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Platelet-rich plasma decreases fibroblastic activity and woven bone formation with no significant immunohistochemical effect on long-bone healing: An experimental animal study with radiological outcomes

İbrahim Deniz Canbeyli[1], Rahmi Can Akgun[2], Orcun Sahin[2], Aysen Terzi[3] and İsmail Cengiz Tuncay[2]

Abstract

Purpose: This study aimed to analyze the immunohistochemical effect of platelet-rich plasma (PRP) on healing of long-bone fractures in terms of bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), the Ki-67 proliferation index, and radiological and histological analyses. **Methods:** Sixteen adult rabbits, whose right femoral diaphysis was fractured and fixed with Kirschner wires, were randomly divided into two groups, control and PRP (groups A and B, respectively). PRP was given to group B at 1 week postoperatively, and all animals were euthanized after 12 weeks. Radiographic evaluations were performed periodically. Cortical callus formation, chondroid and woven bone area percentages, osteoblastic and fibroblastic activities, and mature bone formation were examined. The depths of BMP-2 and VEGF staining were measured. The Ki-67 proliferation index was also calculated. **Results:** The mean radiological union score of group B was significantly higher than that of group A. There were also statistically significant differences between groups A and B in terms of cortical callus formation, woven bone area percentage, fibroblast proliferation, and mature bone formation. Group B had significantly more cortical callus and mature bone formation with less woven bone and fibroblast proliferation. Immunohistochemical analysis revealed no statistically significant difference between the groups in terms of BMP-2 and VEGF staining and the Ki-67 index. **Conclusions:** PRP had no effect on BMP-2 or VEGF levels with no increase in the Ki-67 proliferation index, although its application had a positive effect on bone healing by increasing callus and mature bone formation with decreased woven bone and fibroblast proliferation.

Keywords

fracture healing, immunohistopathology, long-bone fracture, platelet-rich plasma

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Introduction

Bone healing is still a major orthopedic problem, despite improvements in both biological and biomechanical treatment modalities.[1] It has been reported that up to 10% of all fractures either fail to heal or demonstrate a delay in healing. This failure of healing increases health-care costs with prolonged hospital stays, multiple surgeries, and associated complications. Therefore, in clinical practice, it is crucial to understand the fundamentals of bone repair processes and to prevent nonunion or delayed union.

[1] Department of Orthopaedics and Traumatology, Faculty of Medicine, Kırıkkale University, Kırıkkale, Turkey
[2] Department of Orthopaedics and Traumatology, Faculty of Medicine, Baskent University, Ankara, Turkey
[3] Department of Pathology, Faculty of Medicine, Baskent University, Ankara, Turkey

Corresponding author:
Orcun Sahin, Department of Orthopaedics and Traumatology, Faculty of Medicine, Baskent University, M. Fevzi Çakmak Cd. 10. Sk. No:40 Bahçelievler, 06490 Ankara, Turkey.
Email: drorcunsahin@gmail.com

There have been numerous clinical and experimental reports with regard to bone repair processes, and autologous bone grafting as adjuncts combined with surgical techniques is widely accepted as the gold standard technique for the treatment of nonunion or delayed union.[2] The application of platelet-rich plasma (PRP) is currently one of the most popular approaches for treatment of pathologies in cartilage, tendons, and bone. PRP is an autologous concentrate of platelets obtained directly from peripheral venous blood of the patient, which is produced by the centrifugation of whole blood.[3] Studies have shown that platelets carry alpha granules that contain growth factors, angiogenetic factors, and pro-inflammatory cytokines. They also bear fibrin for induction of angiogenesis.[4] Platelets play a significant role in bone healing by promoting early inflammation and repair stages of the process.[5,6] Nevertheless, there is very scarce and inconclusive data about the exact physiological effect of PRP on bone healing. To understand this effect, more histological and immunohistochemical studies should be performed and comparative data must be obtained for definitive conclusions. This is one of the first studies that analyze the in-depth immunohistochemical effect of PRP on "acute bone healing" with histological and radiological outcomes.

Hence, the purpose of this study was to analyze the effect of PRP on "acute bone healing" process in long-bone fractures through radiological, histopathological, and immunohistochemical evaluations of bone morphogenic protein-2 (BMP-2) for new bone formation, vascular endothelial growth factor (VEGF) for angiogenesis, and Ki-67 protein for cell proliferation and to compare the outcomes with a control group.

Materials and methods

Study groups

This study employed 16 New Zealand-type adult rabbits (age: 1.5–2 years; weight: 2.5–3.2 kg) and was approved by the Institutional Ethics Committee in accordance with the Guide for the Care and Use of the Laboratory Animals principles (approval number: D13/27). The rabbits were randomly divided into two groups after their right femoral diaphysis was fractured and fixed with Kirschner (K) wires:

1. Group A ($n = 8$) as the control in which rabbits did not receive any adjuvant treatment for bone healing.
2. Group B ($n = 8$) was injected with PRP into the fracture site of the right femur.

