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ORIGINAL ARTICLE

# Tetracalcium phosphate treatment on experimental fracture model in rats

Burak Kaymaz, MD[1], Onur Yılmaz, MD[2], Ali Osman Taşova, MD[1], Doğukan Anapa, MD[1]

[1]Department of Orthopedics and Traumatology, Çanakkale Onsekiz Mart University Faculty of Medicine, Çanakkale, Turkey
[2]Department of Orthopedics and Traumatology, Çanakkale State Hospital, Çanakkale, Turkey

## ABSTRACT

**Objectives:** This study aims to investigate the efficacy of tetracalcium phosphate (TTCP) on fracture healing in rat femurs.

**Materials and methods:** Forty-two female Wistar Albino rats were randomized into two groups (Group 1 and Group 2, n=21 for each). The left femur of all animals was fractured by osteotomy after deep anesthesia with ketamine. Additional procedure was not applied to the rats in Group 1. Rats in Group 2, following osteotomy, were applied to the fracture line approximately 2 mL TTCP. The animals were sacrificed at Weeks 1, 2, and 3 after surgery (seven animals were sacrificed from each group each week) and the broken femurs were removed. The femurs were examined first radiographically and second histopathologically.

**Results:** Radiologically, callus maturity and bone union increased with time in both groups. However, no significant differences were found regarding callus maturity and bone union in weekly comparisons (anteroposterior plain: p=0.53, p=0.37, p=0.42, lateral plain: p=0.26, p=0.42, p=0.87). Histopathologically, the fractures healed normally as the weeks progressed in both groups. The histological scores of Group 2 were higher at Weeks 1, 2, and 3. In the evaluation, no significant difference was found between the groups in terms of histological scores except for the first week (p=0.024, p=104, p=462, respectively).

**Conclusion:** Although there was no statistically significant difference in the histological evaluation of both groups, except for the first week, the histological scores of Group 2, which underwent TTCP in all weeks, were higher. According to the results of this study, we believe that TTCP may be beneficial, particularly in the early stages of fracture healing.

***Keywords:*** Fracture, fracture healing, tetracalcium phosphate.

Fracture healing is the result of highly ordered physiological and cellular pathways to restore the structure and function of broken bones. Therefore, bone, unlike other types of tissues, heals when the damaged area of the bone is completely reshaped biochemically and biomechanically. Many effective mechanisms such as systemic biological factors, biochemical factors, hormonal factors, and biomechanical factors affect fracture healing.[1,2] A problem that occurs in this process may result in nonunion or late union.[3,4] Bone healing problems are common, particularly in fractures with bone defects. Numerous negative mechanical factors such as excess motion at the fracture site, a large interfragmentary gap and loss of blood supply affect the development of nonunion.[5] On the other hand, although the infection does not cause nonunion on its own, it creates a predisposition by creating similar factors that cause nonunion. Factors such as old age, cachexia and malnutrition, steroids or anticoagulants, anti-inflammatory agents, burns, and radiation may contribute to nonunion, but are not primary causes of nonunion.[6] In the literature, the effects of many minerals and drugs on fracture healing have been investigated. Many treatment methods such as electrical stimulation, low intensity ultrasonography, extracorporeal shock wave therapy and many drugs such as vitamin C, vitamin D, parathyroid hormone,

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**Correspondence**: Onur Yılmaz, MD. Çanakkale Onsekiz Mart Üniversitesi Tıp Fakültesi Ortopedi ve Travmatoloji Anabilim Dalı, 17020 Çanakkale, Türkiye.

E-mail: onurylmz52@gmail.com

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montelukast, and boron have been used to stimulate and help to heal fracture healing.[7-14]

Tetracalcium phosphate (TTCP) is a component used in the formation of some hydroxyapatite calcium phosphate cements used to repair bone defects.[15] Injectable osteoconductive calcium phosphate cements are produced in many types and features, and are used in addition to internal fixation for the treatment of selected fractures. These cements harden at normal physiological pH and body temperature, unlike polymethylmethacrylate (PMMA) cements, which cause local cellular destruction due to the high temperature reaching up to 60°C with the exothermic reaction it generates while hardening. Therefore, it does not cause local cell death or denature the body's proteins.[16] It, then, continues to crystallize and harden *in vivo*, reaching 50% of the ultimate compressive strength within 1 h and 80% of the ultimate compressive strength within 4 h. Therefore, it provides mechanical support when applied to areas with bone defects. As the final crystal structure is 50 to 60% porous and negatively charged, it can bind to proteins and circulating endogenous growth factors can bind to bone cement that stimulates bone healing and remodeling. After application, it is replaced by host bone over time *in vivo* with cell-mediated remodeling.[16,17] An important advantage of TTCP cements is that it reduces the need for bone grafts, thereby eliminating significant donor site morbidity associated with bone graft procedures. Their general use is to support the detection of the applied metal implant and require implant support. They cannot provide broken fixation alone and show biomechanically weak resistance to shear forces in particular.[17]

In this experimental study, we aimed to investigate the effect of TTCP, which is known to have remodeling potential, on fracture healing.

