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Sustained BMP-2 release and platelet rich fibrin synergistically promote tendon-bone healing after anterior cruciate ligament reconstruction in rat

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Abstract. – OBJECTIVE: Anterior cruciate ligament (ACL) injuries which cause knee disabilities remain a clinical challenge due to the compromised tendon-bone repair. Multiple strategies have been proposed to treat the tendon-bone injuries, and the combination of these therapies hold great potential to achieve synergistic effects.

MATERIALS AND METHODS: We built PLGA-BMP-2 (bone morphogenetic protein 2) system and confirmed the sustained release of BMP-2 both *in vitro* and *in vivo*. We then applied different therapies to treat rat ACL reconstruction. We collected the tissue sample and analyzed the BMP-2 concentration both *in serum* and in injured sites. We tested the mRNA expression of genes that were related to inflammation, tissue repair and bone formation in damaged tissues. We also analyzed the protein levels of some genes associated with tendon formation and check the function of newly generated bone through biomechanical test.

RESULTS: We found that, compared to monotherapies, simultaneous utilization of sustained BMP-2 release and platelet rich-fibrin (PRF) after anterior cruciate ligament reconstruction showed better therapeutic effects on tendon-bone healing in rat. This combined therapy efficiently enhanced the levels of growth factors that favor the angiogenesis and relieved the inflammatory responses in the injured sites. Of note, the combined therapy efficiently promoted the signals associated with bone formation and tendon regeneration.

CONCLUSIONS: We demonstrated that the combined therapy with BMP-2 and PRF achieves synergistic effects on tendon-bone healing and holds great potential for the treatment of ACL reconstruction.

Key Words

Anterior cruciate ligament, BMP-2, Platelet rich fibrin, Reconstruction, Rats.

Introduction

The anterior cruciate ligament (ACL) plays important roles in maintaining the normal structure and function of knee, while ACL injuries can influence physiological property of the joint and lead to knee disabilities[1,2]. ACL reconstruction surgery using soft-tissue autografts has been the golden standard to treat the disease[3-5]; however, the outcomes of the surgery are still largely dependent on the efficiency of tendon-bone healing between the graft and the host bone[6-9]. Multiple factors such as the lack of physiological fibro cartilaginous transition restoration, bone and cartilage damage, a low level of angiogenesis and the loss of collagen fiber across the tendon-bone interface hinder the efficiency of tendon-bone healing[3-5,8,9]. Therefore, to improve the therapeutic effects of ACL reconstruction surgery, it is very important to improve the efficiency of current therapies. Considering the repair of tendon-bone interface involved the regeneration of different tissues including tendon, bone, cartilage and fibrous cartilage, several studies have demonstrated that the combined therapies may provide better therapeutic effects on tendon-bone healing[8]. In a rabbit model, the combination of stem cells and platelet-rich plasma has been found to achieve a synergistic effect on tendon-bone healing[8]. In another study, compare to MSCs genetically modified with basic fibroblast growth factor (bFGF) or bone morphogenetic protein 2 (BMP-2) alone, MSCs genetically modified with both bFGF and BMP-2 can better enhance the tissue healing process after ACL reconstruction[5]. These reseaches indicated that the combination of different therapies hold great potential to improve the tendon-bone repair. Our aim was to evaluate the beneficial effect of combined therapy with BMP-2 and platelet rich fibrin. BMP-2 plays important roles in regulating the development of bone, cartilage and angiogenesis[10-12]. Upon bone damage, BMP-2 can promote bone formation through upregulating the recruitment and differentiation of bone progenitor cells[10,12,13]. After ACL reconstruction, a high concentration of BMP-2 was observed near the bone, which lasted for at least 12 weeks[5,14]. Furthermore, additional BMP-2 administration was found to pro-

