Combined Therapy of Platelet-Rich Plasma and Basic Fibroblast Growth Factor Using Gelatin-Hydrogel Sheet for Rotator Cuff Healing in Rat Models

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Research Article

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Combined therapy of platelet-rich plasma and basic fibroblast growth factor using gelatin-hydrogel sheet for rotator cuff healing in rat models

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Abstract

Introduction: Excellent outcomes of arthroscopic rotator cuff repair for small and medium tears have been recently reported. However, re-tears after surgery have been a common complication after surgical repair of large and massive rotator cuff tears and often occur in early postoperative phase. It was previously reported that basic fibroblast growth factor and platelet-rich plasma enhanced rotator cuff tear healing. We hypothesized that this combined therapy could enhance rotator cuff healing after rotator cuff repair in a rat model. This study aimed to evaluate the efficacy of combined therapy of platelet-rich plasma and basic fibroblast growth factor with gelatin-hydrogel sheet.

Methods: To create a rotator cuff defect, the infraspinatus tendon of Sprague Dawley rat was resected from the greater tuberosity. The infraspinatus tendons were repaired and covered with gelatin-hydrogel sheet impregnated with PBS (control group), basic fibroblast growth factor (bFGF group), platelet-rich plasma (PRP group), or both basic fibroblast growth factor and platelet-rich plasma (combined group). Histological examinations were conducted using hematoxylin and eosin, safranin O, and immunofluorescence staining such as Isolectin B4, type II collagen. For mechanical analysis, ultimate failure load of the tendon-humeral head complex was evaluated at 6 weeks postoperatively.

Results: In the hematoxylin and eosin staining, the tendon maturing score of the

combined group was higher than that of the control group at postoperative 2 weeks. In the safranin O staining, stronger proteoglycan staining was observed in the combined group compared with the other groups at postoperative 2 weeks. Vascular staining with isolectin B4 in 3 treatment groups was significantly higher than that in the control group. Type II collagen expression in the combined group was significantly higher than those in the other groups. The ultimate failure load of the combined group was significantly higher than that of the control group.

Conclusion: Combined therapy of basic fibroblast growth factor and platelet-rich plasma promoted angiogenesis; tendon maturing and fibrocartilage regeneration at the enthesis, which could be enhance the mechanical strength. It was suggested that basic fibroblast growth factor and platelet-rich plasma enhance both tendon and bone-tendon junction healing, and basic fibroblast growth factor and platelet-rich plasma were synergistic.

Key words： rotator cuff tear, basic fibroblast growth factor (bFGF), platelet-rich plasma (PRP), rotator cuff repair, rat model, infraspinatus tendon

Introduction: Rotator cuff tear is common in the elderly population and the outcome of surgical treatment using arthroscopic rotator cuff repair for small cuff tear is satisfactory. (1) (2) In contrast, surgical treatment of large and massive cuff tear is still challenging because of re-tear after surgery. Rotator cuff re-tear is a common complication after surgery that usually occurs in early postoperative phase. (3) (4) The re-tear ratio is reportedly 14%~66% and re-tear can lead to loss of shoulder motion and shoulder function, and recurrence of pain. (4) (5) (6) (7) (8) To prevent re-tear after surgery, enhancement of biological healing and enforcement of mechanical strength at the repaired site could be required. Footprint augmentation with fascia lata graft is clinically reported for large and massive rotator cuff tears. (9) Using fascia lata graft, collagen expression enhancement was observed at the repaired site. Compared with single-row repair, better mechanical property in fascia lata grafting is also reported. (10) However, harvesting fascia lata can cause donor site morbidity such as pain, hematoma formation, and scar formation. The use of tissue engineering technique is an attractive tool to solve this problem. There are several growth factors that could enhance the tendon healing process. (11) Basic fibroblast growth factor (bFGF) expression is reported to be upregulated at healing site 1 week after supraspinatus repair. (12) (13) It was reported that Platelet-rich plasma (PRP) also enhanced the healing process after rotator cuff repair in a mouse model. (14) These investigations were performed by a single administration of growth factors

without any carriers. Considering the drug-delivery system, carrier of these growth factors could be important for better effect of drug. Gelatin is a biodegradable polymer extensively used for medical purposes and its biosafety and biocompatibility have been demonstrated through long clinical application and a number of tissue engineering studies.(15-19) Gelatin-hydrogel combined with growth factor successfully enhanced the tissue regeneration of fracture, ligament injury, and fibrocartilage. (20) (21) We speculated that controlled release of bFGF and PRP might enhance the regeneration of repaired site, as growth factors can affect the surrounding tissue in longer period. In this study, gelatin-hydrogel sheet (GHS) with/without bFGF and PRP is transplanted to the rotator cuff repaired site in a rat rotator cuff injury model. We hypothesized that this combined therapy could enhance rotator cuff healing after rotator cuff repair in a rat model. This study aimed to evaluate the efficacy of combined therapy of platelet-rich plasma and basic fibroblast growth factor with gelatin-hydrogel sheet.

Materials and Methods

All animal experiments were approved by the committee of our institute.