## MATERIALS AND METHODS

### Animals

Ethical approval was obtained from the Çanakkale Onsekiz Mart University University Experimental Research Application and Research Center (date/no: 2019/09-05). All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (revised, 1985). Forty-two female Wistar Albino rats weighing approximately 250 to 350 g were included. During the experimental procedure, all rats were housed under standard laboratory conditions with an artificial 12-h light/dark cycle. They were caged individually under controlled temperature (22±1°C) and relative humidity and allowed free access to food and water in polycarbonate units. The rats were observed for seven days in the animal care laboratory to exclude any possibility of underlying disease.

### Surgical technique and experimental design

The rats were operated on following an intraperitoneal injection of ketamine hydrochloride (30 mg/kg, Ketalar®, Eczacıbaşı, Istanbul, Turkey) and xylazine anesthesia (10 mg/kg, Xylazinbio®, Bioveta, Ankara, Turkey), as described by Bonnarens and Einhorn.[18] A femoral channel was prepared by means of a 1-mm Kirschner wire (Hipokrat®, Izmir, Turkey) that was inserted through the femoral condyles, and a 0.8 mm Kirschner wire (Hipokrat®, Izmir, Turkey) was inserted into this channel. Then, the animals

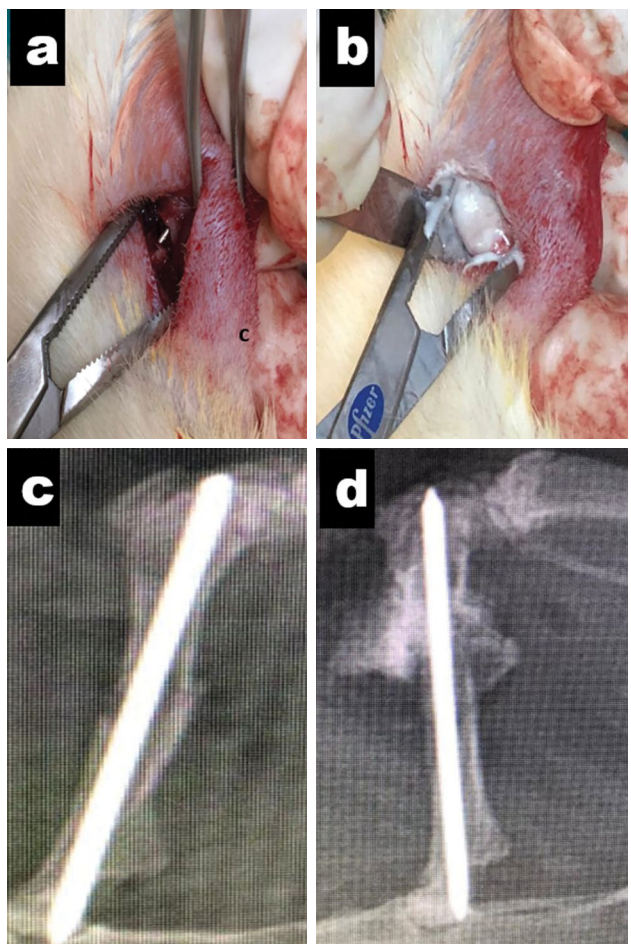


**FIGURE 1. (a)** Fracture formation in the femur and **(b)** application of TTCP. **(c)** Confirmation of the postoperative fracture formation by radiological imaging and **(d)** the radiological appearance of TTCP applied to the fracture site.
TTCP: Tetracalcium phosphate.