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mote tendon-bone healing[2,15-17]. However, the biological instability of BMP-2 limits the application of this molecule[2,5,15,16]. To overcome this obstacle, a sustained release system by poly(lactic-co-glycolic acid) (PLGA) or gene modified stem cells has been developed to provide long acting BMP-2 release at the injured sites. Platelet rich fibrin (PRF) is a fibrin biomaterial that contains leukocytes, platelets and multiple growth factors. Due to the specific composition and matrix architectures, PRF hold potentials to treat wound healing and promote tissue repair[18-20]. Previous studies[18-21] have demonstrated that the utilization of PRF can enhance bone repair, angiogenesis and tendon healing. Although BMP-2 and PRF can separately promote the integration of tendon-bone interface, whether the combination of these two therapies can achieve a better effect on tendon-bone repair is still uncertain and further investigation is merited. The purpose of this investigation was to demonstrate the efficiency of combined therapy with sustained released BMP-2 and PRF in promoting tendon-bone healing. We hypothesized that the simultaneous application of BMP-2 and PRF could achieve a synergistic effect on tissue repair after ACL reconstruction in rat.

Materials and Methods

Preparation of Platelet Rich Fibrin

For PRF preparation, 10 mL blood were collected from the precaval vein and stored in tubes without anticoagulants. Samples were then immediately centrifuged at 3000 rpm for 10 minutes. The samples were let to stand for 3 min. The supernatant and the red blood cell at tube bottom were discarded; the rest part was collected as PRF clots.

Anterior Cruciate Ligament (ACL) Reconstruction Rat Model

This study was approved by the Animal Ethics Committee of Xiaoshan Traditional Chinese Hospital Animal Center. The 2-month-old Sprague-Dawley (SD) rats were maintained in Zhejiang Chinese Medical University Laboratory Animal Research Center. The ACL reconstruction rat model was established as previously reported. Briefly, the animals were anesthetized by the intravenous injection of 5% pentobarbital (30 mg/kg) and aseptically prepared for surgery. The animals were then fixed in the supine position to ensure that the knees were able to flex freely to 90°. The hind legs were shaved to expose the knee joint by a lateral parapatellar arthrotomy. Anterior cruciate ligament was then excised from its femoral and tibial insertions. A 2-mm-diameter drill was used to create tunnels between femur and tibia through the original ACL footprints. The grafts were then sutured to the femoral side periosteum by using 2-0 Ethibond (Ethicon, Shanghai, China). The graft was then implanted into the bone tunnels and sutured to the tibial side with slight tensioning of the graft. The wound should be sutured in layers. After the surgery, the animals were returned to the cage. The animals were injected with penicillin (50 mg/kg) intramuscularly for 7 days to avoid further infection.

Preparation of BMP-2 Contained PLGA

Poly (lactic-co-glycolic acid) (PLGA, Boehringer Inglheim, Germany) was purchased and the microparticles were fabricated as previously described by using water-in-oil-in water (W1-O-W2) double-emulsion-solvent-extraction method. Accordingly, different dose of BMP-2 was added to a solution of PLGA and PLGA-PEG-PLGA (90%:10% respectively) in dichloromethane. The mixture was emulsified to form the water in oil emulsion. This emulsion was further mixed with an aqueous solution of polyvinyl alcohol (PVA) (0.3%) and then homogenized for 2 min at 2,000 rpm. The double emulsion was stirred magnetically at 300 rpm for at least 4 hours and then filtered, washed and lyophilized to generate microparticles. The resulted microparticles were stored at -20°C for further investigation. We first confirmed the encapsulation efficiency of BMP-2 in PLGA. 20 mg microparticles were reconstituted in phosphate-buffered saline (PBS) (pH 7.4), then the samples were vortexed at 100 rpm for 36 hours under 37°C. After the centrifugation, the supernatant was collected, filtrated and stored at -20°C for further analysis. Encapsulation efficiency is calculated as: (BMP-2 in the particles)/(total BMP-2)*100%. We next tested the release rate of BMP-2 in PLGA. 10 mg BMP-2-containing PLGA were reconstituted in 1 ml phosphate-buffered saline (PBS); after the 30 minutes of centrifugation at 800 rpm, the supernatant was collected and stored at -20°C for further analysis. The PLGA microparticles were reconstituted with 1 ml fresh phosphate-buffered saline (PBS) in a fresh tube and stored under 4°C for future experiment. The PLGA-PRF complexes were obtained through mixing BMP-2-containing PLGA and PRF in a vacuum environment, and PRF will be absorbed into the pore of the PLGA.