PRP preparation: PRP was prepared by double-spin method. (22) Sprague Dawley (SD) rats were anesthetized with isoflurane (Wako, Tokyo, Japan) and intraperitoneal injection of pentobarbital sodium (50 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan). Briefly, rat bloods (10 ml) were collected into tubes containing acid-citrate-dextrose solution and centrifuged for 7 min at 450 g and 4°C. Next, the yellow plasma with buffy coat was centrifuged for 5 min at 1600 g and 4°C. The platelet pellet was collected and the thrombolytic pellet in 1 ml of plasma was used as PRP. The density of platelets in the *PRP:* PRP prepared was increased by a factor of 5 when compared with that of the original blood (1.5-2.0*10^8/ml plasma). To activate PRP for the release of growth factors, the PRP preparation was mixed with CaCl2 solution at concentrations of 2 wt.% at a ratio of 7:1 by volume and then left for 1 h at 37°C.

bFGF: An aqueous solution of human recombinant bFGF (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) was diluted with physiological saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to give a solution concentration of 500 μg/ml according to the previous report. (13)

GHS preparation: Pig skin gelatin with a molecular weight of 100,000 Da and an isoelectric point of 5.0 was supplied by Nitta Gelatin Co., Ltd. (Osaka, Japan). After

preparing with 5 wt% aqueous solution of gelatin-hydrogel, the solution was cast into polystyrene dish as thinly as possible and frozen at -80°C in deep freezer. After the solution was frozen, freeze drying was performed for 48h. Freeze-drying gelatin-hydrogel was cut into 2 mm × 2 mm segments. The segments were cross-linked by dehydrothermal treatment at 140 °C for 48 h in a vacuum oven. (23)

A solution of 5 ul of PRP or bFGF was dropped onto a GHS for impregnation. Similarly, empty gelatin hydrogels without bFGF were prepared by adding PBS to the solution. Finally, 4 kinds of GHS were created according to experimental groups as PBS (control), PRP, bFGF, and combined groups (PRP and bFGF).

GHS transplantation: In this study, 40 SD rats (12-week-old) with a mean weight of 250 g (CLEA Japan, Inc., Tokyo, Japan) were used. All operations were performed under sterile conditions and anesthesia with isoflurane (Wako), intraperitoneal injection of pentobarbital sodium (50 mg/kg; Kyoritsu Seiyaku), and subcutaneous injection of lidocaine (2.5 mg/kg, Xylocaine®; AstraZeneca, London, UK) at the surgical site. The animals were placed in a lateral position, and a 1 cm incision was made over the lateral border of the acromion. A small portion of the deltoid muscle was divided to expose the underlying acromion and the infraspinatus tendon. The infraspinatus tendon was carefully identified (Figure 1A) and cut off at the insertion to the greater tuberosity (Figure 1B). The footprint was abraded to remove normal enthesis. In the right shoulders, tendons were

repaired by a transosseous technique using 4-0 nylon suture, and the repaired site was covered with GHS (Figure 1C, D). Four kinds of GHS were transplanted in each group (n=10 each group). The deltoid and skin were closed with 4-0 nylon. After transplantation, all rats were immediately allowed to move freely within their own cage in laminar flow rack. Rats were euthanized with overdose of isoflurane and intraperitoneal injection of pentobarbital sodium at the indicated times.

Histological Examination

For the histological examination, 6 rats from each group were sacrificed at 2 weeks after transplantation. The scapular-humeral complexes were harvested and quickly embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) and stored at −80 °C for histochemical and immunohistochemical staining as described below. Tissue sections were stained with hematoxylin and eosin (H&E) and safranin O for histological characterization of tissue composition, and the histological findings were evaluated at 2 points: the tendon proper and tendon insertion using light microscopy. Watkins et al. reported the tendon maturing scoring system to quantitatively evaluate the regenerated tendon. (24) Six histological parameters, such as cellularity, fibrocytes, vascularity, fiber diameter, parallel cells, and parallel fibers, were evaluated to identify the characteristics of the maturity of cellular and intercellular constituents. In the safranin O staining, proteoglycan content was calculated as a percentage of the pixels of each

tendon–bone interface (positive/total pixels) using Adobe Photoshop CC 2015 software (Adobe Systems Incorporated, San Jose, USA). In immunofluorescence staining, isolectin B4 antibody (Vector Laboratories, Burlingame, CA), a rat-specific endothelial marker, was used to access the regenerated capillaries and neovascularity. Type II collagen (Col2) antibody (Cosmo Bio Co., Ltd., Tokyo, Japan) was used to assess fibrocartilage regeneration. Antibodies were used at a 1:100 dilution, and staining was performed at room temperature for 1 h. DAPI solution was applied for 5 min for nuclear staining. After staining, we evaluated the number of positively stained cells in 5 randomly selected fields.

Mechanical analysis: For mechanical analysis, ultimate failure load of the tendon-humeral head complex was evaluated at 6 weeks postoperatively.

Six weeks after surgery, 4 rats from each group were euthanized, and shoulders were biomechanically tested. All soft tissues except the infraspinatus tendon-humeral complex were carefully removed before the biomechanical tests. The prepared infraspinatus-humeral complex was mounted in a conventional tensile tester (model AGIS 5kN; Shimadzu, Kyoto, Japan). The humerus was embedded in an aluminum tube using polymethylmethacrylate. Testing was performed with the shoulder at 60° of abduction in a testing machine. The humerus was clamped with its long axis in the horizontal plane. The proximal end of the infraspinatus tendon was glued between 2 pieces of sandpaper.