were randomized into two groups (Group 1 and Group 2, n= 21 for each). Group 1 was selected as the control group and Group 2 as the TTCP applied group. To constitute fracture after surgery, the osteotomy method was used.[19] The area near the femur was cleaned and shaved for surgery, and an incision was made through the skin across the lateral aspect of the thigh. The fascia latae between the gluteus superficialis and biceps femoris muscles were cut to separate the two muscles, exposing the femur. Subsequently, fractures were created in the left femur diaphysis of the rats using an osteotome and confirmed using a spacer (Figure 1a). The rats in Group 2, following osteotomy, were applied to the fracture line approximately 2 mL of TTCP (Grandus® B-One, Permed Health Products, Çanakkale, Turkey) (Figure 1b). Following the clinical examination, the fracture created with osteotomy was confirmed radiologically (Figure 1c and 1d). The fascia was sutured using a sterile synthetic absorbable suture (Pegelak 3/0, Doğsan, Istanbul, Turkey) followed by the skin with a non-absorbable suture (Propilen 2/0, Doğsan, Istanbul, Turkey). For preoperative antibiotic prophylaxis, 10 mg/kg cefazolin sodium (Cezol 500 mg, Deva, Istanbul, Turkey) was administered subcutaneously. The same antibiotic used preoperatively was also administered subcutaneously at 45 mg/kg/day for three days after surgery. Each animal was housed in its own cage and monitored daily for infection and mobility. Following surgery, the rats were allowed to resume normal activity and were given unrestricted access to food. All animals survived both the fracture procedures throughout the study period. Seven animals from each group were sacrificed by cervical dislocation following ketamine anesthesia (50 mg/kg) at Weeks 1, 2, and 3 the operation. This method has been recommended by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes.[20] The

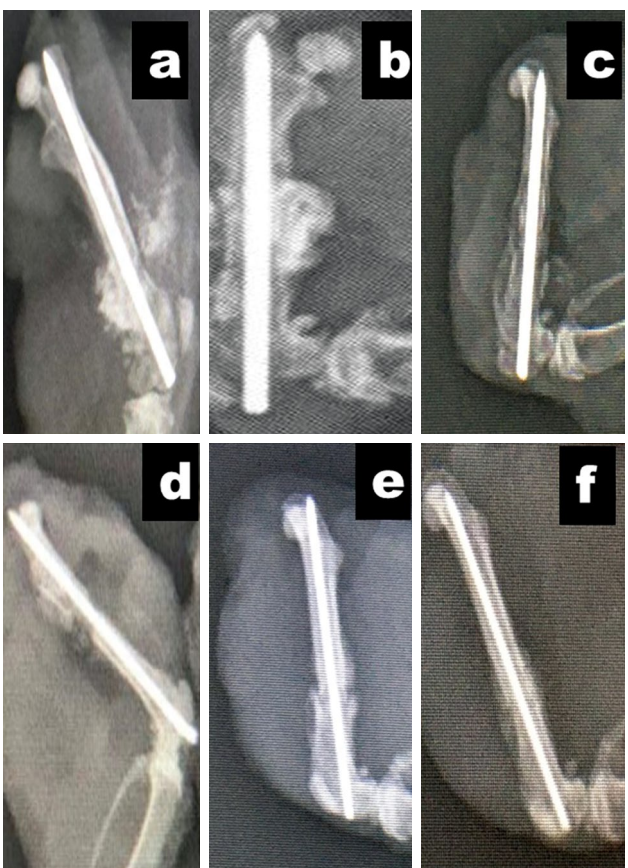


**FIGURE 2.** Radiographic lateral assessment of rat fracture healing femoral radiograms of the rats. **(a)** Week 1 in Group 2, **(b)** Week 2 in Group 2, **(c)** Week 3 in Group 2, **(d)** Week 1 in Group 1, **(e)** Week 2 in Group 2, **(f)** Week 3 in Group 2. Callus maturation and bone union becomes more mature as weeks elapsed.

