Immunohistological effects of transforming growth factor-β via platelet-rich plasma on segmental bone defects: an animal study

Aziz ATİK[1]* , **Serdar SARGIN**[1] , **Gökhan MERİÇ**[2] , **Cengiz CEYLAN**[3]

[1]Department of Orthopaedics and Traumatology, Balıkesir University Medical Faculty, Balıkesir, Turkey
[2]Department of Orthopaedics and Traumatology, Yeditepe University Medical Faculty, İstanbul, Turkey
[3]Department of Surgery, Balıkesir University Veterinary Faculty, Balıkesir, Turkey

Abstract: Bone healing is a different and unique process that results in complete bone tissue, rather than ending in scar formation. However, more significant bone defects may fail to regenerate and forms scary granulomatous tissue. This study aimed to investigate the efficacy of transforming growth factor-β (TGF - β) via platelet rich plasma (PRP) in rabbit tibia bones with segmental bone defects by radiological and immunohistological methods. Tibial segmental defects were performed to adult New Zealand rabbits (n = 10) on both legs. 0.5 mL of PRP was administered to the defect zone on one leg (PRP group), while the other leg was closed without PRP administration (control group). Following six weeks of observation samples were sent for immunohistological examination. According to Allen's fracture healing score, the mean score was higher in the PRP group compared to the control group (3.2 vs. 1.3). Immunohistological evaluations revealed that in the control group, samples were mostly at the early stages of bone development, whereas in the PRP group, later stages of development were able to be observed (P < 0.05). PRP administration increases bone union, reduces healing time, and it may provide a more cost-effective treatment with fewer complications in segmental bone defects.

Key words: Platelet-rich plasma, transforming growth factor, segmental bone defect, rabbit, tibia

1. Introduction

Bone healing is a different and unique process that results in complete bone tissue, rather than ending in scar formation. However, more significant bone defects may fail to regenerate thoroughly and terminate in regeneration that forms scary granulomatous tissue, especially for reasons such as traumas, tumors, infections, congenital anomalies, which may negatively affect peripheral healing structures. It is for sure that autologous bone grafting is the gold standard in reconstructing these defects. Nevertheless, additional incision necessity, donor site morbidity, complications during harvesting autologous bone may put surgeons in trouble [1]. Besides, the formation of new bone is a long process and brings complication risks such as surgical infections, joint stiffness, soft tissue contractures. In order to reduce the risk of these complications, the treatment period should be shortened by accelerating bone formation. Regenerative medicine helps to overcome these shortcomings [2], but due to potential risks of infection, allergic reactions, and transmission of pathogens, the administration of heterologous materials may not be preferred.

Platelet-rich plasma (PRP) is a plateletpheresis-derived autologous concentration of platelets in a small volume of plasma that includes many growth factors [3,4], which have been shown to have therapeutic effects on fracture healing [5,6,7]. PRP acts as fibrin gel (bio-glue), provides homeostasis during and after surgery, and acts as a tissue adhesive [8]. Since it is autologous, it is not allergic, and there is no risk of rejection. It accelerates the wound healing process with the secretory growth factors in its content [9]. Additionally, the small amount of leukocytes and the antibody proteolytic enzymes inside its content, makes it act as a physiological antibiotic in addition to its osteoinductive activity [10]. Its plasma contains hormones, biotransformed vitamins, and other nutrients [11]. Because of these features, PRP is widely used in clinical applications of bone, tendon, ligament, cartilage tissue injuries, and osteoarthritis [12].

The cytoplasmic granules of platelets contain several growth factors such as transforming growth factors (TGF - β1, TGF - β2, TGF - β3), platelet-derived growth factors (PDGF αα, PDGF ββ, and PDGF αβ), vascular endothelial growth factor (VEGF), and epithelial growth factor (EGF)

* Correspondence: azizatik@yahoo.com

[7,13,14]. PRP helps neo-collagenous tissue formation with its TGFs, neo-vascularization with EGF and VEGF, and extracellular matrix formation with PDGF [15,16]. In particular, TGF-β1, TGF-β3, growth/differentiation factor 5 (GDF5), bone morphogenetic proteins (BMP-2 and BMP-7) are factors that stimulate hyaline cartilage and fibrous cartilage development [17]. These factors cause chondrocyte hypertrophy, collagen type X, alkaline phosphatase, and osteocalcin expression [17]. Especially TGF and BMP-2 are the growth factors that provide chondrogenic differentiation [18]. Additionally, deposition of the matrix in the cartilage tissue, chondrogenic differentiation, and reduction of the inhibitory effect of interleukin-1 (IL-1), which inhibits proteoglycan synthesis are among the essential functions of PRP [19,20].

TGF-β is a growth factor that regulates many biological activities, such as cell reproduction, differentiation, adhesion, migration, and apoptosis [21]. It stimulates the proliferation of fibroblasts and mesenchymal stem cells (MSCs) [22]. In addition to platelets, TGF-β is stored in the bone as well, since it is produced by osteoblasts, chondroblasts, and macrophages in the bone. It is highly synthesized during endochondral ossification and regulates chondrogenesis by differentiating osteogenic and chondrogenic MSCs [23]. During bone healing, TGF-β1 regulates osteoblast mitosis, while TGF-β2 and β3 regulate chondrogenesis. TGF-β2 is highly secreted in proliferation, hypertrophy, and mineralization phases [24]. In the first stages of osteogenesis, TGF allows the stem cells to differentiate into osteoblasts, but due to the increase of bone cell resources in the late period, osteoblast formation, mineralization, and differentiation are inhibited [25]. BMPs belong to the TGF-β family, and they enable the stem cells to be transformed into chondroblasts for enchondral ossification, and into the osteoblastic stem cells for membranous ossification [26]. Committed osteoblastic stem cells form pre-osteoblast I and II, and these cells, with the help of BMP, insulin-like growth factor (IGF), and TGFs, are transformed into osteoblasts, and membranous ossification occurs [27].