After surgery, six rabbits from each group recovered without any postoperative signs of infection, wound complications, or other systemic problems. The remaining two rabbits from each group were omitted because of wound complications causing systemic septicemia. Hence, a total of 12 rats (6 rabbits in each group) were analyzed for the final evaluation. All animals were euthanized after 12 weeks of follow-up by an overdose of sodium pentobarbital. Immediately after euthanizing, the right femur from each animal was placed in cold 70% ethanol and processed for histological evaluation.

Surgical procedure and PRP application

After proper cleaning and sterilization of the surgical area, a 3-cm lateral longitudinal incision was made on the right femoral diaphysis of the rabbits under dissociative anesthesia induced by 50 mg/kg ketamine and 7 mg/kg xylazine. After mid-shaft exposure of the femoral bone, an oblique fracture line was created in the diaphysis by multi-drilling and an osteotome. Oblique fracture line was preferred for an easier reduction and fixation with a K-wire and to make the area of histopathological examination more prominent via a long fracture line. Then, the fracture was fixed with an intramedullary 2.0-mm K-wire entering through the trochanter major to the intercondylar notch. The rabbits were kept in clean cages postoperatively, and antibiotic prophylaxis was applied by intramuscular injections of 50 mg/kg ampicillin twice a day for 5 days.

To analyze the effect of PRP on "acute bone healing process", at 1 week postoperatively, 1 ml of autologous PRP gel prepared from 5 ml of venous autologous blood was injected percutaneously into the fracture line under fluoroscopy guidance in all rabbits of group B. Then, 5 ml of blood was collected from central veins of the ear into tubes containing 3.8% sodium citrate.[7] PRP was obtained from the anticoagulated blood by the double centrifugation technique as described previously.[7] First, centrifugation was performed at 150 g for 20 min. At the second stage, the supernatant obtained from the first centrifugation was recentrifuged at 450 g for 10 min. The PRP at the bottom of tubes was then aspirated. Platelet counts before and after the procedure were performed automatically using a hematology analyzer (Advia 120, Bayer B.V., Mijdrecht, the Netherlands). The platelet counts prior to PRP preparation were 9.6–15.4 $\times$ 10^4 cells. After preparation of PRP, the platelet counts were increased to 22.9–48 $\times$ 10^4 cells. PRP was used as a PRP gel for the experiment. Therefore, before injection of PRP at the fracture site, the aspirates were mixed with 10% calcium chloride at a ratio of 1:0.15 and 100 U/ml bovine thrombin for activation of the cells at the time of injection.

Radiographic evaluation

Radiographic evaluations [anteroposterior(AP) and lateral plain X-ray of the femur] were performed following the surgical procedure at week 0 (immediately after surgery), week 4 (3 weeks after PRP application), week 8, and week 12 (at euthanization). All plain X-rays (65 kVp, 7.5 mA, and 0.25 s) were evaluated by a blinded observer experienced in musculoskeletal trauma. Digital radiographs of all animals were downloaded to the picture archiving and

Table 1. Radiological union score.[8]

No cortical bridging	0
Cortical bridging only on one radiograph (AP or lateral)	1
Cortical bridging on both radiographs	2
Fracture line not visualized	3

AP: anteroposterior.

Table 2. Histopathological grading of callus formation.

Grade 0	No callus formation
Grade 1	Callus formation in at least one cortex with no continuation
Grade 2	Callus formation in at least one cortex with continuation
Grade 3	Callus formation in both cortices

Table 3. Histological evaluation of osteoblastic activity.

Grade 0	No osteoblasts were seen in the magnification area
Grade 1	Osteoblasts were seen only by detailed examination of the cells in the magnification area
Grade 2	Osteoblasts were easily seen even in small magnifications
Grade 3	Osteoblasts form clusters

communication system and evaluated with a scoring system including the most commonly used criteria of radiographic bone healing (Table 1).[8] In this scoring system, union was scored according to cortical bridging in AP and lateral plain radiographs and fracture line visualization with a maximum score of 0 (no cortical bridging) and a maximum score of 3 (no fracture visualization). Data obtained by analysis according to the radiological criteria at week 12 were then analyzed statistically.

Histopathological analysis

After euthanization, all right femurs were resected 1 cm proximal and 1 cm distal to the fracture line and sent for histopathological examination. Femoral bone samples were fixed in 10% formaldehyde and transferred to 20% formic acid for decalcification.[9] The specimens were embedded in paraffin, and sections of 5 μm thickness were prepared on slides. After deparaffinization, the sections were stained with hematoxylin and eosin. Cortical callus formation, chondroid and woven bone area percentages, osteoblastic and fibroblastic activities, and mature bone formation were examined for histopathological evaluation under a light microscope at 10× magnification.