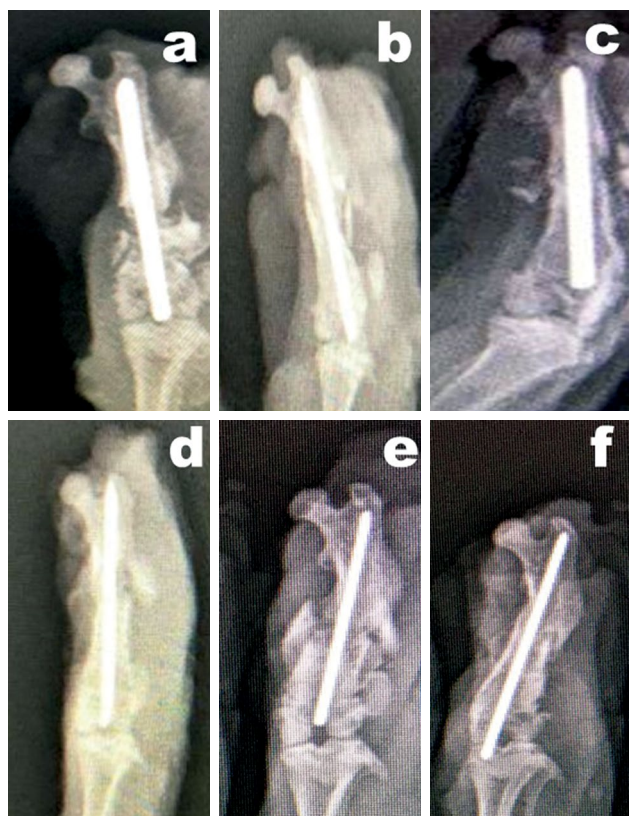


**FIGURE 3.** Radiographic anteroposterior assessment of rat fracture healing femoral radiograms of the rats. **(a)** Week 1 in Group 2, **(b)** Week 2 in Group 2, **(c)** Week 3 in Group 2, **(d)** Week 1 in Group 1, **(e)** Week 2 in Group 2, **(f)** Week 3 in Group 2. Callus maturation and bone union becomes more mature as weeks elapsed.

broken femurs of the animals sacrificed at Weeks 1, 2, and 3 after surgery were disarticulated from the hip and knee joints in order not to damage the callus tissue. The soft tissues on the removed femurs were gently stripped from the bone without damaging the callus tissue, and the names of the groups to which