Within the elucidation of all these data, this study aimed to investigate the efficacy of PRP, particularly the role of TGF in rabbit tibia bones with segmental bone defects, by immunohistological methods.

2. Materials and Methods

This study was initiated after obtaining approval from the local Ethics Committee for Animal Research (approval no./date: B30CAU00506-050.04-122/04 December 2014). Principles of "laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed. Male and female (n = 5 for each sex), adult (1 year of age), New Zealand rabbits (n = 10), with bodyweights ranging approximately between 2000–2500 g, were obtained and identified with numbers. Rabbits were housed in individual cages with free access to ad libitum food and water. On the day of surgery, anesthesia was applied intramuscularly with 3 mg/kg xylazine (Bayer Corporation, Whippany, New Jersey, USA) and 35 mg/kg ketamine hydrochloride (Pfizer Inc., Parke Davis, New York, USA). Following the anesthesia, both tibias were shaved, disinfected, and the skin incisions were made over the medial aspect of the tibias in the sterile surgery room. Tibial diaphyses were exposed following the separation of muscles and stripping periosteum. Osteotomies were performed using an electric micro-saw, immediately distal to the tibiofibular synostosis line. A 4 mm bony block was removed to form a segmental defect in a standard manner. The defect was stabilized using a mono-lateral external fixator and four K-wires instead of internal plate fixation to avoid surrounding tissue harm bias. Unlike the human tibia, the rabbit tibia may be subjected to nonaxial shear forces during gait. Therefore, casts were applied over the fixators above the knee level using cotton pads and gypsum.

In the meantime, 8 mL of peripheral venous blood was collected from the auricular vein of each rabbit and separated using a standard PRP kit to obtain autologous PRP. Before closing the wound in layers, 0.5 mL of PRP was administered to the segmental defect on one leg (PRP group), while the wound on the other leg was closed without PRP administration (control group). In the PRP group, PRP was administered to half of the rabbits in the right leg and the other half in the left leg in order to eliminate the extremity side bias. The remaining portion of the PRP sample was sent to the laboratory to confirm that the platelet count is over 1 million/mL [28,29,30].

While rabbits were still anesthetized, intra-operative x-ray images were taken to confirm the placement of fixators, defect, and alignments. For each rabbit, the rabbit ID and the PRP administered side were recorded. Antibiotic prophylaxis of cefazolin sodium (25 mg/kg intramuscular) was initiated (GlaxoSmithKline plc., Wales, UK) and continued for three days with three administrations each day. Postoperative pain was managed by subcutaneous injection of 4 mg/kg carprofen (Zoetis Services Llc, Parsippany, NJ, USA) twice daily for seven days. For each rabbit, weekly x-ray examinations were conducted to check callus development (Figure 1). Skin sutures were removed two weeks after the surgery. Following six weeks of observation, rabbits were sacrificed by 100 mg/kg intravenous injection of sodium thiopenthal (Abbott Laboratories, Illinois, USA), and tibias were removed and sent for histological examination.

Bony tissue samples taken for histological examinations were fixed in 10 % neutral buffered formalin for 72 hours. For decalcification of bone tissue samples, solutions of formic acid (125 mL of 90 % formic acid + 125 mL distilled water) and sodium citrate (50 g of sodium citrate + 250
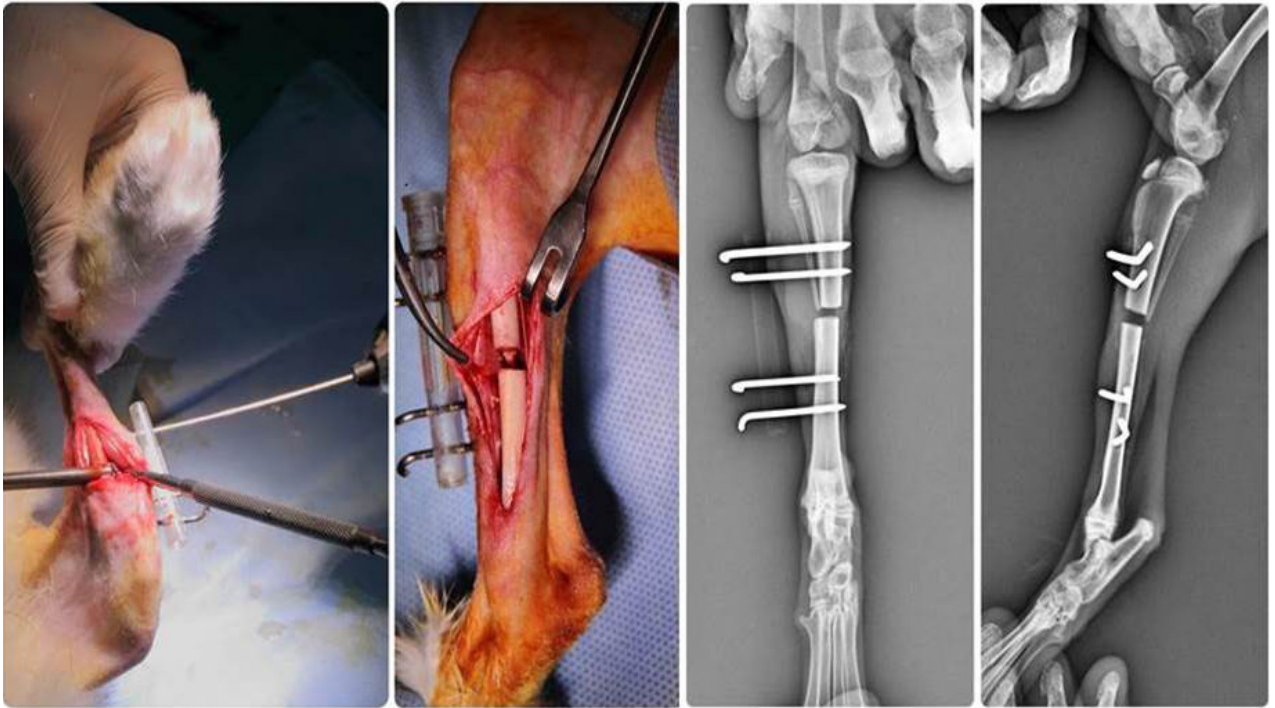
Figure 1. Peroperative and follow-up x-ray images.