The Analysis of the Gene Expression in Tissue

After the euthanization of animals, the tissue samples were collected. The samples were stored in liquid nitrogen immediately after weighing. Pre-cold pestles were used to grind the tissue sample in the presence of the liquid nitrogen. Next, the samples were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA) to extract RNA. The reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed to measure the gene expression. The primers used were listed here: β-actin, forward 5'-TTCCAGCCTTCCTTCTTGGG-3', reverse 5'- TGTTGGCATAGAGGTCTTTACGG-3'; Osterix forward, 5'-ATGGCGTCCTCTCTGCTTG-3', reverse 5'-TGAAAGGTCAGCGTATGGCTT -3'; Runx2 forward 5'-ATGCTTCATTCGCCTCACAAA-3', reverse 5'-GCACTCACTGACTCGGTTGG-3'; Osteocalcin forward 5'-GCCCTGAGTCTGACAAAGGTA-3', reverse 5'- GGTGATGGCCAAGACTAAGG-3'; VEGF forward 5'-GCACATAGAGAGAATGAGCTTCC-3', reverse 5'- CTCCGCTCTGAACAAGGCT-3'; TGF-β forward 5'- CTCCCGTGGCTTCTAGTGC-3', reverse 5'-GCCTTAGTTTGGACAGGATCTG-3'. TNF-α forward 5'-CCCTCACACTCAGATCATCTTCT-3', reverse 5'-GCTACGACGTGGGCTACAG-3'; IL-1β forward 5'-GCAACTGTTCCTGAACTCAACT-3', reverse 5'-ATCTTTTGGGGTCCGTCAACT-3'; BMP-7 forward 5'-ACGGACAGGGCTTCTCCTAC-3', reverse 5'- ATGGTGGTATCGAGGGTGGAA-3'; OPN forward 5'-AGCAAGAAACTCTTCCAAGCAA-3', reverse 5'-GTGAGATTCGTCAGATTCATCCG-3'; col1α forward 5'-GCTCCTCTTAGGGGCCACT-3', reverse 5'-CCACGTCTCACCATTGGG -3'.

Statistical Analysis

All experiments in our study were performed for at least three times. The results were 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

The Construction of PLGA-BMP-2 System for Sustained BMP-2 Release

To investigate whether platelet rich fibrin (PRF) and sustainedly released BMP-2 could synergistically alleviate tendon-bone damage that caused by anterior cruciate ligament (ACL) reconstruction, we first constructed BMP-2-containing poly(lactic-co-glycolic acid) (PLGA) (PLGA-BMP-2) by which BMP-2 could maintain the sustained release for a long term. Then, we tested the entrapment efficiency (EE) of BMP-2 in the PLGA and the dynamic of BMP-2 release in PLGA-BMP-2 system *in vitro*. We found that the entrapment efficiency was very high (the EE was counted as loading efficiency/ theoretical loading efficiency* 100%) in different BMP-2 concentration conditions (Figure 1A).