The sandpaper-tendon complex was clamped vertically. The biomechanical testing protocol that we used was similar to that described by Galatz et al. (25) and Mikolyzk et al. (26) Specimens were subjected to a preload of 0.2 N and were preconditioned for 5 cycles to 0.38 mm of displacement (approximately 5% of gage length at a rate of 0.1 mm/s). A stress relaxation test was then performed for 300 s at 0.38 mm of displacement followed by 300 s of recovery. Specimens were then tested to failure in tension at a rate of 0.1 mm/s. The ultimate failure load was determined for each specimen.

Statistical analysis: All data are expressed as mean values ± standard deviations. One-way ANOVA analysis followed by Tukey-Kramer analysis was performed for comparison of 4 groups. $P < 0.05$ was considered statistically significant. SPSS (version 23.0; IBM Corporation, Armonk, NY) were used for data analysis.

Results

Histological analysis: In H&E staining, tendon maturing scores in control, bFGF, PRP, and combined groups were 67±0.47, 8.67±0.47, 8.67±1.25, and 11.3±1.25, respectively (Figure 2). The score in the combined group was significantly higher than that in the control group at postoperative 2 weeks ($p < 0.05$). The quantitative analysis with safranin O staining at the tendon–bone junction showed scores of 7.2±0.69 %, 9.3±1.9 %, 16.2±2.9 %, and 25.7±2.2 % in control, bFGF, PRP, and combined groups, respectively (Figure 3). The significantly stronger proteoglycan staining was observed at the repaired enthesis in the combined group compared with the other groups at postoperative 2 weeks ($p < 0.05$). The percentages of positive cells in vascular staining with isolectin B4 in control, bFGF, PRP, and combined groups were 1.4±0.38 %, 3.4±0.41 %, 3.0±0.22 %, and 3.7±0.43 %, respectively (Figure 4). The 3 groups with the growth factors showed significantly higher vascular expression than in the control group ($p < 0.05$). Finally, Col2 expression in control, bFGF, PRP, and combined groups were 0.34±0.12 %, 0.95±0.53 %, 4.7±2.0 %, and 14.6±1.9 %, respectively (Figure 5). The combined group showed significantly higher expression than those in the other groups ($p < 0.05$).

Mechanical analysis: The ultimate failure load in control, bFGF, PRP, and combined groups were 10.5±2.8 N, 15.0±1.4 N, 15.5±2.6 N, and 21.0±5.5 N, respectively (Figure 6). The combined group showed significantly higher failure load than that of the other

201 groups ($p < 0.05$).

202

Discussion

There were many laboratory experiments that used growth factors for tendon healing. Local administration of bFGF enhanced tendon–bone interface healing. (12) Tokunaga et al. reported that the use of bFGF stimulates the proliferation of tenogenic progenitor cells leading to higher expression of tenogenic markers in a rat model (13)PRP contains various growth factors such as platelet-derived growth factor-B, transforming growth factorβ-1, vascular endothelial growth factor, and epithelial growth factor. (27) After PRP activation, the platelets in PRP release various growth factors through the degranulation of α-granules. Then the growth factors exhibit various biological activities. It was reported that PRP administration into the articular cartilage defect enhanced cartilage regeneration. (28) The positive effect of PRP after rotator cuff repair in a rat model has also been reported. (29) In spite of good results with growth factors in laboratory study, clinical situation might differ because dose and healing duration is different in animal experiments. The drug-delivery system of growth factors is considered as a key to improve tissue regeneration. Once the growth factor in solution form is injected, biological activity is not always expected because the growth factor activity is unstable due to rapid enzymatic digestion or deactivation. Intra-articular injection of the growth factor solution can be easily diluted after shoulder joint surgery. Therefore, drug-delivery system of growth factors is essential to efficiently improve biological functions. The use of GHS has been

222 successful in the controlled release of various growth factors and PRP. (30) Impregnation
223 of GHS with growth factors and the subsequent adsorption using electrostatic force allow
224 for the preservation of biological activity. Therefore, growth factors with preserved
225 biological activity can be released in a sustained manner until degradation of GHS occurs.
226 This GHS was degraded for 2 weeks, and sustained release of the encapsulated growth
227 factors occurred. (14) Kabuto et al. compared intra-articular injection of Bone
228 Morphogenetic Protein-7 (BMP-7) and GHS impregnated with BMP-7 in a rat rotator
229 cuff repair model. (31) Slow release of BMP-7 was observed in GHS group up to 3 weeks
230 after surgery yielding better tissue regeneration. In this study, we used bFGF and PRP as
231 growth factors for rotator cuff regeneration. Among the various kinds of growth factors,
232 we chose these 2 factors as these are approved growth factors for clinical use in our
233 country. Administration of single factor with GHS showed better histological outcome
234 compared with the control group. Moreover, use of both factors showed the best
235 histological and mechanical property. In a study of mouse ischemic limb, dual release of
236 PRP and bFGF impregnated in the biodegradable gelatin-hydrogel granules promoted not
237 only angiogenesis but also maturation of blood vessels. (22)In this study, combined bFGF
238 and PRP therapy promoted angiogenesis at the tendon–bone interface, which might lead
239 to better tendon and fibrocartilage regeneration compared with the control.
240 This study has several limitations. First, this rat model was an acute rotator cuff injury

model. The animal models may differ from chronic human rotator cuff injury. Second, the anatomy between the rat shoulder and that of humans are different, and the short rotator cuff muscles of rat do not form a rotator cuff that is similar to humans. Third, rats have greater healing capacity than humans; hence the tendon–bone healing process progressed faster than that in humans. Finally, the dose of growth factors was determined according to previous report and we did not optimize its dose as growth factor composition in PRP might differ from patient to patient.