mL of distilled water) were prepared and mixed in equal volume. Bone samples were kept in a mixture of formic acid sodium citrate solution for one week. The solution was changed daily to improve decalcification. The decalcified tissues were washed in running tap water for 24 h, passed through alcohol, cleared with xylene, and embedded in paraffin longitudinally. Five sections of 6 microns were taken from each tissue sample. Hematoxylin and eosin staining was used for examining the histological structure of the bone tissue, whereas Safranin O-Fast Green staining was preferred to investigate hyaline cartilage and collagen structure in the area of calcification.

Moreover, TGF-β containing cells in tissue samples were immunohistochemically stained with the streptavidin-biotin method. Inflammation, angiogenesis, fibrous tissue, collagen fiber, safranin O-positive cartilage tissue, chondrocyte hypertrophy, and newly formed bone tissue density were evaluated in terms of histological changes, and scoring was performed. Image analysis was performed using a personal computer, a camera, software (Olympus SC30), and high-powered light microscopy (CX-41, Olympus). A seven-point semiquantitative scoring system (modified Allen's grading) [31,32] was used for the evaluation of fracture healing. The adopted scoring system is presented in Table 1.

All data were presented as mean. Non-parametric analyses (Kruskal–Wallis and Mann–Whitney tests) were used due to the small sample size to compare the differences between control and PRP groups. P-values less than 0.05 were considered significant.

3. Results

During the follow-up period, one rabbit died on day 8, another rabbit died on day 16, and both were excluded from the study. The results of the remaining eight rabbits (totally 8 specimens for control group, and 8 specimens for PRP group) were presented in this paper. In the tibial defected zone of the rabbits, histological findings of different intensities were obtained between PRP and control groups. The mean grade score was higher in the PRP group compared to the control group, according to Allen's fracture healing score (3.2 vs. 1.3) (P < 0.05). In both groups, an increased number of fibroblasts in the callus area, fibrocartilaginous tissue, hyaline cartilage proliferation, and soft and hard callus area formation characterized by hypertrophic chondrocytes were observed (Figure 2– 5). Besides, many newly formed small blood vessels and proliferated cells around these vessels were also observed (Figure 4). The rabbits in the PRP group were generally in grades 3 and 4, according to Allen's grading (Table 2).

In the defected zone of the control group, vascular fragmentation and scattered bone marrow were observed, but rare appearance of inflammatory cells, increase of intense fibrous tissue, vascular invasions, and very little hyaline cartilage proliferation indicated newly developing

Table 1. Allen's fracture healing scoring.

| Healing	Score
Nonunion (fibrous tissues)	0
Incomplete cartilage union (cartilage with some fibrous tissues)	1
Complete cartilage union (entirely cartilage)	2
Incomplete bony union with phase of ossification (predominantly cartilage with some trabecular bone)	3
Incomplete bony union with intermediate phase of ossification (equal amounts of cartilage and trabecular bone)	4
Incomplete bony union with late phase of ossification (predominantly trabecular bone with some cartilage)	5
Complete bony union (entirely bone)	6
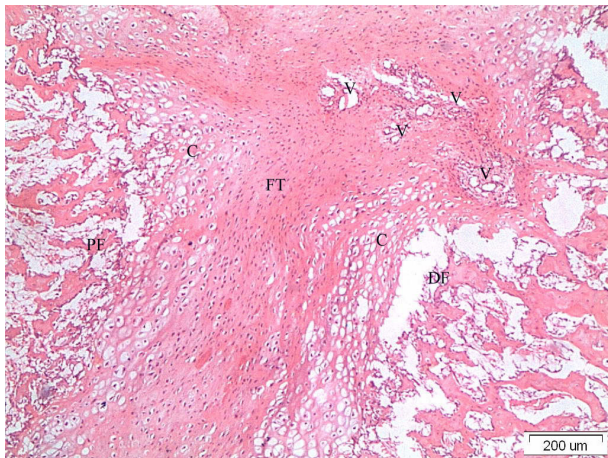
Figure 2. Micrograph of grade 1 in the control group. It displays the formation of severe vascular invasion and proliferative fibrous tissue, hyaline cartilage tissue. V: vascular invasions, C: hyaline cartilage, PF: proximal fracture of tibia, DF: distal fracture of the tibia. H&E staining.
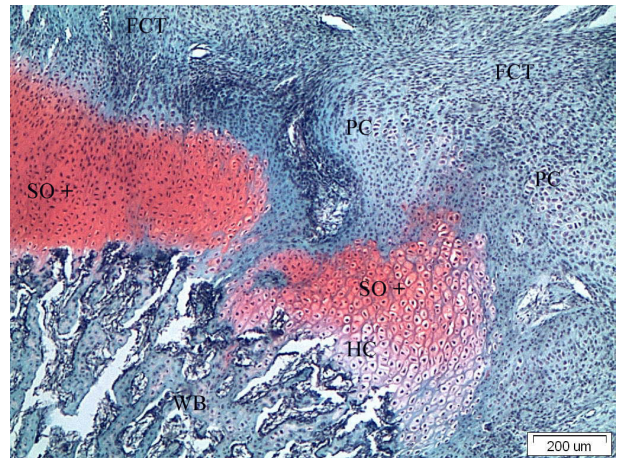