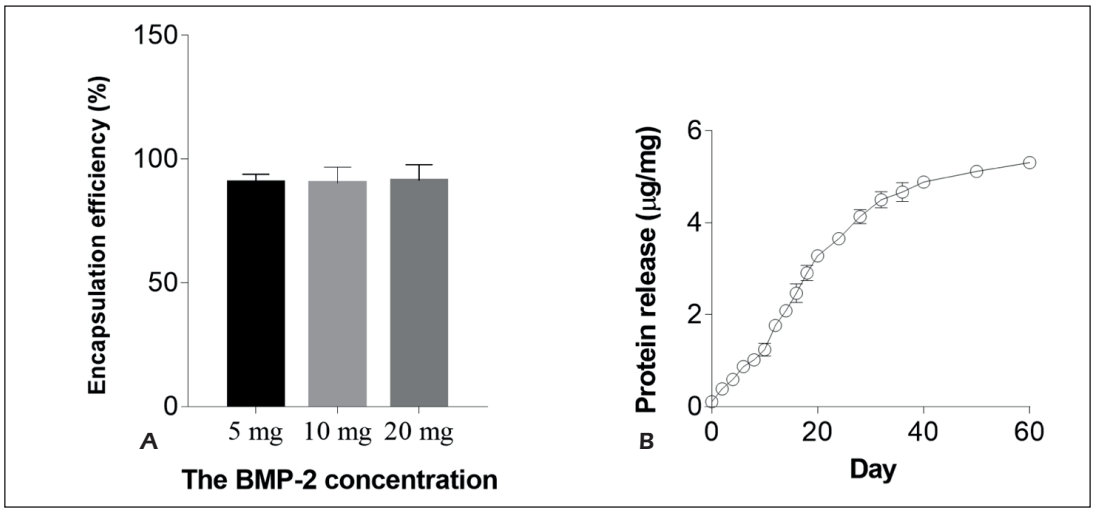

Figure 1. The property of BMP-2-contained PLGA microparticles. **A**, The entrapment efficiency of BMP-2 in PLGA system was calculated as: loading efficiency/ theoretical loading efficiency* 100%. **B**, the sustainedly released BMP-2 was collected from the supernatant and the concentration of BMP-2 was determined by ELISA.

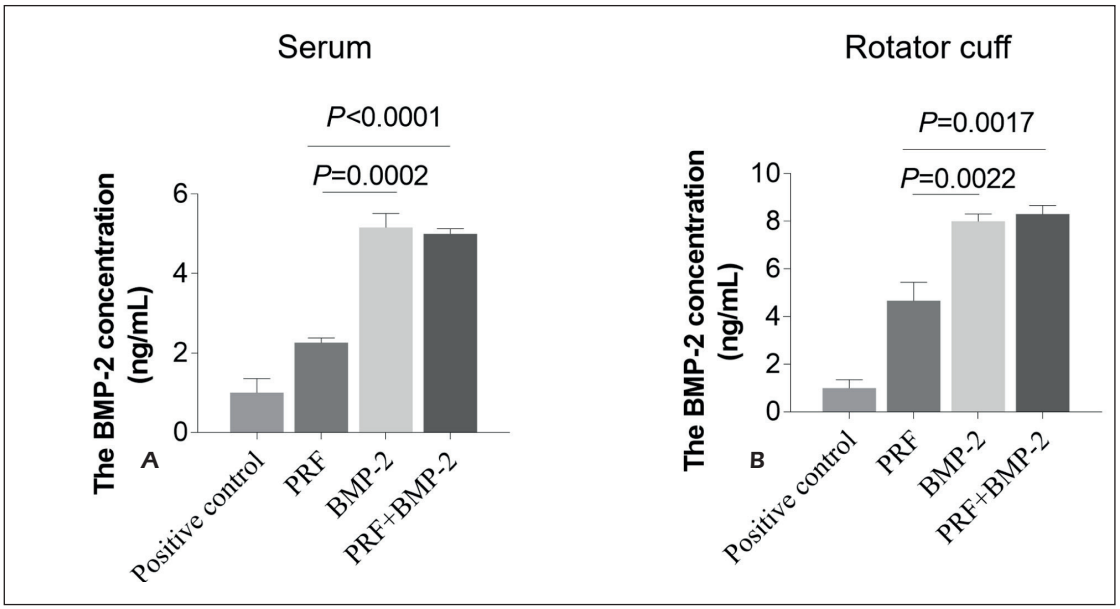

Figure 2. BMP-2 in PLGA system can be released for a long time. About 4 weeks after model induction and treatment, the animals were euthanized. The serum and the injured tissues were then collected to test the BMP-2 concentration. **A**, The BMP-2 concentration in serum was determined directly by ELISA. **B**, The tissue was grinded in the presence of liquid nitrogen and the molecule was resuspended in PBS for BMP-2 test.

We also found that the PLGA system maintained sustained BMP-2 release for about 40 days (Figure 1B). These data revealed that the PLGA-BMP-2 system was efficient and had the potential to provide BMP-2 in certain disease microenvironments.