Chondroid and woven bone area percentages were measured using a grid system that divided the section into equal areas. Cortical callus formation was graded by the grading system shown in Table 2. Osteoblastic activity was scored according to the evaluation criteria shown in Table 3. Fibroblasts within the best representative area of callus formation at 10× magnification were counted and graded as 0–50 = 1, 50–100 = 2, and >100 = 3. Mature bone formation was evaluated as none (grade 0) and present (grade 1).

Immunohistochemical analysis

For immunohistochemical analysis of growth factor and cytokine concentrations at the fracture site, the depths of anti-BMP-2 (monoclonal antihuman Pro-BMP-2 antibody, (MAB2260, R & D Systems, Inc., Minneapolis, Minnesota, USA) and VEGF (AB-293-NA, R & D Systems, Inc.) antibody staining were measured.[10] They were graded immunohistochemically as *none* (0), *mild* (1), *moderate* (2), and *strong* (3) staining according to the depth of staining. The Ki-67 proliferation index was also calculated by counting cells showing nuclear positivity within 100 proliferative chondrocytes and osteoblasts in the area of most intense bone healing.[11] Immunohistochemical staining for evaluation of the Ki-67 proliferation index as well as BMP-2 and VEGF was performed according to a standard protocol.[12,13] Positivity of immunostaining was assessed as follows: negative to 0 (0–5% positive cells), 1 (5–20% positive cells), 2 (20–50% positive cells), and 3 (50–100% positive cells). Immuno-stained cells were evaluated using a light microscope (Eclipse E400 light microscope; Nikon, Tokyo, Japan).

Statistical analysis

All data were analyzed with SPSS 17.0 statistical software (SSPS Inc., Chicago, Illinois, USA). Conformity of the data to a normal distribution was evaluated by the Shapiro–Wilk test. Homogeneity of variances was analyzed by the Levene's test. Mean vascular proliferation variables, which met the assumptions of the parametric tests, were compared between two independent groups by the Student's *t*-test. When the assumptions of the parametric tests were not met with respect to the other variables, a comparison of the median of two independent groups was made by the Mann–Whitney *U* test. Data are shown as the mean ± standard deviation, median, minimum and maximum values, and interquartile range. In terms of mature bone formation, the groups were compared by the paired ratio test. A value of $p < 0.05$ was considered as statistically significant.

Results

There were no intraoperative complications during the surgical procedure. Following the PRP injections, no early or late complications including systemic or local side effects were observed during the follow-up period.

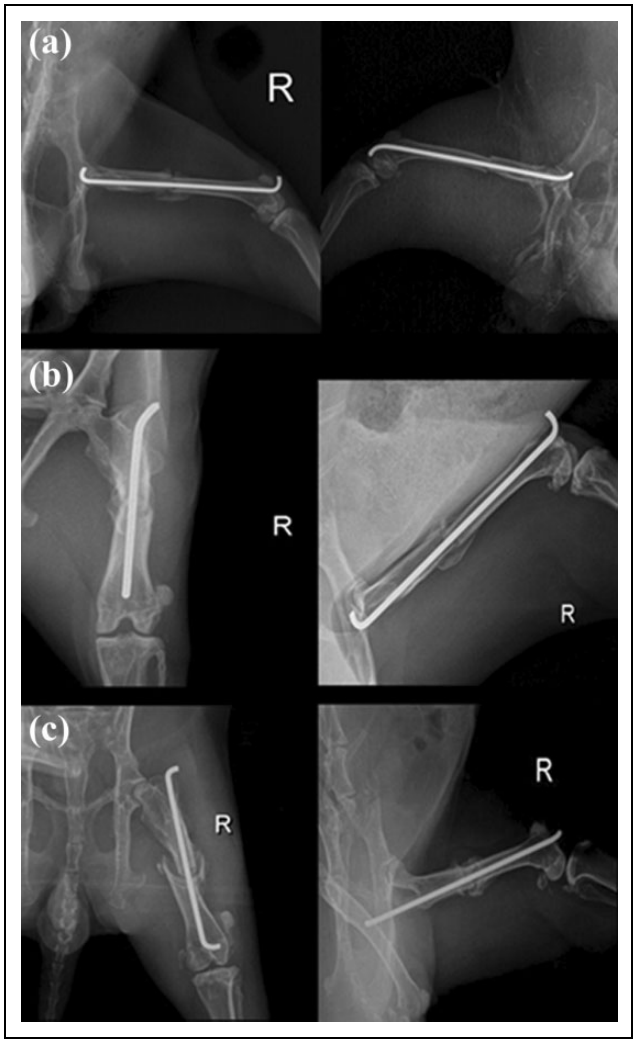

Figure 1. AP and lateral radiographs of group A at week 4 (a), week 8 (b), and week 12 (c) after surgery. AP: anteroposterior.