Figure 3. Micrograph of grade 1 in the control group. It shows the formation of safranin O positive hyaline cartilage and network of immature bone (woven bone) associated with endochondral ossification. SO +: safranin O positive hyaline cartilage, FCT: proliferative fibro-cartilaginous tissue, HC: hypertrophied chondrocytes, PC: proliferative chondrocytes, WB : remodeling woven bone. Fast green safranin O staining.

callus formation (Figure 2). Most of the specimens in the control group were found to have fibroblasts that form a soft callus, chondrocyte-like cells, and some safranin-positive proliferative hyaline cartilage tissue (Figure 3). According to these findings, it was concluded that the callus areas were generally grades 0 and 2 in the control group (Table 2).

In the PRP group, hypertrophic chondrocytes indicating the terminal ossification stage were abundant. Lamellar bone tissue was observed in the hard callus areas of the fracture ends, which was a sign of the remodeling process (Figure 4,5). In the PRP group, a more considerable amount of hard callus (woven bone) and a small amount of soft callus containing fibrocartilaginous tissue showed that endochondral ossification had started earlier than the control group. Although the trabecular bone was developed by endochondral ossification in the

PRP group, it was determined that the callus zone did not consist entirely of mature bone cells, instead, the gap between fracture ends was mostly filled with hyaline cartilage model (Figure 5). At the ends of the lamellar bones, a minimal amount of fibrocartilaginous tissue with membranous ossification was observed, whereas, in the trabecular bones, callus formation began with an endochondral ossification (Figure 4, 5).

Safranin-O staining showed an intensive positivity in the areas with soft callus, and in the areas of cartilage matrix with mineralized hard callus, Safranin-O staining showed partial positivity. It was concluded that these areas consisted of hypertrophic chondrocytes and partially cartilage tissue matrix (Figure 5). In the control group, findings such as an abundant increase in fibrous tissue
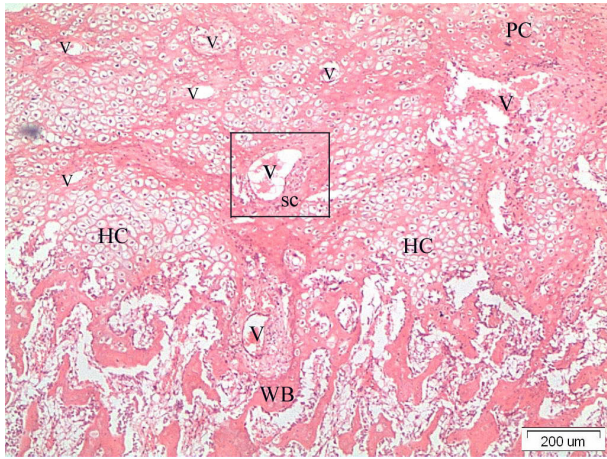
Figure 4. Micrograph of grade 3 in the PRP treated rabbit. It displays the formation of vascular invasion, stem cells, proliferative and hypertrophied chondrocytes, and network of immature bone (woven bone) associated with endochondral ossification. V: vascular invasions, SC: stem cells, HC: hypertrophied chondrocytes, PC: proliferative chondrocytes, WB: remodeling woven bone. Hematoxylin and Eosin staining.
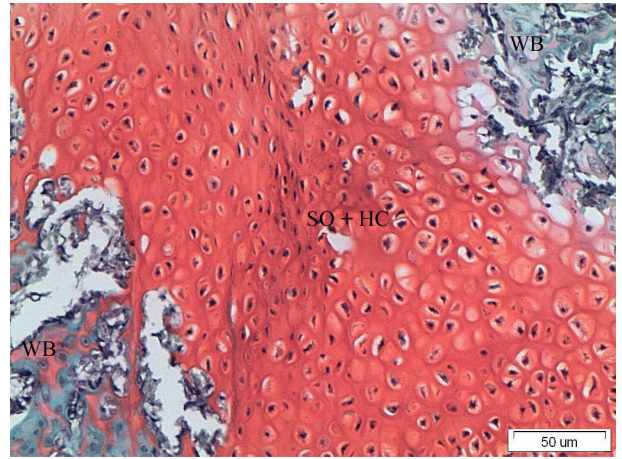

Figure 5. Micrograph of grade 4 in the PRP treated rabbit. It shows the formation of hyaline cartilage and network of immature bone (woven bone) associated with endochondral ossification. SO + HC: safranin O positive hyaline chondrocytes, WB: remodeling woven bone. Fast green safranin O staining.

and the formation of angiogenesis in addition to the presence of a small amount of hyaline cartilage tissue were considered as an indicator of the slow development of the endochondral ossification process.