PLGA-BMP-2 System Maintain Sustained BMP-2 Release in Rat Suffering ACL Reconstruction

To test our hypothesis *in vivo*, we built the ACL reconstruction model in rat and treated these animals with different therapies (PRF alone, PLGA-BMP-2 alone, PRF plus PLGA-BMP-2), the animals without treatment were used as positive control. At the 4th week after the surgery and treatment, we euthanized the animals and collected the tissue samples (blood and damaged tissues) for further analysis. We first tested the BMP-2 concentration both *in serum* and in damaged tissues. The data showed that the rats treated with PLGA-BMP-2 and PRF+PLGA-BMP-2 had much higher BMP-2 levels in their serum and damage sites than positive control group and PRF group (Figure 2), suggesting that PLGA-BMP-2 system effectively delivered BMP-2 in these animals for at least four weeks.

The Therapeutic Effects of Different Therapies on ACL Reconstruction

The alleviation of inflammation is needed during the regeneration of damaged tissues. To verify the effects of different therapies on the immune status during ACL reconstruction, we tested the mRNA expression of inflammatory cytokines TNF-α and IL-1β in damaged tissues. We found that the expression of these two important inflammation-related genes decreased significantly in combined therapy group (PRF plus PLGA-BMP-2), while the single therapies (PRF alone, PLGA-BMP-2 alone) did not show such effects (Figure 3A). This result indicated that the combined therapy with PRF and PLGA-BMP-2, other than single therapies, could effectively promote the alleviation of inflammation in damaged tissues. Besides inflammation, the endogenous production of growth factors supporting the regeneration of tendon, bone and other tissues played critical roles in efficient ACL reconstruction. Thus, we also tested the expression of genes that were related to the angiogenesis and tendon-bone healing such as VEGF and TGF-β. The upregulation of these genes is supposed to be related to efficient tendon-bone repair through enhancing the regeneration of tendon, bone and blood vessels. We found that the single therapies