Conclusion

Combined bFGF and PRP therapy promoted angiogenesis, tendon maturing and fibrocartilage regeneration at the enthesis and the mechanical strength. PRP and bFGF enhance both tendon and bone-tendon junction healing, and b-FGF and PRP were synergistic. The combined therapy of PRP and bFGF using GHS could synergistically work and enhanced rotator cuff healing after rotator cuff repair.

Abbreviations

PBS: Phosphate buffered saline; PRP: platelet-rich plasma; bFGF: basic fibroblast growth factor; GHS: gelatin-hydrogel sheet; BMP-7: Bone Morphogenetic Protein-7

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Availability of data and materials

Not applicable. All data are presented in the manuscript.

Authors' contributions

Set up research was done by TK. Animal surgery was done by TK, TK, KY and SM. Data analysis (Histology, Mechanial analysis) was done by TK, YM and AI. Statistical Analysis was done by TK, HN and TM. Manuscript preparation was done by TK, YM, AI and TN. Supervisor was done by YT and RK.

Ethics approval and consent to participate

The research protocol was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (Permission number 170701.)

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1

A: Infraspinatus tendon was identified. B: Infraspinatus tendon was cut off at the insertion to the greater tuberosity. C: Gelatin-hydrogel sheet (GHS). D: Infraspinatus tendon was repaired by a transosseous technique, and the repaired site was covered with GHS. (E) Scheme of ISP (infraspinatus tendon) repair with GHS. Yellow square is transplanted GHS. (H, humeral head)

Figure 2

H&E staining at 2 weeks postoperatively, A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: Tendon maturing score. The tendon maturing score of the combined group was higher than the control group.

Figure 3

Safranin O staining at 2 weeks postoperatively, A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group. E: Proteoglycan content was calculated as a percentage of the pixels of each tendon–bone interface (positive/total pixels).

Significantly stronger proteoglycan staining was observed at the repaired enthesis in combined group compared with other groups.

Figure 4

Immunofluorescence staining: isolectin B4 at 2 weeks postoperatively (A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: The number of positively stained cells in 5 randomly selected fields. Vascular staining with isolectin B4 in treatment groups was significantly higher than that in the control group.

Figure 5

Immunofluorescence staining: type II collagen at 2 weeks postoperatively (A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: The number of positively stained cells in 5 randomly selected fields. Type II collagen in the combined group was significantly higher than that in other groups.

Figure 6

The ultimate failure load of the combined group was significantly higher than that of the control group at 6 weeks after surgery.