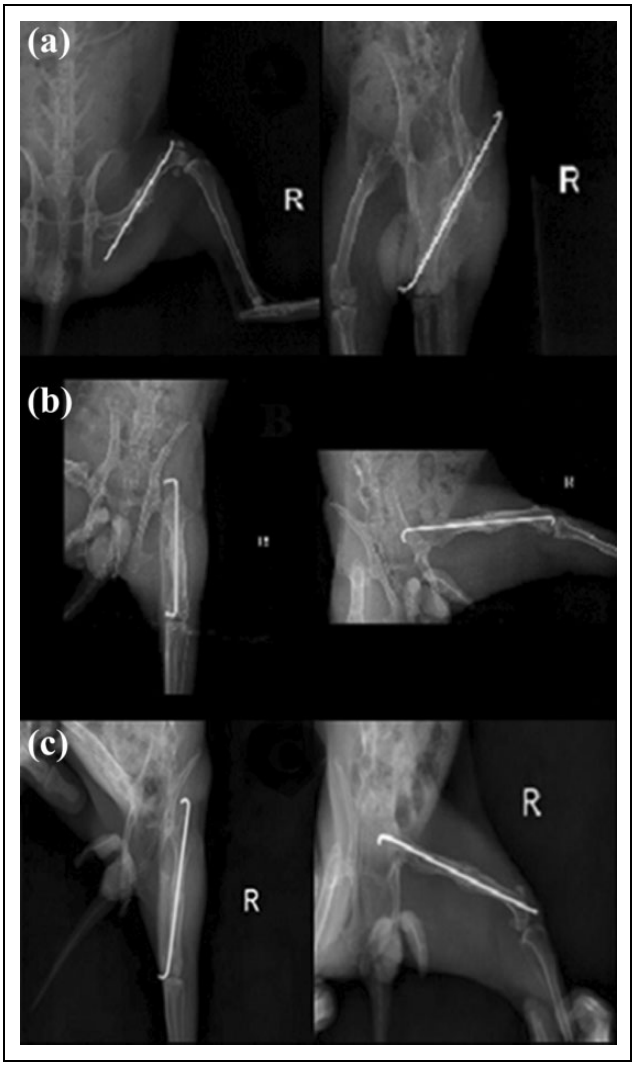

Figure 2. AP and lateral radiographs of group B at week 4 (a), week 8 (b), and week 12 (c) after surgery. The callus formation was more prominent in group B. AP: anteroposterior.

Radiological evaluation

The mean radiological union scores of groups A and B were 1.2 (range: 0–3) and 2.7 (range: 2–3), respectively. Control radiographs at weeks 4, 8, and 12 showed delayed union of the fracture with less callus formation in group A compared with group B. Delayed union described as delay in bone bridging when compared to an adequate control group.[14] However, fracture union with callus formation was achieved in all rabbits of group B (Figures 1 and 2). In contrast, radiological nonunion was determined in two rabbits of group A. The findings obtained according to the radiological evaluation are summarized in Table 4. Based on the radiological union scores, the mean score of group B was significantly higher than that of group A ($p < 0.01$).

Histological evaluation

Histological results and statistical comparisons between the groups are summarized in Tables 4 and 5, respectively. There were statistically significant differences between groups A and B in terms of cortical callus formation, woven bone area percentage, fibroblast proliferation, and mature bone formation ($p < 0.01$). Conversely, there was no significant difference in the chondroid area percentage or osteoblastic proliferation ($p = 0.154$ and $p = 0.336$, respectively)

Although histological analysis revealed that cortical callus formation was irregular in group A, it was more regularly oriented in group B (Figure 3). Additionally, group A had more extensive new woven bone formation in the intramedullary area compared with group B that had significantly more mature bone formation ($p < 0.01$). Fibroblast proliferation was also intensified within the granulation tissue of group A (Figure 3). At 4× magnification, fibroblast-rich areas were easier to be determined, and there were a greater number of fibroblasts in group A. In contrast, fibroblast-rich areas in group B were more difficult to identify because they were significantly fewer in number.

Table 4. Radiological and histological results of the study groups.

Group	Callus formation grade	Chondroid area	Woven bone area	Osteoblastic activity grade	Fibroblast proliferation	Mature bone formation	Radiological union score
A	1	5%	55%	1	3	0	0
A	1	1%	60%	3	2	0	0
A	2	3%	45%	2	3	1	1
A	2	3%	45%	2	3	1	2
A	1	3%	40%	2	3	0	3
A	1	3%	60%	2	2	0	1
B	3	10%	30%	2	1	1	2
B	3	1%	25%	2	1	1	2
B	3	<1%	30%	3	1	1	3
B	3	<1%	30%	3	2	1	3
B	3	<1%	15%	2	1	1	3
B	3	3%	30%	2	1	1	3

Table 5. Mean values and statistical comparison results of the histological parameters.