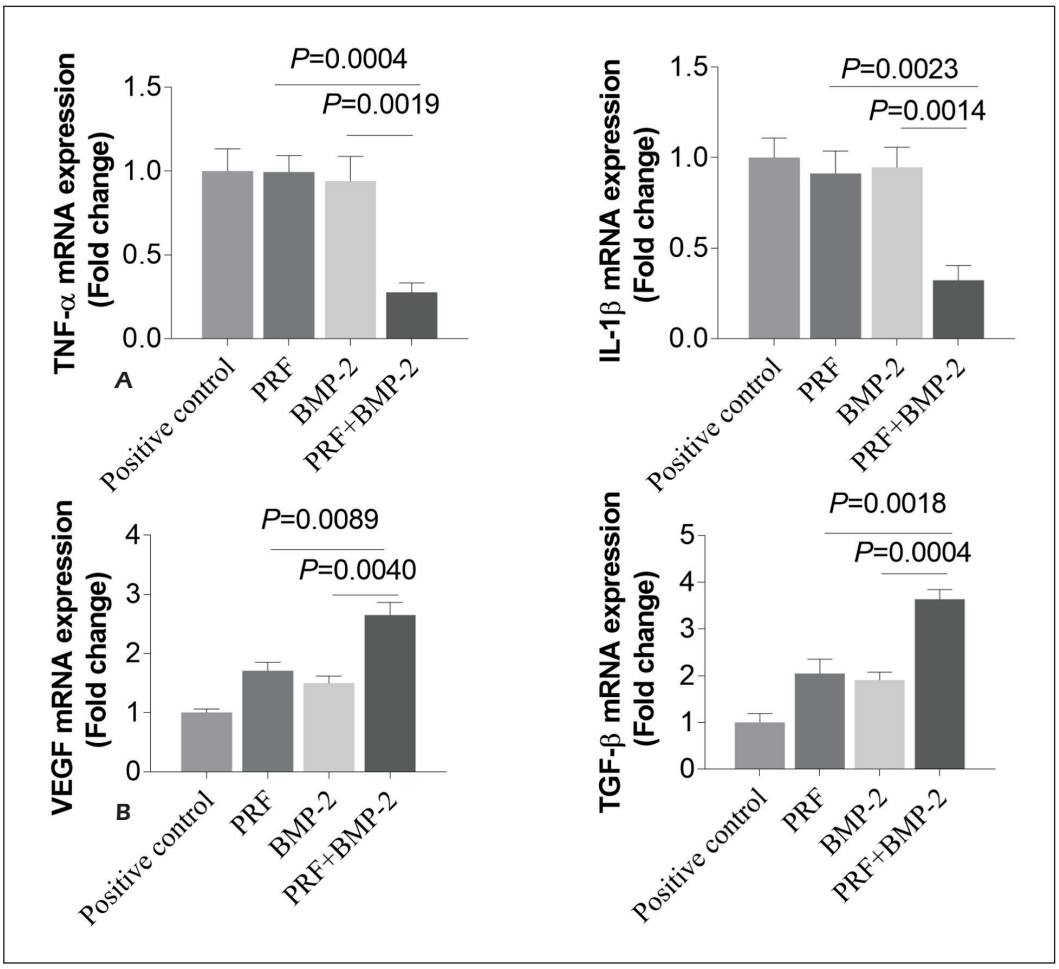

Figure 3. Combined therapy alleviates inflammation and promotes growth factor expression during ACL reconstruction. The animals were euthanized at 4 weeks after the model induction and treatment. The tissue was grinded in the presence of liquid nitrogen, TRIzol was added to lyse the samples. After extracting the RNA in the TRIzol, the RT-PCR assays were performed to measure the mRNA expression of (**A**) TNF-α and IL-1β; (**B**) VEGF and TGF-β.

could promote the expression of these genes, while combined therapy was more powerful to enhance the expression of these genes (Figure 3B). Collectively, the analysis of gene expression mentioned above demonstrated that PRF and PLGA-BMP-2 could work synergistically to alleviate the local inflammation and promote the tissue repair -related signals, thus holding potential to better enhance the tendon-bone healing after ACL reconstruction.

Combined Therapy with PRF and PLGA-BMP-2 Promoted the Signals Related to Bone Formation and Tendon Regeneration

Next, we tested the status of bone formation and tendon regeneration by analyzing the gene expression *in vivo*. We found that compared to control group and monotreatment groups, combined therapy significantly enhanced the mRNA expression of Osterix, Runx2, OCN (osteocalcin), OPN (osteopontin) and Col1α (collagen 1α) (Figure 4A), all of which were supposed to benefit bone formation. On the other hand, the tendon generation-related protein such as Col I (collagen I), Col II (collagen II), Col III (collagen III), TNMD (tenomodulin), SCX (scleraxis), Shc and P-ERK1/2 were significantly enhanced by combined therapy other than single therapies (Figure 4B). These data collectively revealed that PRF and PLGA-BMP-2 could work together to induce stronger signals for bone and tendon regeneration *in vivo*. Furthermore, we analyzed the maximal loads and stiffness of the newly generated bone by using biomechanical testing at 4 weeks and 8 weeks after treatment, and found that the bone from rat that was treated with