Figures

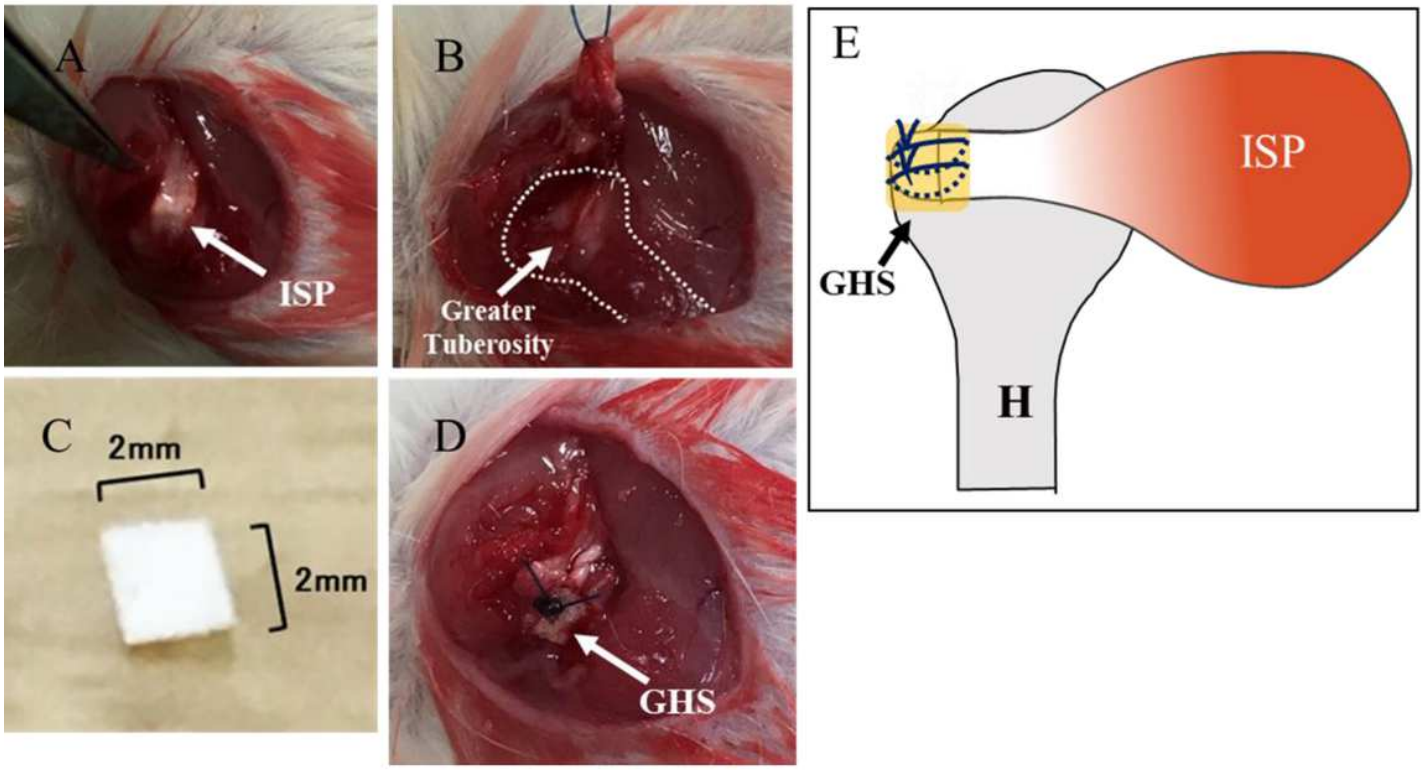

Figure 1

A: Infraspinatus tendon was identified. B: Infraspinatus tendon was cut off at the insertion to the greater tuberosity. C: Gelatin-hydrogel sheet (GHS). D: Infraspinatus tendon was repaired by a transosseous technique, and the repaired site was covered with GHS. (E) Scheme of ISP (infraspinatus tendon) repair with GHS. Yellow square is transplanted GHS. (H, humeral head)

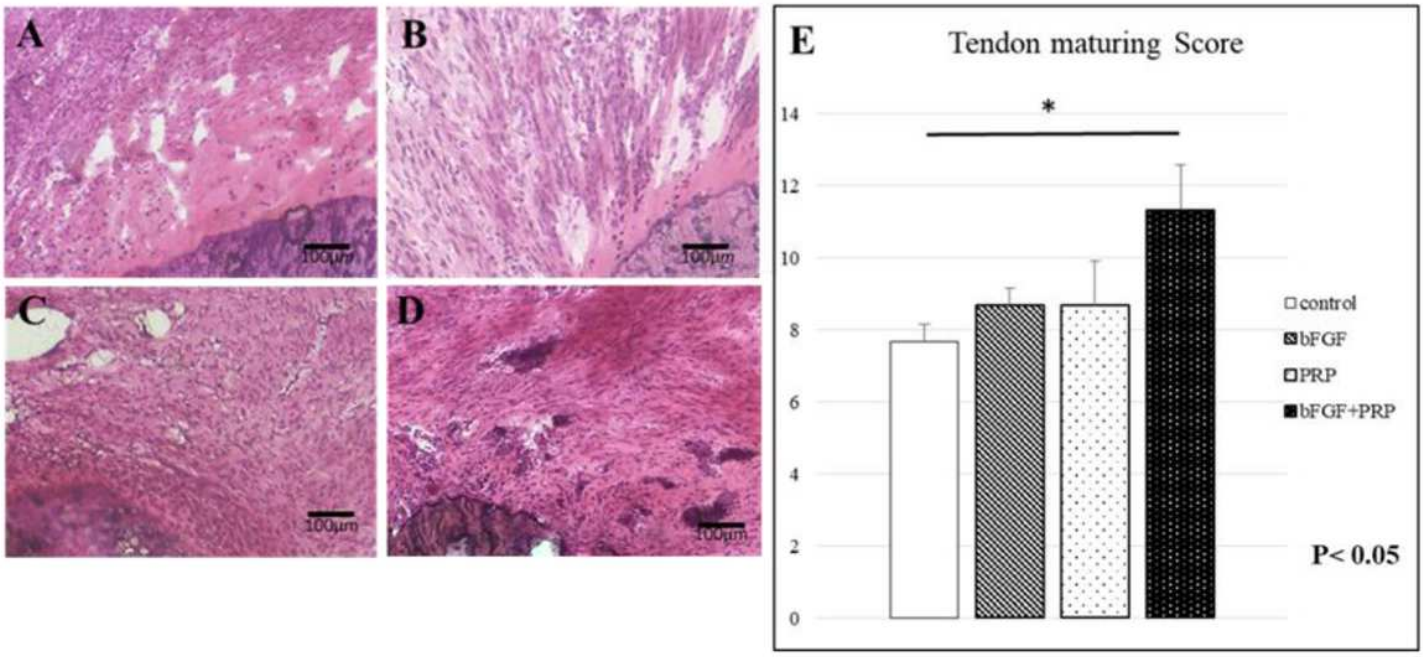

Figure 2

H&E staining at 2 weeks postoperatively, A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: Tendon maturing score. The tendon maturing score of the combined group was higher than the control group.

