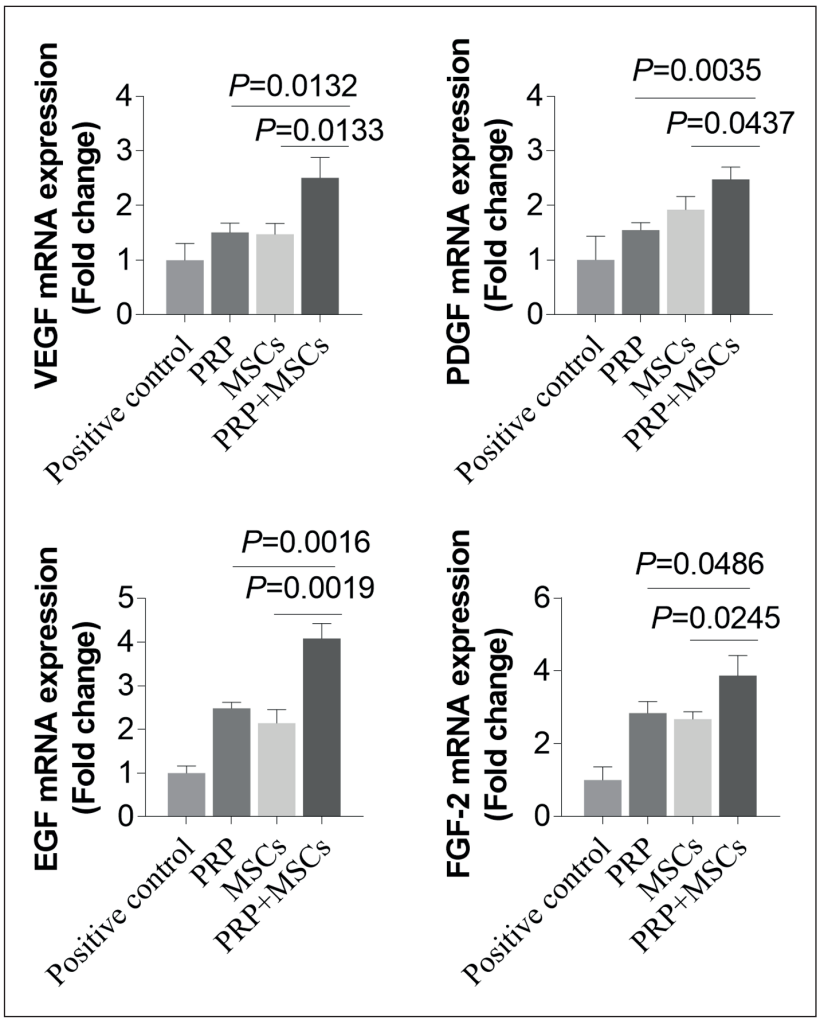

Figure 5. Combined therapy promoted the expression of growth factors in injured tissues. The tissue samples were collected at 4 weeks after surgery and treatment. Then, tissues were grinded with liquid nitrogen, lysed with TRIzol, and total RNA were extracted for the gene expression analysis of VEGF, PDGF, EGF, and FGF-2.

or graft transplantation, the combination of different therapies targeting both tendon and bone/cartilage is needed. Mesenchymal stem cells have been proved to produce a high amount of growth factors and immunoregulatory molecules; thus, these cells have been applied to treat tissue damage and immune disorders[15,17]. These cells are also found to be effective in enhancing the repair and regeneration of tendon and bone[18]. However, the therapeutic effect of MSCs is not stable, and the combination of MSCs with other therapies should achieve better outcomes. On the other hand, platelet-rich plasma (PRP) contains various growth factors which could promote angiogenesis and tissue regeneration, thus holding great potential to treat tendon-bone injuries[28,29]. The combination of these two therapies is supposed to hold potential to better promote the tissue recovery after rotator cuff injuries. In our studies, we first found that PRP can regulate the gene expression and resistance to cell death. Next, we applied different treatments in rat model and perform gene analysis, as well as biomechanical test, to compare the therapeutic effects of these strategies. Based on our investigations, we demonstrated that the combination of PRP and MSCs can better improve the tendon-bone repair and enhance the biomechanical property of the newly regenerated tissue than monotherapies.

Conclusions

There are lots of strategies which are proposed to enhance tendon-bone healing. Due to complexity of tendon-bone injuries, the combined therapy should be specially modified for specific situations. Our researches revealed that PRP could influence the gene expression, apoptosis-resistance, and osteogenic differentiation of MSCs.