Histological parameters	Group A (mean ± SD)	Group B (mean ± SD)	*p* Value
Cortical callus form.	1.33 ± 0.52	3 ± 0.0	<0.01[a]
Chondroid area	3 ± 1.26	2.3 ± 3.93	0.154
Woven bone area	50.83 ± 8.61	26.67 ± 6.05	<0.01[a]
Osteoblast prolif.	2 ± 0.63	2.33 ± 0.52	0.336
Fibroblast prolif.	2.67 ± 0.52	1.17 ± 0.41	<0.01[a]
Mature bone form.	0.33 ± 0.13	1 ± 0.0	<0.01[a]

SD: standard deviation, form: formation, prolif: proliferation.
[a]Statistically significant results.

Immunohistochemical evaluation

Mean values of the groups and statistical comparisons of the immunohistochemical results are shown in Table 6. There was no statistically significant difference between the groups in terms of BMP-2 and VEGF staining ($p = 0.999$ and $p = 0.471$, respectively). At 10× magnification under a light microscope, the depths of BMP-2 and VEGF staining were similar in both groups as shown in Figure 4. Similar results with no statistically significant difference ($p = 0.197$) in Ki-67 staining was also found in both groups (Figure 4).

Discussion

This is one of the first studies that has specifically analyzed the immunohistochemical effect of PRP on long-bone healing. Although the literature includes numerous clinical and experimental studies about PRP application and its positive histological effect on long-bone healing with increased cell proliferation of osteoblasts and fibroblasts with more callus formation, the exact immunohistochemical mechanism of action is still a matter of discussion. Hence, in the current study, the specific effects of PRP on long-bone healing in terms of BMP-2 and VEGF together with the Ki-67

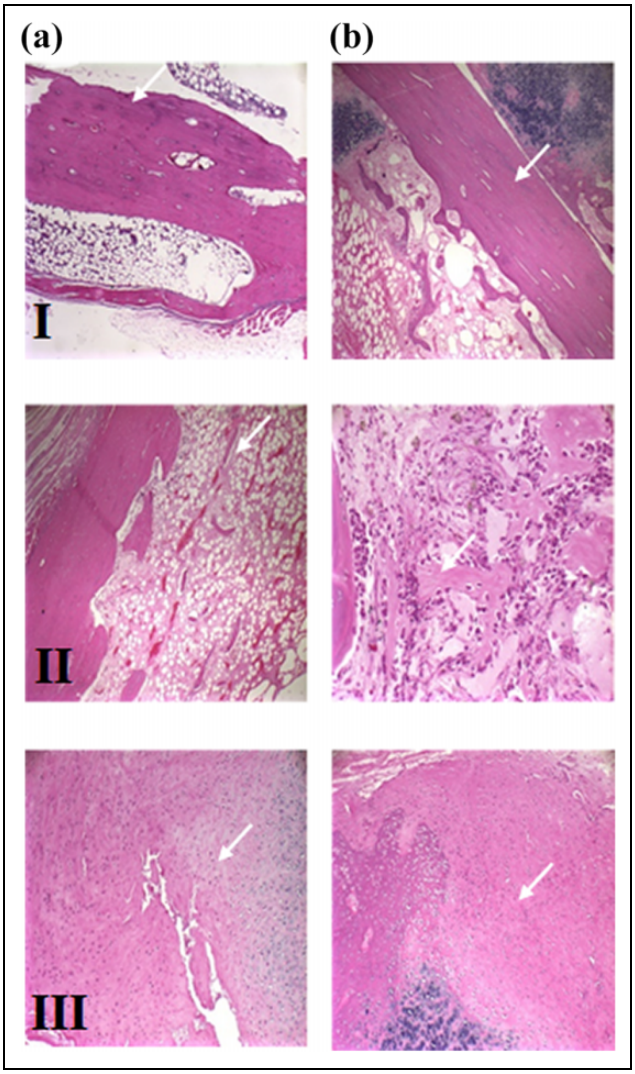

Figure 3. Fracture samples with H & E staining under light microscopy at ×10 magnification. (a) Group A and (b) group B. (I) The callus formation was more prominent in group B. (II) More vascular proliferation was detected in group B. (III) Fibroblast proliferation in group A was more prominent than in group B. H & E: hematoxylin and eosin.

Table 6. Immunohistochemical outcomes of the study groups with statistical comparison results.