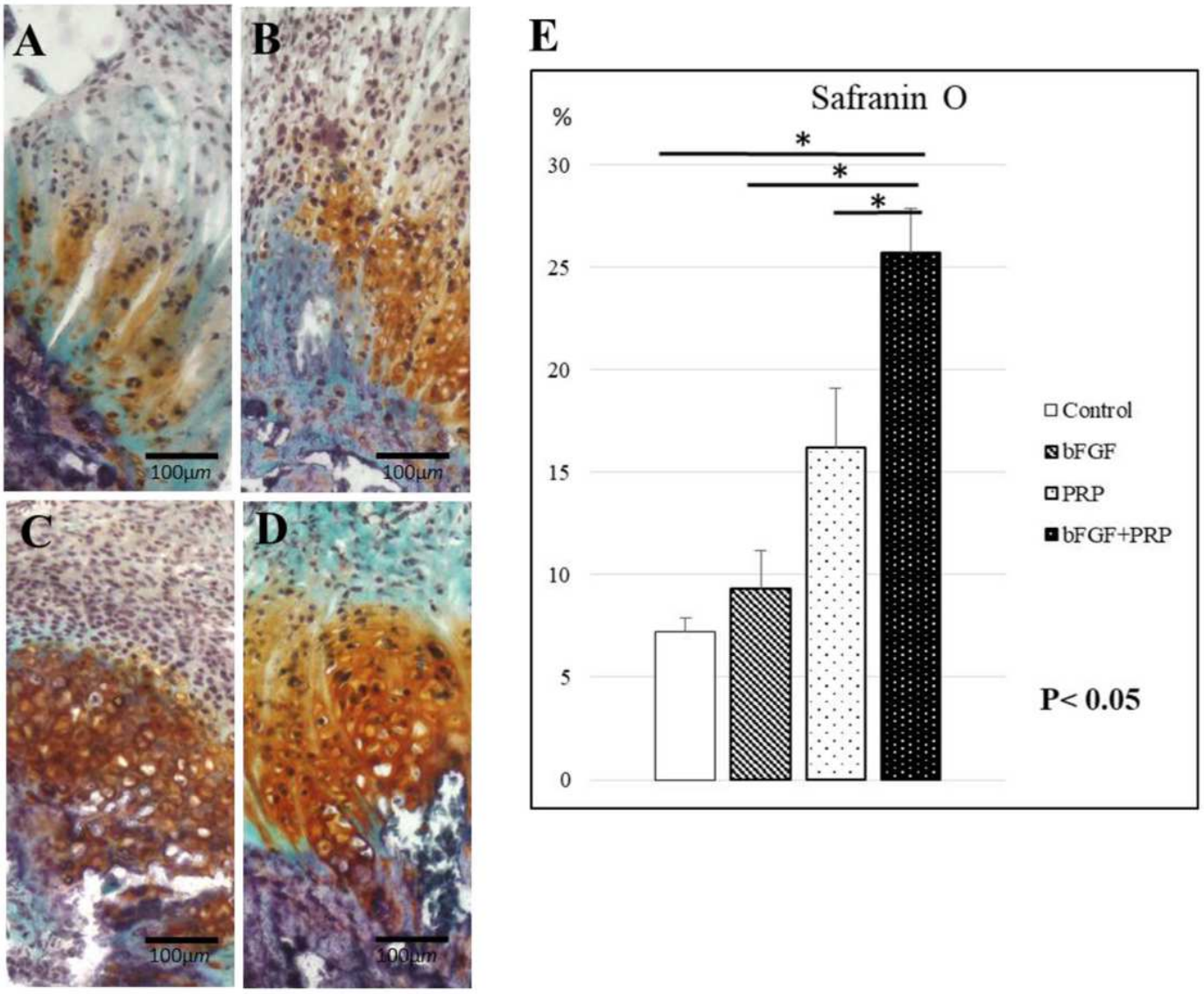

Figure 3

Safranin O staining at 2 weeks postoperatively, A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group. E: Proteoglycan content was calculated as a percentage of the pixels of each tendon–bone interface (positive/total pixels). Significantly stronger proteoglycan staining was observed at the repaired enthesis in combined group compared with other groups.