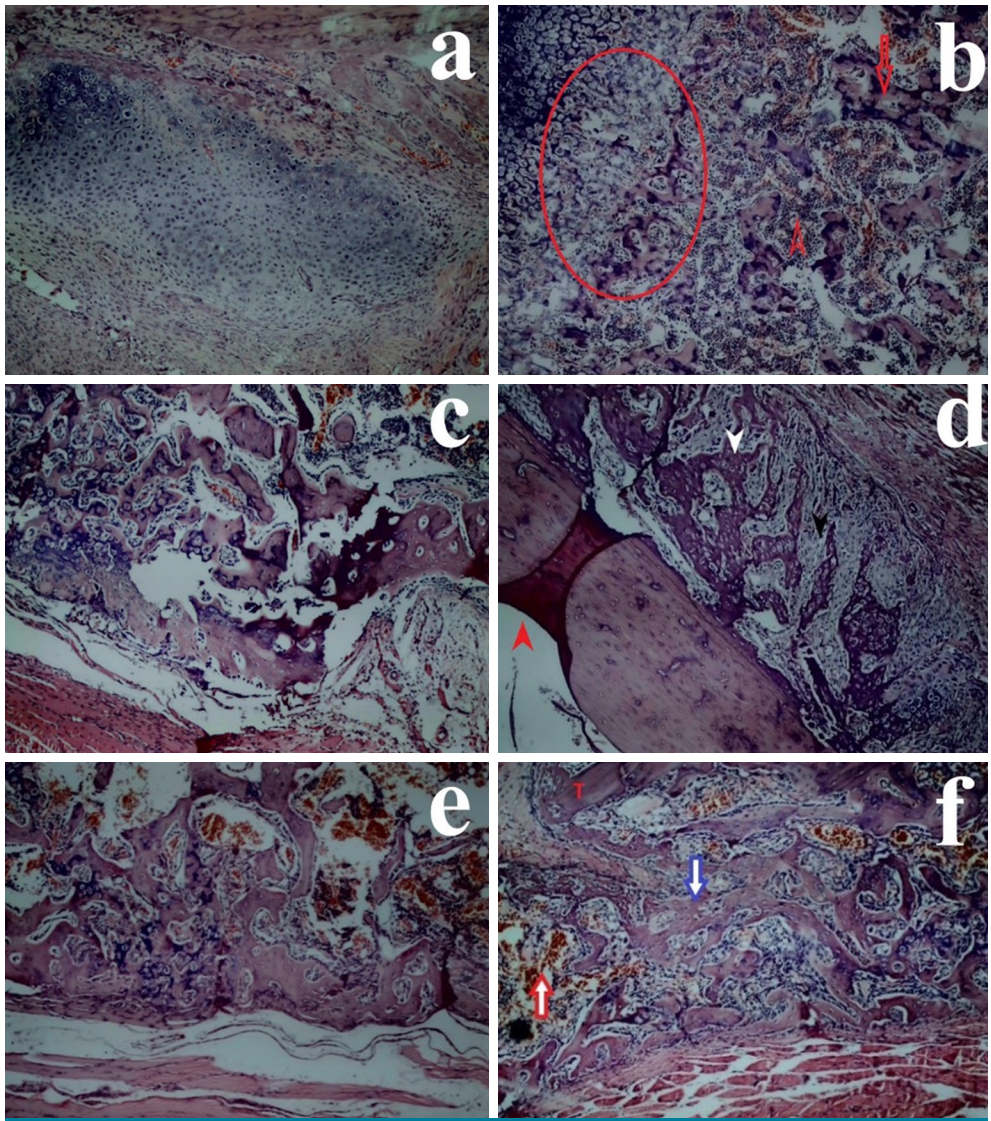


**FIGURE 4.** In comparison of Group 2 and Group 1 within each week of the rats sacrificed at Weeks 1, 2, and 3, Group 2 was found to be higher in histological scoring than Group 1. There was no significant difference between the groups, except for the first week. **(a)** Group 1, Week 1, sections showing callus consisting entirely of cartilage in Grade V callus formation (H&E, ×40). **(b)** Group 2, Week 1, sections showing callus characterized by equal cartilage and immature bone in Grade VII callus formation (H&E, ×40) as well as endochondral bone formation. Red arrow shows calcified trabecular bone and the point of red arrow shows hypercellularity between trabeculae **(c)** Group 1, Week 2, sections showing immature bone callus with a small amount of cartilage in Grade VIII callus formation (H&E, ×40). **(d)** Group 2, Week 2, sections showing callus characterized by immature bone formation in Grade IX callus formation (H&E, ×40). Red arrow shows healing area among minimum gap. White arrow shows new bone matrix and anastomosis of trabeculae. Black arrow shows active osteoblasts synthesizing bone matrix and fusiform progenitor cells **(e)** Group 1, Week 3, sections showing mature bone formation in a Grade X callus formation (H&E, ×10). **(f)** Group 2, Week 3, sections showing mature bone formation in Grade X callus formation (H&E, ×40). It can be seen that early-stage intramembranous ossification occurs. Red arrow shows defect area, hemorrhage, and vanished bone matrix. Blue arrow shows new matrix and lamellar bone formation. T shows old trabeculae.

**TABLE I**
Histological grading of callus tissue

| Grade | Histological findings of callus |
|---|---|
| 1 | Fibrous tissue |
| 2 | More fibrous tissue |
| 3 | Equal fibrous and cartilage tissue |
| 4 | More cartilage and little fibrous tissue |
| 5 | Cartilage tissue |
| 6 | More cartilage and little immature bone |
| 7 | Equal cartilage tissue and immature bone |
| 8 | More immature bone and cartilage tissue |
| 9 | Callus with immature bone |
| 10 | Callus with mature bone |

they belong were classified and numbered and placed in pathology containers containing 10% formalin solution for histopathological evaluation.

### Radiographic evaluation

Posteroanterior and lateral plain X-rays (X-ray system; FUJIFILM Corp., Minato-ku, Tokyo, Japan) of the femur removed from the animals sacrificed at Weeks 1, 2, and 3 after surgery were commented by two orthopedists who were blinded to the nature of the present study. They were asked to evaluate callus maturity according to classification of Goldberg et al.[21] Using this classification, Stage 1 indicates nonunion, Stage 2 indicates possible union, and Stage 3 indicates radiographic union. The mean radiologic scores were calculated for both groups. The anteroposterior (Figure 2) and lateral radiographs (Figure 3) of all groups were evaluated separately.

### Histopathological analyses

Histological evaluations were conducted after clinical and radiological assessments. The bones were fixed in 10% formaldehyde solution. The specimens were decalcified in 10% acetic acid, 85% sodium chloride, and 10% formaldehyde solution for 72 h. After decalcification, longitudinal sections were taken from the fracture site, including the proximal and distal sites of the fracture. All slides were stained with standard hematoxylin and eosin (H&E) methods and evaluated by a pathologist who was blinded to the groups (Figure 4). Fracture healing was graded (I-X), as described by Huo et al.[22] (Table I), based on the ratios of the fibrous tissue, fibrocartilage, cartilage, and bone areas in the fracture site.