Immunohistochemical parameters	Group A (mean ± SD (min–max))	Group B (mean ± SD (min–max))	*p* Value
BMP-2	1.67 ± 0.46 (1–3)	1.41 ± 0.82 (0–3)	0.999
VEGF	2.43 ± 1.36 (0–3)	2.32 ± 1.93 (0–3)	0.471
Ki-67 PI	1.83 ± 2.61 (0–3)	1.92 ± 6.05 (1–3)	0.197

SD: standard deviation; BMP-2: bone morphogenetic protein-2; PI: proliferation index; VEGF: vascular endothelial growth factor.

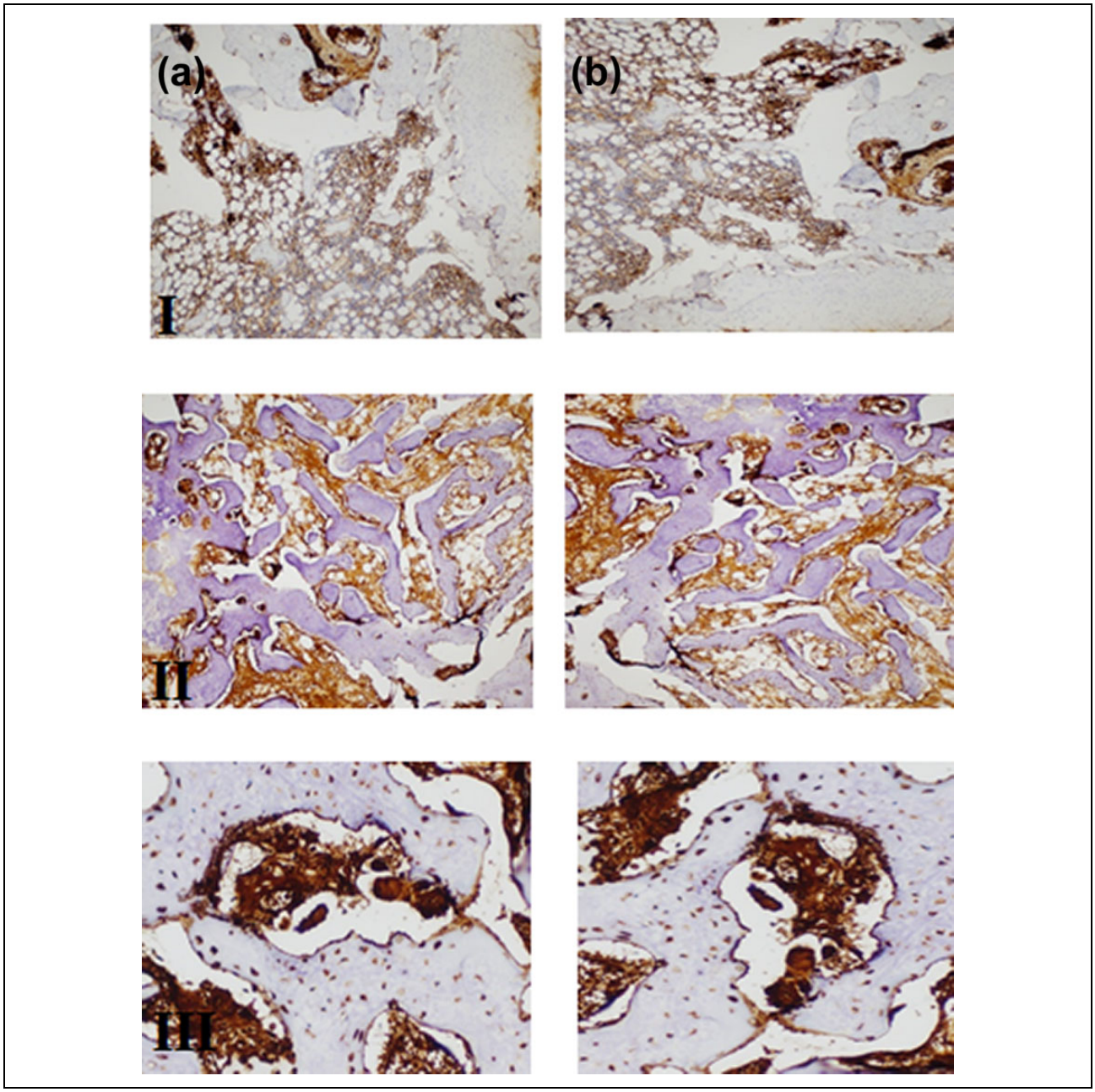

Figure 4. Immunohistochemical analysis of the right femoral bone samples at ×10 magnification under light microscopy. (a) Group A and (b) group B. The depths of (I) BMP-2 and (II) VEGF staining and (III) Ki-67 nuclear positivity are provided. There were no significant differences between the groups for all staining. BMP-2: bone morphogenetic protein-2; VEGF: vascular endothelial growth factor.

proliferation index were analyzed to understand the pathways of PRP in long-bone healing.