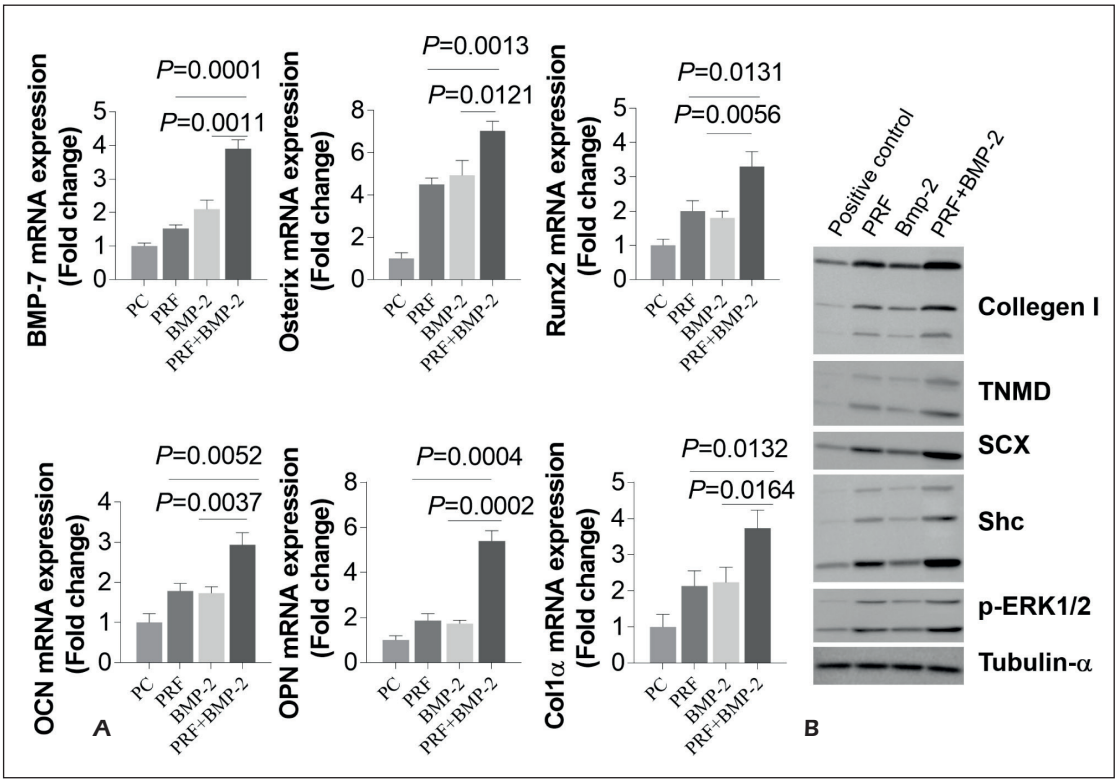

Figure 4. Combined therapy enhances signals related to bone formation and tendon generation. The tissue samples were collected after euthanizing animals. The tissues were grinded in the presence of liquid nitrogen. mRNA was extracted by TRIzol and proteins were extracted by RIPA lysis buffer. **A**, The mRNA expression of BMP-7, Osterix, Runx2, OCN, OPN, and Col1α were measured by RT-PCR analysis. **B**, The protein levels of collagen I, TNMD, SCX, Shc and p-ERK1/2 were tested by Western blot.

combined therapy showed much higher maximal loads (Figure 5A). The stiffness of the bone was also determined and the data showed that the bone from combined therapy treated group have significantly higher stiffness than those from single treatment groups (Figure 5B). These data revealed that compared to single therapies, the combined therapy with PRF and PLGA-BMP-2 could better enhance the tendon-bone repair *in vivo*. In summary, we confirmed that PRF and PLGA-BMP-2 could work synergistically to promote tendon-bone repair in rat ACL reconstruction model, and these studies will provide important information to improve the related clinical strategies.

Discussion

The normal biological structure of tendon-bone interface guarantees the physiological functions of anterior cruciate ligament (ACL)[1,2]. In ACL reconstruction, the efficiency of tendon-bone healing determines the outcomes of the surgical treatment[6-9]. Up to now, different treatment therapies have been proposed. Various factors like transforming growth factor beta, basic fibroblast growth factor, connective tissue growth factor and bone morphogenetic proteins are proved to play important roles in tendon repair or bone formation[22,23]. Besides, new innovative drugs such as stem cells and platelet concentrates are also potential choices for tendon-bone repair[23-25]. However, the modification and improvement of these strategies requires further investigations. In this study, we tested the effects of the combined therapies of BMP-2 and PRF on ACL reconstruction model in rat and found the combination of these two treatments can better promote tendon-bone healing process. As we all know, tendon-bone interface region was composed of four distinct parts: tendon, uncalcified fibrocartilage, calcified fibrocartilage and bone[8,23]. The recovery of all parts is interactive, influencing the outcomes of ACL reconstruction. However, the repair of each of these four zones relies on different factors and mechanisms. The poor supply of required growth