Above all, in immunohistochemical staining, proliferative chondroblasts and fibroblast-like cells in the callus area were stained TGF-β immunopositive with the streptavidin-biotin method. On the other hand, hypertrophic chondrocytes were immunonegative. In the woven bone tissue chondroblasts, osteoblasts, osteoid tissue, and in the mature bone tissue, most of the osteocytes were immunopositive (Figure 6 A–6D). In addition, the stem cells, matrix, fibroblasts, and chondroblasts in the areas, in which angiogenesis formated, were intensively detected and stained TGF-β immunopositive (Figure 6A). The proliferative chondroblasts, vascular invasion, and presence of hard callus areas were more pronounced in the PRP group comparing to the control group, which resulted in an increased TGF-β immune positive matrix and cell density. Other histological properties were similar in both groups in terms of immunopositivity.

According to Allen's fracture healing grading, a higher mean grade score was observed in the PRP group comparing to the control group, suggesting faster healing with the administration of PRP (P < 0,05).

4. Discussion

Formation of new bone is a long process and brings complication risks, which can be reduced by shortening the treatment period. Several methods, including

Table 2. Mean fracture healing scores in rabbit tibia after distraction.

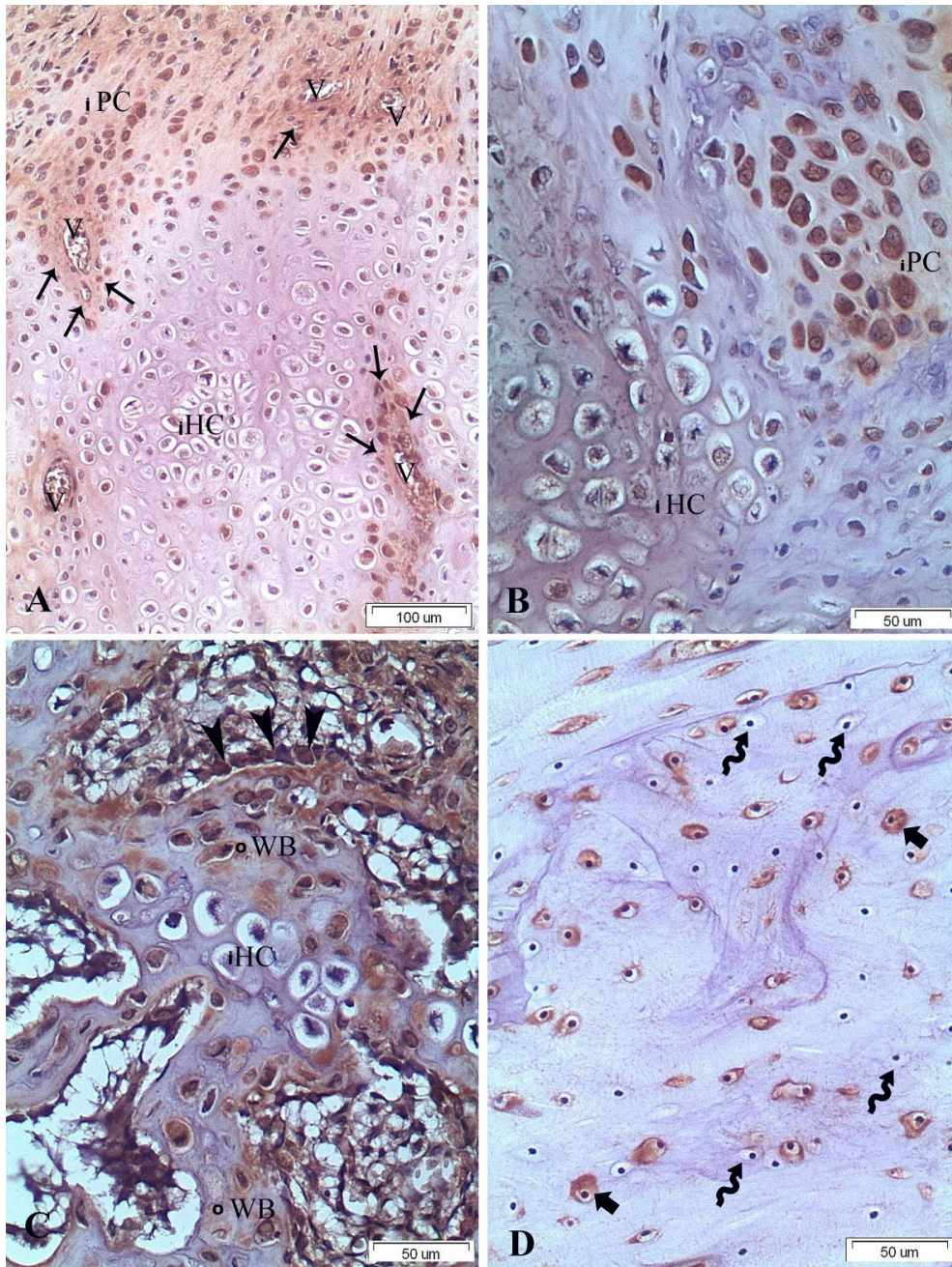| Allen's fracture healing score	Control group (n = 4)	PRP group (n = 4)
Fibrous tissues	3	0
Cartilage with some fibrous tissues	4	1
Entirely cartilage	2	3
Predominantly cartilage with some trabecular bone	0	4
Equal amounts of cartilage and trabecular bone	0	3
Predominantly trabecular bone with some cartilage	0	0
Entirely bone	0	0
PRP: Platelet rich plasma		