In the last decade, PRP application has gained broad popularity in orthopedics with various studies analyzing the role of high concentrations of platelets in histological, mechanical, and clinical aspects of fracture healing. The most commonly accepted scientific data about the effectiveness of PRP is the release of high concentrations of various platelet-derived biological agents including platelet-derived growth factor (PDGF), transforming growth factor beta, and interleukin [1.3,12–15] It is generally accepted that these bioactive agents play a considerable role in inflammation, neovascularization with vascular remodeling, differentiation of mesenchymal cells, and synthesis and remodeling of both bone and cartilage tissues.[16] Nevertheless, the exact physiological pathways of this considerable role of PRP is still a matter of discussion. Hence, in our study, we determined whether PRP had any effect on bone repair with regard to the immunohistochemical mechanism of action including BMP-2 and VEGF production and the Ki-67 proliferative index.

Although numerous benefits ascribed to PRP and the promising results reported for its therapeutic potential, the clinical outcomes are heterogeneous and sometimes contradictory due to both the application of different protocols and the lack of standardization in PRP preparation procedures. This has led to incomparable results and inconclusive data about the real efficacy of PRP. Among the several variables affecting PRP efficacy, platelet activation is a crucial step that might influence the availability of bioactive molecules.[11,12] Currently, there is lack of evidence on the most suitable method for PRP activation, and the choice of strategy for activation is mainly based on practical reasons rather than supported by studies. Although different activation steps before PRP administration are analyzed in various studies, the most commonly used method is the addition of thrombin and/or calcium chloride ($CaCl_2$). In a study by Cavallo et al., different activation methods for PRP were compared with regard to the release of growth factors and as a conclusion it was stated that $CaCl_2$ induced a progressive release of growth factors increasing up to 24 h.[17] We believe that, activation of PRP is the most critical step for the effectiveness of PRP application. Therefore, in our study, all PRPs were activated with both thrombin and $CaCl_2$ to increase the efficacy of PRP. Nevertheless, as a limitation of our study, a separate study group that only includes thrombin and $CaCl_2$ should be analyzed in future studies to better understand the effect of PRP.

In the literature, the incidence of traumatic nonunion of femur is approximately 2–8%.[17] To overcome this almost highly common complication, different efforts have been described in the literature so far, including the PRP application. In a prospective clinical study, Malhotra et al. analyzed 30 femoral nonunions treated with PRP application and only 5 of them accepted as failure after 4 months of follow-up.[18] So, the authors concluded that PRP is a safe and effective treatment for the treatment of long-bone nonunion. In a recent meta-analysis by Gianakos et al., 29 different articles from the literature were analyzed, and 89% of the studies reported significant improvement in earlier bone healing after PRP application.[19] In our study, fracture union with callus formation was achieved in all rabbits in group B (PRP group), whereas two nonunions were detected in the control group. So, we believe that PRP has a positive effect on acute fracture healing. In the current study, we just analyze the effect of PRP on acute bone healing. Future studies are needed comparing PRP with autologous bone grafting for the treatment of long-bone nonunions or delayed unions.

Although there were numerous clinical and biomechanical studies analyzing the femoral nonunions in humans, there are very few clinically useful reports that describe femoral fracture repair and prognosis in rabbits.[9,11,20] Although different fracture fixation techniques have been advocated for rabbit femurs, these techniques are usually in the context of an experimental procedure rather than treatment of the fracture. Additionally, the conformation of the rear limbs and their excessive muscle mass complicates immobilization process in these animals.[12,20] It is also stated in the literature that low-bone density and large muscle mass of rabbits may predispose frequent fractures and/or non-unions. In the current study, there were two nonunions in group A. We believe that, apart from the specific features of the rabbits, insufficient fixation and immobilization may lead to these nonunions.

Histological effect of PRP on bone healing

Previous studies clearly show that exogenous application of thrombocyte-related growth factors induces proliferation of osteoblasts in cell culture.[6] In animal studies, increased osteoblastic proliferation and activity are also found with induction of various thrombocyte-related growth factors.[7,21] These studies emphasize that increases in differentiation, proliferation, and metabolic activity of osteoblasts is strongly related to PDGF and other proteins secreted from platelets, leading to the activation of intracellular signaling pathways for matrix synthesis and cellular differentiation.[22] Our study does not have comparable results with the literature. In our study, we found no significant difference in osteoblastic proliferation after PRP injection. We believe that PRP has a major effect on the early inflammatory phase of fracture healing, including the formation of a fracture hematoma and proliferation of mesenchymal stem cells derived from the bone marrow, without any direct effect on osteoblastic differentiation.