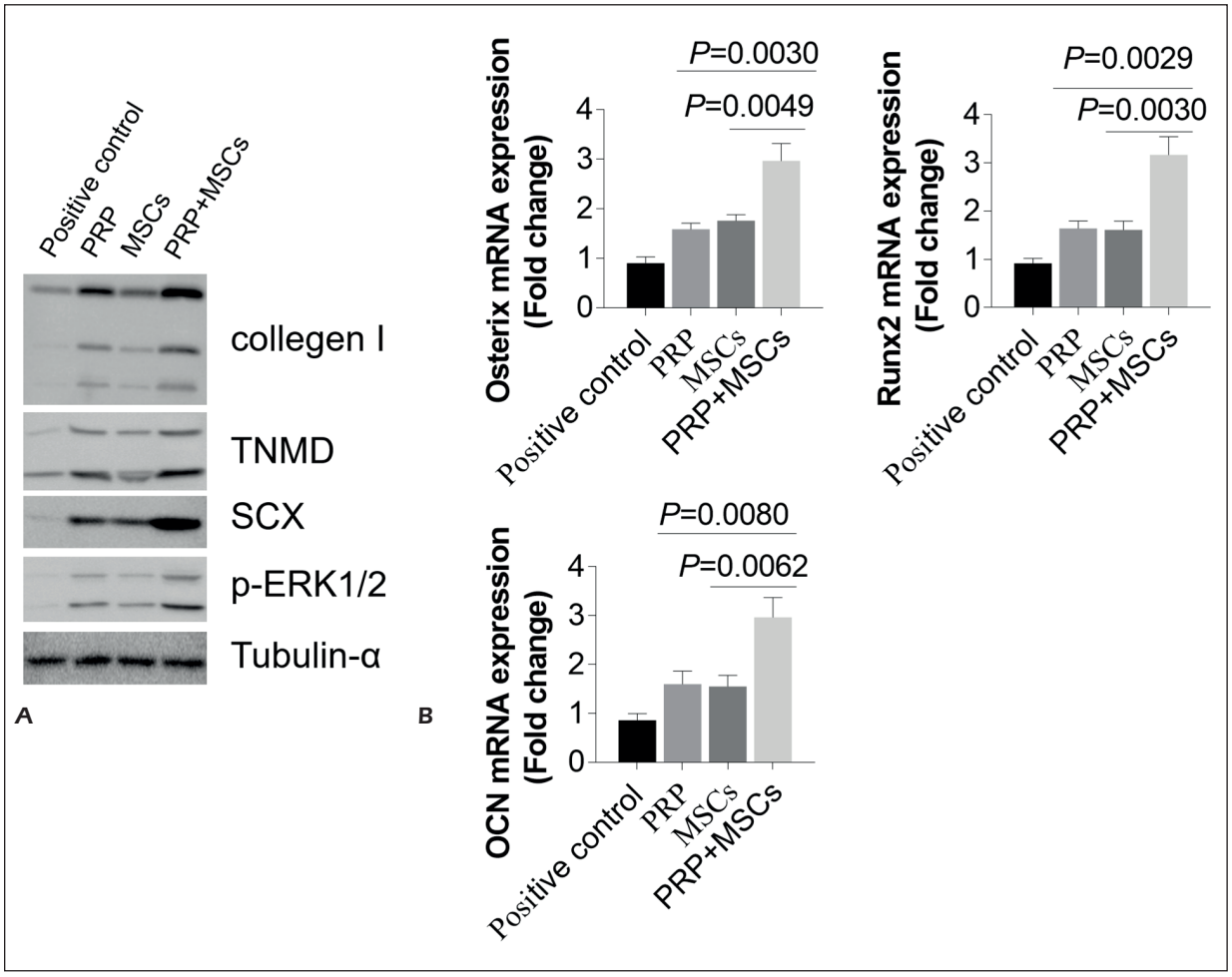

Figure 6. PRP and MSCs cooperated to enhance the tissue regeneration signals. The animals were euthanize to collect the tissues at 4 weeks after the model induction and treatment. Firstly, tissues were grinded with liquid nitrogen. **A**, A part of samples were lysed with RIPA lysis buffer and the protein was collected to determine the protein levels of collagen I, TNMD, SCX, and p-ERK1/2 through Western blot. **B**, Other samples were treated with TRIzol to extract total RNA, and mRNA expression of Osterix, Runx2, and OCN was measured through RT-PCR test.

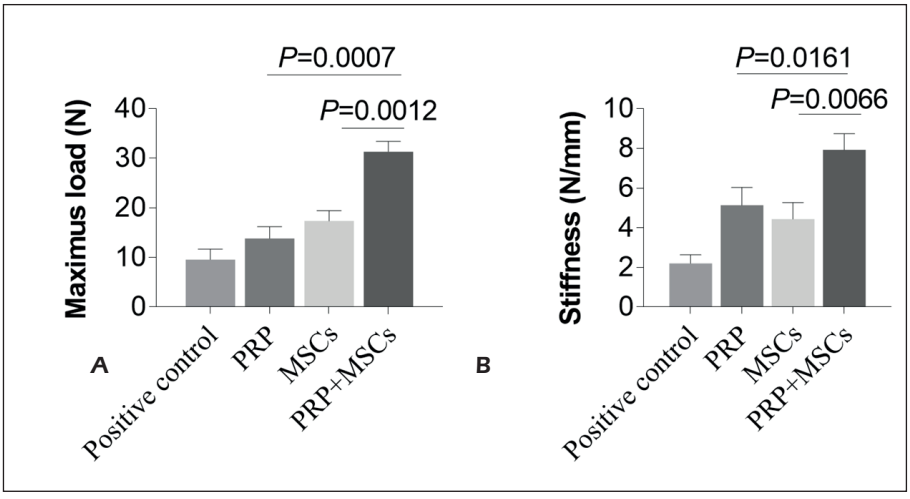

Figure 7. Mechanical test of tendon-bone repair. 4 weeks after treatment, the tissue samples were collected for the mechanical test. **A**, The maximum load of the collected samples was determined. **B**, Samples collected at 4 weeks were used to evaluate the stiffness.

Moreover, the combination of PRP and MSCs could achieve synergistic effects on tendon-bone healing in a rat supraspinatus repair model. Such information will help to improve the efficiency of rotator cuff tear treatment and hold great promise for the clinical application.

Acknowledgments

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Conflict of Interests

The authors declare that they have no conflict of interest.

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