Figure 6. Transforming growth factor-beta immunopositive cells in the PRP treated rabbit. It shows positive cells in the callus zone. iHC: immunonegative hypertrophic chondrocytes, iPC: immunopositive proliferative chondrocytes, oWB: osteoblast-like immunopositive cells in woven bone, V: vascular invasion, arrows: immunopositive stem cells in the vascular invasion areas, bold arrows: immunopositive osteocytes, curved arrows: immunonegative osteocytes. Streptavidin biotin peroxidase staining.

intramedullary nailing, low-intensity ultrasound, recombinant growth hormone, were proposed in order to reduce treatment time. PRP is developed from autologous blood, and there are published studies about fracture healing [5,6,7]. However, there are also controversial results in the literature regarding the effect of PRP on bone regeneration [33,34].

Among all these studies, TGF-β and PRP effects on segmental bone defects were rarely analyzed. In our study, histological findings were in different intensities between control and PRP groups. We saw endochondral ossification primarily occurred in the trabecular bone areas of defected tibias and intramembranous bone tissue formation in the lamellar bone regions. Besides,

hypertrophic chondrocytes, together with the Safranin-O positive matrix, were observed in the calcified newly formed hard callus areas. These data made us think about the possible effect of TGF-β in PRP. So, in this study, we aimed to investigate the effect of TGF-β via PRP in segmental bone defects of rabbit tibia.

TGF-β stimulates differentiation of the cell types involved in the healing process [35]. While TGF-β increases mitosis in the bones during fracture healing, it also supports the formation of hyaline cartilage. The presence of TGF-β immunopositive angiogenetic cells and chondroblast density in rabbit tibias treated with PRP may indicate that PRP has a positive effect on cartilage tissue neogenesis via TGF-β. Besides, systemic and local administration of TGF-β1 has been shown to enhance bone remodeling and fracture healing in animal models [36,37].

Marx et al. reported that the administration of PRP to grafts might accelerate bone maturation rate 1.6 to 2.1 times compared to grafts without PRP [38]. They mentioned that fundamental growth factors such as PDGF and TGF-β enhance the rate of bone formation and increase the amount of bone formed. The role of growth factors such as TGF-β, IGF-1, and basic fibroblast growth factor (bFGF), has been investigated in many studies [39,40,41]. Farhadieh et al. studied the roles of growth factors such as TGF-β, IGF-1, bFGF, and reported that the presence of growth factors (IGF-1 and bFGF) in the union region with high concentration might lead to osteoblast proliferation and formation from precursor mesenchymal cells [39].

Zimmermann et al. prospectively assessed systemic changes in TGF-β levels of patients with delayed healing and nonunion of long bone fractures [42]. He found that in both normal and nonhealing groups, the serum level of TGF-β1 increased within the first two weeks after fracture, but the delayed healing group had a faster decline of serum concentration between 2 and 4 weeks after the trauma and its level was significantly lower in the delayed fracture-healing group at four weeks. Systemic levels of TGF-β were found to vary based on smoking status, age, sex, diabetes mellitus, and chronic alcohol abuse at different time points [43]. Moreover, Sarahrudi found no significant differences in the TGF-β1 concentrations of delayed and normal fracture healing groups [44]. So, an immunohistological investigation was performed in our study to eliminate such conditions.

Does exogenous TGF-β work on the fracture site? Lind et al. applied 10 μg/day TGF-β to rabbit tibias for six weeks and found increased maximal bending strength and callus formation, but they reported no effect on bending-stiffness, bone mineral content, cortical thickness or Haversian canal diameter [45]. Again, immunohistological investigations were needed to reveal more definite conclusions.

Srouji et al. investigated bone regeneration induced by TGF-β and IGF-1-containing hydrogel scaffolds in rat tibia defect models [46]. They found that scaffolds containing TGF-β could function both osteoinductive and osteoconductive as a matrix for TGF-β, and a site for bone osteointegration. Nevertheless, exogenous TGF may be hard to provide, so PRP seems to be the most affordable way of the belief "treat the person with the person himself".

In a similar animal study model, Kanthan et al. reported good outcomes with PRP in larger bone defects [47]. They reported enhancements in the repair of delayed bone unions involving critical-sized defects with PRP. They concluded that a bone-replacement material was needed for PRP to be efficient in a 2 cm - defect model of rabbit tibiae. However, their defect was five times larger than our model. Thus, further studies may reveal how large defect size needs graft with PRP.

Despite promising results, this present study has several limitations: the number of subjects would be higher, biped animals would be chosen instead of quadruped animals to mimic human behavior more, and biomechanical tests would be applied. These may all be inspirations for future works.