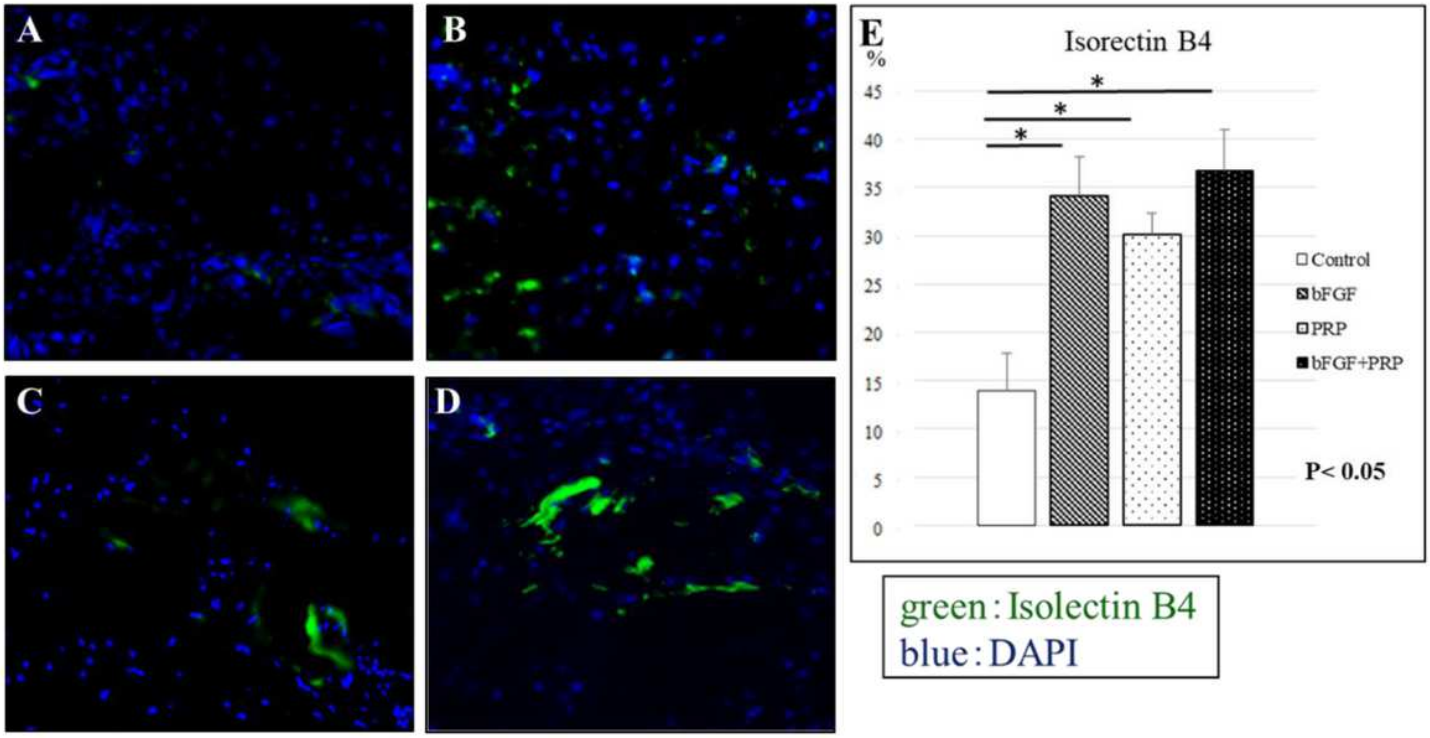

Figure 4

Immunofluorescence staining: isolectin B4 at 2 weeks postoperatively (A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: The number of positively stained cells in randomly selected fields. Vascular staining with isolectin B4 in treatment groups was significantly higher than that in the control group.