Callus formation with lamellar (mature) bone is one of the most critical points for fracture union. All studies about this issue specifically focus on this aspect to determine the quality of fracture union. Almost all studies of PRP application for bone healing emphasize the positive effect of PRP on callus formation. However, there are controversial

results in the literature about the formation of woven versus lamellar bone after PRP application. In a study by Simman et al., a significantly increased amount of callus formation was detected in healing bone after PRP application.[23] It has also been reported that significantly less woven bone and a higher rate of lamellar bone are seen in the fracture zone after PRP treatment. In contrast, Sarkar et al. found no histologically or histochemically significant differences between PRP treatment and the control in terms of woven and lamellar bone formation.[24] For callus formation, we obtained comparable results with the literature. In the current study, callus formation in the PRP group was more evident and extensive compared with the control group. Additionally, there was more woven bone histologically in the control group compared with the PRP group that had more advanced lamellar bone formation in the fracture zone. Therefore, we believe that PRP has a direct positive effect on callus and lamellar bone formation.

It is very well documented that fibroblastic invasion of the fracture zone, which is seen in the early stages of bone healing, is replaced by osteoblasts and chondroblasts at later stages.[23] This cellular differentiation is one of the major steps of bone healing, which leads to endochondral new bone formation. Although fibroblastic proliferation after PRP application has not been analyzed frequently, our study has comparable results with the literature. The PRP group had a lower number of fibroblasts compared with the control group in the current study, demonstrating a more advanced stage of fracture healing in the PRP group.[23]

Immunohistochemical effect of PRP on bone healing

The literature contains various studies analyzing different biochemical markers that promote bone healing. Among these, BMPs and VEGF are the most commonly used markers which induce angiogenesis and bone formation.[25,26] In a review article by Barrena et al., it was clearly stated that neovascularization of fracture zone is crucial for successful bone healing, providing oxygen and delivering progenitor cells, and BMPs and VEGF are key osteogenic and angiogenic factors in this process.[25] Ki-67 proliferation index is also one of the most commonly used markers that specifically demonstrates cellular proliferation. In a study by Scholzen et al., it was stated that the monoclonal antibodies that react with the Ki-67 equivalent protein from rodents extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research.[27] Hence, in our study, we preferred to use BMP, VEGF, and Ki-67 index for the immunohistochemical analysis.

One of the bioactive agents detected at the fracture site after PRP injection is BMP-2. BMP-2 is a signaling molecule that influences cell division, matrix synthesis, and tissue differentiation by recruiting mesenchymal stem cells from the surrounding muscle, bone marrow, and blood vessels and by differentiating these cells into osteoblasts to form either bone directly or cartilage cells that subsequently differentiate into bone cells. The literature has inconclusive data about the effect of BMP-2 on bone healing. In a meta-analysis by Garrison et al., in 2010, 11 randomized controlled trials of BMP treatment for fracture healing in skeletally mature adults were analyzed. In conclusion, it was stated that there is limited evidence for the use of BMP-2 for bone healing.[28] Additionally, although there have been various studies and systematic reviews about BMP usage in bone healing, there is no single article that specifically reports the interaction between PRP and BMP. In our study, we evaluated BMP-2 staining at week 12 for comparison with the control group. There was no significant difference in BMP staining between the groups. The reason for this result may be the timing of the analysis. BMP-2 expression increases and reaches plateau at the second week of normal fracture healing and subsequently decreases for 2–4 weeks until the initiation of ossification. Nevertheless, in the current study, BMP-2 staining was analyzed after 12 weeks. The characteristic changes in BMP-2 concentrations may explain why we did not observe different BMP-2 levels between the two groups.

VEGF is a potent angiogenic factor that was first described as an essential growth factor for vascular endothelial cells. Although its detailed effects on bone metabolism remain unclear, it is believed to have a major role in extracellular matrix remodeling, angiogenesis, and bone formation. In the literature, there have been numerous studies of PRP and its VEGF content. In a review article by Alsousou et al., it was clearly stated that VEGF is an important signaling molecule in PRP, which stimulates angiogenesis and cell migration.[29] Nevertheless, to our knowledge, there is no study that has specifically analyzed VEGF expression after PRP application for long-bone healing. In our study, we found no significant difference in VEGF staining between the groups. The harvested material was stained for VEGF after 12 weeks of fracture formation. Therefore, we believe that VEGF may not have an effect on long-bone healing, especially in late phases of fracture union.

We concluded that, although PRP application had a positive effect on bone healing by increasing callus and mature bone formation with decreased woven bone and fibroblast proliferation,[30,31] it had no effect on BMP-2 or VEGF levels with no increase in the Ki-67 proliferation index. Hence, the current study demonstrates that PRP does not increase chondrocyte functions or endochondral ossification without any further increase in osteoblastic functions and angiogenesis. Additional studies are needed with a larger study population and different time points for evaluations.

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