5. Conclusion
In this study, the effectiveness of TGF-β via PRP on segmental bone defect healing was investigated. PRP administration increases bone union, reduces the healing time, and with these advantages, it may provide a more cost-effective treatment with fewer complications in the treatment of segmental bone defects.

Conflict of Interest
The authors have no conflict of interest to declare.

References

1. Rawashdeh MA, Telfah H. Secondary alveolar bone grafting: the dilemma of donor site selection and morbidity. British Journal of Oral Maxillofacial Surgery 2008; 46 (8): 665- 670.

2. Jeong W, Kim YS, Roh TS, Kang EH, Jung BK, et al. The effect of combination therapy on critical-size bone defects using non-activated platelet-rich plasma and adipose-derived stem cells. Child's Nervous System 2019: 1-7.

3. Marx RE. Platelet-rich plasma: evidence to support its use. Journal of Oral and Maxillofacial Surgery 2004; 62 (4): 48 - 496.

4. Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T. Platelet-rich plasma enhances human osteoblast-like cell proliferation and differentiation. Journal of Oral Maxillofacial Surgery 2005; 63 (3): 362-369.

5. Rai B, Oest ME, Dupont KM, Ho KH, Teoh SH, et al. Combination of platelet-rich plasma with polycaprolactone-tricalcium phosphate scaffolds for segmental bone defect repair. Journal of Biomedical Material Research Part A. 2007; 81 (4): 888-899.

6. Sarkar MR, Augat P, Shefelbine SJ, Schorlemmer S, Huber-Lang M, et al. Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials 2006; 27 (9): 1817-1823.

7. Kitoh H, Kitakoji T, Tsuchiya H, Mitsuyama H, Nakamura H, et al. Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis-a preliminary result of three cases. Bone 2004; 35 (4): 892-898.

8. Cole BJ, Seroyer ST, Filardo G, Bajaj S, Fortier LA. Platelet-rich plasma: where are we now and where are we going? Sports Health 2010; 2 (3): 203-210.

9. Chicharro-Alcantara D, Rubio-Zaragoza M, Damia-Gimenez E, Carrillo-Poveda JM, Cuervo-Serrato B, et al. Platelet Rich Plasma: New Insights for Cutaneous Wound Healing Management. Journal of Functional Biomaterials 2018; 9 (1).

10. Drago L, Bortolin M, Vassena C, Taschieri S, Del Fabbro M. Antimicrobial activity of pure platelet-rich plasma against microorganisms isolated from oral cavity. BMC Microbiology 2013; 13: 47.

11. Comella K, Bell W. First-in-man intraglandular implantation of stromal vascular fraction and adipose-derived stem cells plus platelet-rich plasma in irradiation-induced gland damage: a case study. International Medical Case Reports Journal 2017; 10: 295-299.

12. Fu W, Li Q, Li J. Application of platelet-rich plasma in clinical orthopedics. Chinese Journal of Reparative and Reconstructive Surgery 2014; 28 (10): 1311-1316.

13. Landesberg R, Roy M, Glickman RS. Quantification of growth factor levels using a simplified method of platelet-rich plasma gel preparation. Journal of Oral Maxillofacial Surgery 2000; 58 (3): 297-300.

14. Hermeto LC, Rossi R, Padua SB, Pontes ER, Santana AE. Comparative study between fibrin glue and platelet rich plasma in dogs skin grafts. Acta Cirurgica Brasiliera 2012; 27 (11): 789-794.

15. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: from basic science to clinical applications. American Journal of Sports Medicine 2009; 37 (11): 2259-2272.

16. Pavlovic V, Ciric M, Jovanovic V, Stojanovic P. Platelet Rich Plasma: a short overview of certain bioactive components. Open Medical (Wars) 2016; 11 (1): 242-247.

17. Murphy MK, Huey DJ, Hu JC, Athanasiou KA. TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells. Stem Cells 2015; 33 (3): 762-773.

18. Mehlhorn AT, Niemeyer P, Kaschte K, Muller L, Finkenzeller G, et al. Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. Cell Proliferation 2007; 40 (6): 809-823.

19. Ramezanifard R, Kabiri M, Hanaee Ahvaz H. Effects of platelet rich plasma and chondrocyte co-culture on MSC chondrogenesis, hypertrophy and pathological responses. Experimental and Clinical Sciences, International Online Journal for Advances in Sciences 2017; 16: 1031-1045.

20. Liou JJ, Rothrauff BB, Alexander PG, Tuan RS. Effect of platelet-rich plasma on chondrogenic differentiation of adipose- and bone marrow-derived mesenchymal stem cells. Tissue English Part A. 2018; 24 (19-20) : 1432-1443.

21. Wu MY, Hill CS. Tgf-beta superfamily signaling in embryonic development and homeostasis. Developmental Cell 2009; 16 (3): 329-343.

22. Ghosh D, McGrail DJ, Dawson MR. TGF-beta1 Pretreatment improves the function of mesenchymal stem cells in the wound bed. Frontiers in Cell and Developmental Biology. 2017; 5: 28.

23. Somoza RA, Welter JF, Correa D, Caplan AI. Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. Tissue Engineering Part B Reviews 2014; 20 (6): 596-608.

24. Malhotra A, Pelletier MH, Yu Y, Walsh WR. Can platelet-rich plasma (PRP) improve bone healing? A comparison between the theory and experimental outcomes. Archives of Orthopedics and Trauma Surgery 2013 ; 133 (2) : 153 - 165.