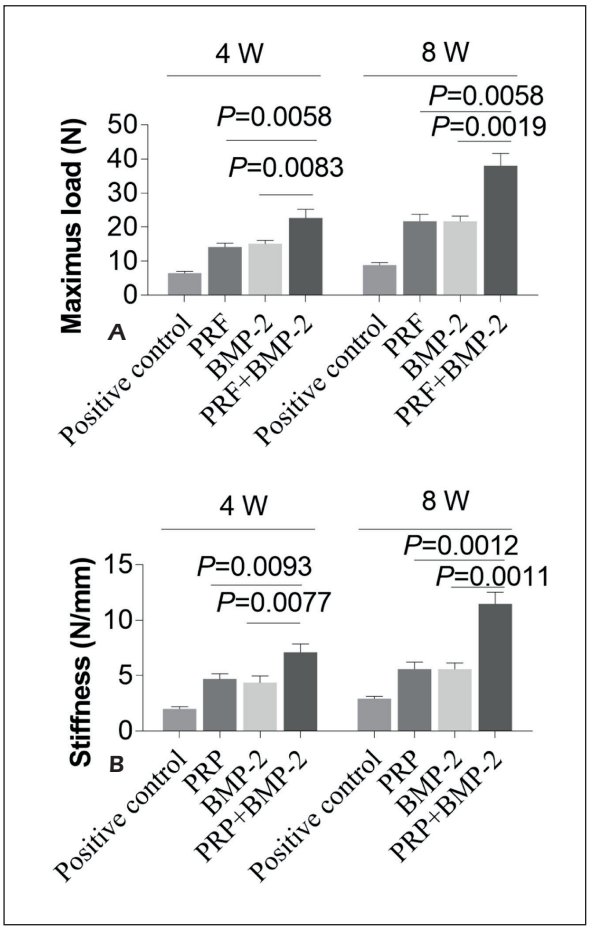

Figure 5. Mechanical examination of tendon-bone healing in the rat model at 4 and 8 weeks after model induction and treatment. The tissue samples were separately collected at 4 and 8 weeks after surgery and treatment. **A**, Samples were collected at 4 and 8 weeks and applied for maximum load analysis. **B**, The stiffness of samples from different groups at 4 and 8 weeks was determined. PC: positive control.

factor limits the regeneration of both tendon and bone. To further improve the efficiency of the tendon-bone healing after surgical treatment and graft transplantation, combine different therapies to target tendon regeneration, bone/cartilage formation and angiogenesis is needed. BMP-2 is a powerful molecule in enhancing angiogenesis and promoting the bone/cartilage formation[10-12], and the administration of BMP-2 in animals suffering from joint injury has proved it to be an effective strategy. However, BMP-2 showed little beneficial effect on tendon repair[26, 27]. On the other hand, PRF composes of multiple growth factors and holds great potential for the tendon tissue regeneration[18-20]. Our data showed that the combination of these two therapies alleviated the local inflammation and enhanced the biomechanical property of the newly regenerated bone. These results indicate that the repair of tendon, bone and blood vascular is interdependent and the strategies targeting the repair of different tissues have great possibilities to improve tendon-bone healing after ACL reconstruction. As discussed previously, various factors and therapies have been found to be salutary to the tendon-bone repair; the combination of other growth factors and therapies may also have potentials to achieve synergistic effect on ACL recovery. The comparison of different combined therapies will help to establish optimal combination and provide more information for the understanding of tendon-bone pathophysiology. Furthermore, besides the treatment targeting tissue repair, the strategies aiming to modulate the immune response and activate the differentiation of local stem cells should also be considered in future clinical application.

Conclusions

We established ACL reconstruction model in rat and compared the beneficial effects of different therapies. By performing gene expression analysis, Western blot and biomechanical testing, it has been demonstrated that the combination of BMP-2 and PRF achieved synergistic effects on promoting tendon-bone healing process. These results will provide important information and potential strategies for the treatment of ACL injuries.

Acknowledgments

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Conflict of Interests

The authors declare no conflict of interests.

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