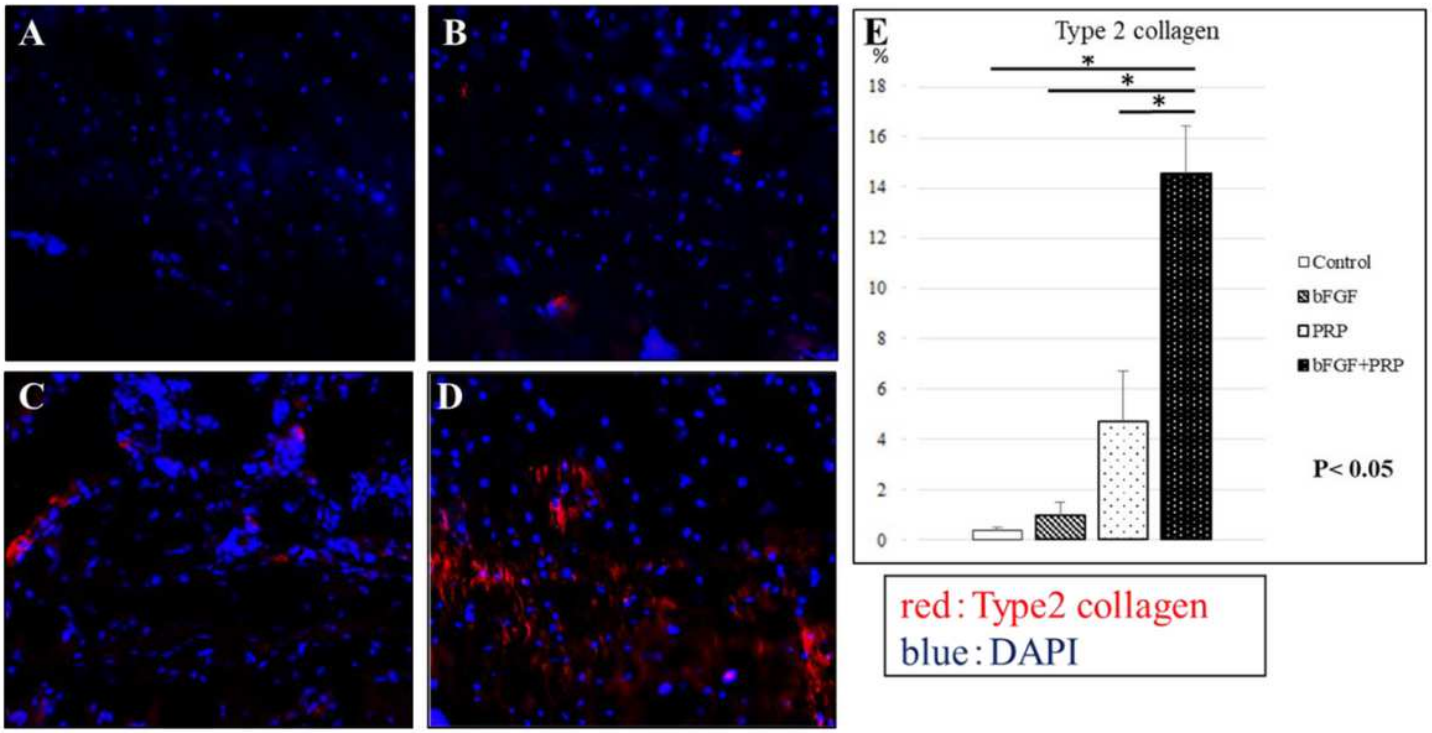

Figure 5

Immunofluorescence staining: type II collagen at 2 weeks postoperatively (A: control group, B: bFGF group, C: PRP group, D: bFGF+PRP combined group). E: The number of positively stained cells in 5 randomly selected fields. Type II collagen in the combined group was significantly higher than that in other groups.

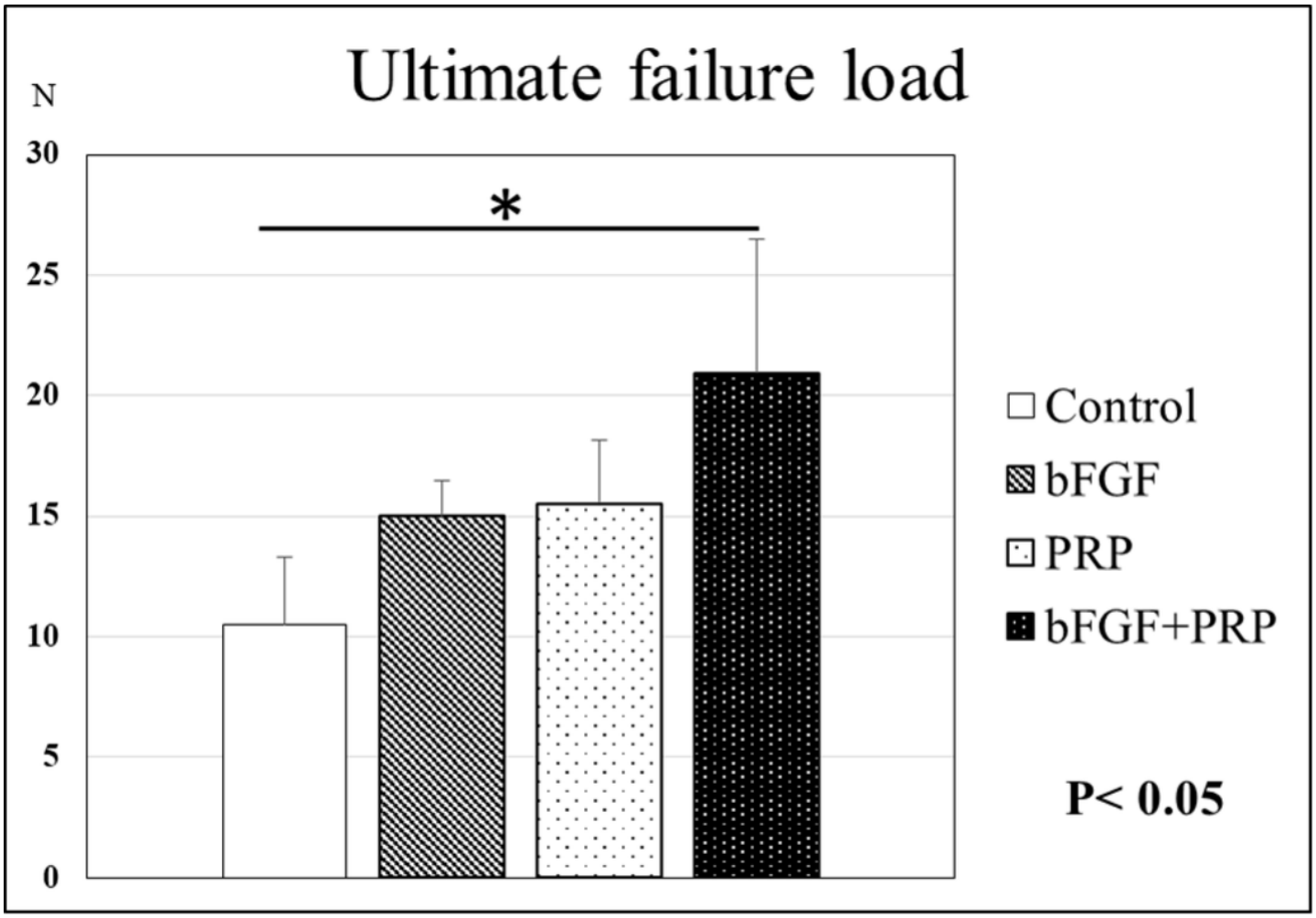

Figure 6

The ultimate failure load of the combined group was significantly higher than that of the control group at 6 weeks after surgery.