25. Kumar GS. Orban's Oral Histology and Embryology. 14 ed: Elsevier (A Divisionof Reed Elsevier India Pvt. Limited); 2015.

26. Rahman MS, Akhtar N, Jamil HM, Banik RS, Asaduzzaman SM. TGF-beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Research. 2015; 3: 15005.

27. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. The Journal of Biochemistry 2010; 147 (1): 35-51.

28. Dhurat R, Sukesh M. Principles and methods of preparation of platelet-rich plasma: a review and author's perspective. Journal of Cutaneous and Aesthetic Surgery. 2014; 7 (4): 189-197.

29. Nagata MJ, Messora MR, Furlaneto FA, Fucini SE, Bosco AF, et al. Effectiveness of two methods for preparation of autologous platelet-rich plasma: an experimental study in rabbits. European Journal of Dentistry 2010; 4 (4): 395-402.

30. Singhal P, Agarwal S, Dhot PS, Sayal SK. Efficacy of platelet-rich plasma in treatment of androgenic alopecia. Asian Journal of Transfusion Science 2015; 9 (2): 159-162.

31. Allen HL, Wase A, Bear WT. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. Acta Orthopaedica Scandinavica 1980; 51 (4): 595-600.

32. Estai MA, Soelaiman IN, Shuid AN, Das S, Ali AM, et al. Histological changes in the fracture callus following the administration of water extract of piper sarmentosum (daun kadok) in estrogen-deficient rats. Iranian Journal of Medical Sciences 2011; 36 (4): 281-288.

33. Swennen GR, Schutyser F, Mueller MC, Kramer FJ, Eulzer C, et al. Effect of platelet-rich-plasma on cranial distraction osteogenesis in sheep: preliminary clinical and radiographic results. International Journal of Oral Maxillofacial Surgery 2005; 34 (3): 294-304.

34. Ali AM, El-Alfy B, Amin M, Nematalla M, El-Shafaey el SA. Can platelet-rich plasma shorten the consolidation phase of distraction osteogenesis? An experimental study. European Journal of Orthopedic Surgery and Traumatology 2015; 25 (3): 543-548.

35. Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. The Journal of Cell Biology 1990; 110 (6): 2195-2207.

36. Wildemann B, Schmidmaier G, Ordel S, Stange R, Haas NP, et al. Cell proliferation and differentiation during fracture healing are influenced by locally applied IGF-I and TGF-beta1: comparison of two proliferation markers, PCNA and BrdU. Journal of Biomedical Materials Research Part B: Applied Biomaterials 2003; 65 (1): 150-156.

37. Schmidmaier G, Wildemann B, Heeger J, Gabelein T, Flyvbjerg A, et al. Improvement of fracture healing by systemic administration of growth hormone and local application of insulin-like growth factor-1 and transforming growth factor-beta1. Bone 2002; 31 (1): 165-172.

38. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, et al. Platelet-rich plasma: Growth factor enhancement for bone grafts. Oral Surgery, Oral Medicine, Oral Pathology, and Oral Radiology. 1998; 85 (6): 638-646.

39. Farhadieh RD, Dickinson R, Yu Y, Gianoutsos MP, Walsh WR. The role of transforming growth factor-beta, insulin-like growth factor I, and basic fibroblast growth factor in distraction osteogenesis of the mandible. Journal of Craniofacial Surgery 1999; 10 (1): 80-86.

40. Liu Z, Luyten FP, Lammens J, Dequeker J. Molecular signaling in bone fracture healing and distraction osteogenesis. Histology and Histopathology 1999; 14 (2): 587-595.

41. Steinbrech DS, Mehrara BJ, Rowe NM, Dudziak ME, Luchs JS, et al. Gene expression of TGF-beta, TGF-beta receptor, and extracellular matrix proteins during membranous bone healing in rats. Plastic and Reconstructive Surgery 2000; 105 (6): 2028-2038.

42. Zimmermann G, Henle P, Küsswetter M, Moghaddam A, Wentzensen A, et al. TGF-β1 as a marker of delayed fracture healing. Bone 2005; 36 (5): 779-785.

43. Kaiser G, Thomas A, Köttstorfer J, Kecht M, Sarahrudi K. Is the expression of transforming growth factor-Beta1 after fracture of long bones solely influenced by the healing process? International Orthopaedics 2012; 36 (10): 2173-2179.

44. Sarahrudi K, Thomas A, Mousavi M, Kaiser G, Kottstorfer J, et al. Elevated transforming growth factor-beta 1 (TGF-beta1) levels in human fracture healing. Injury 2011; 42 (8): 833-837.

45. Lind M, Schumacker B, Soballe K, Keller J, Melsen F, et al. Transforming growth factor-beta enhances fracture healing in rabbit tibiae. Acta Orthopaedica Scandinavica 1993; 64 (5): 553-556.

46. Srouji S, Blumenfeld I, Rachmiel A, Livne E. Bone defect repair in rat tibia by TGF-beta1 and IGF-1 released from hydrogel scaffold. Cell and Tissue Banking 2004; 5 (4): 223- 230.

47. Kanthan SR, Kavitha G, Addi S, Choon DS, Kamarul T. Platelet-rich plasma (PRP) enhances bone healing in non-united critical-sized defects: a preliminary study involving rabbit models. Injury 2011; 42 (8): 782-789.