### Statistical analyses

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). The normality of the distribution for the variables was tested using the Shapiro-Wilk normality test. Non-parametric tests were used for variables without normal distribution. Continuous data without normal distribution were analyzed using the Mann-Whitney U test. Quantitative data were expressed in mean ± standard deviation (SD) or median (min-max). The Cohen's kappa statistical analysis was used to evaluate inter-observer agreement, and the results were interpreted according to Landis and Koch's guidelines with 0.00-0.20 indicating very low agreement, 0.21-0.40 indicating minor agreement, 0.41-0.60 indicating moderate agreement, 0.61-0.80 indicating adequate agreement, and 0.80-1.00 indicating strong agreement.[23] A $p$ value of $<0.05$ was considered statistically significant with 95% confidence interval (CI).

## RESULTS

### Radiographic analysis

Callus formation, remodeling, and bridging bone formation were evaluated on the radiograms of the femur removed at Weeks 1, 2, and 3. The two orthopedists demonstrated adequate inter-observer agreement for anteroposterior and lateral radiographs (kappa=0.627 and 0.656, respectively). Callus maturity and bone union increased every week according to the Goldberg criteria; however, the comparisons of the weekly values of both groups were non-significant (Table II). Radiological examination failed to show any beneficial or harmful effects of TTCP on fracture healing.

**TABLE II**
The mean radiological stages of both groups

| | Radiologic stage of anteroposterior | | | Radiologic stage of lateral | | |
|---|---|---|---|---|---|---|
| | Week 1 | Week 2 | Week 3 | Week 1 | Week 2 | Week 3 |
| Group 1 (n=21) | 1.07 | 1.35 | 1.71 | 0.93 | 1.14 | 1.78 |
| Group 2 (n=21) | 1.14 | 1.57 | 1.92 | 1.2 | 1.43 | 1.85 |
| p-values | 0.53 | 0.37 | 0.42 | 0.26 | 0.42 | 0.87 |

**TABLE III**
Grade of histological score assessment between groups in rats

| Week | Groups | Histopathologic grade | | *p* |
|---|---|---|---|---|
| | | Min-Max | Mean±SD | |
| Week 1 | Group 1 | 5-7 | 6.3±0.8 | 0.024 |
| | Group 2 | 6-9 | 7.9±1.2 | |
| Week 2 | Group 1 | 7-9 | 7.7±0.8 | 0.104 |
| | Group 2 | 7-9 | 8.4±0.8 | |
| Week 3 | Group 1 | 7-10 | 8.7±1.2 | 0.462 |
| | Group 2 | 8-10 | 9.1±0.9 | |

**Histological analysis**

The progression of fracture callus in all samples taken for histopathological examination from both groups was calculated according to weeks. It was seen at Weeks 1, 2, and 3 after surgery that the fractures healed in a remarkably steady fashion in both groups. Histopathological scores of Group 2 were statistically significantly higher in the evaluation made for the first week callus progression and fracture healing (p=0.024). In the evaluation made in terms of callus progression at Weeks 2 and 3, there was no statistically significant difference between the groups, although Group 2 had higher scores than Group 1 (p=0,104, p=0.462). Histopathological examination was not able to exert any stimulating or inhibitory effects of TTCP treatment on fracture healing, except for the first week. The minimum-maximum values, averages and *p* values of all histopathological results at Weeks 1, 2, and 3 postoperatively are summarized in Table 3.

## DISCUSSION

The fracture healing consists of three stages, which are roughly the inflammatory phase, the repair phase, and the remodeling phase, which follow each other and can be intertwined in places.[1] The negativities that may occur in each of these stages would delay or prevent the healing of the fracture and the formation of the mature bone desired. The negative effects of prolonged release of inflammatory cytokines on bone are known; however, short-term controlled inflammatory response is critical in fracture healing. The acute inflammatory response reaches its highest level in the first 24 h and continues until Day 7.[24]

Although many bioactive cements fracture healing has been studied in the current literature, to the best of our knowledge, there is a limited number of experimental studies on the effect of TTCP, the bioactive cement we use in fracture healing. In our study, we examined the effects of TTCP on the healing of fractures in rat femurs. In this study, we believe that TTCP may be useful on fracture healing, particularly in the early stages of fracture healing. In this process, which coincides with the inflammatory phase, which is the first phase of fracture healing, we believe that the implant applied supports fracture stabilization, as well as the osteoconductive effect in the fracture line. The main finding of this study is that the use of TTCP in addition to the intramedullary implant in diaphyseal fractures makes a significant histopathological contribution in the early stages of fracture healing.

Preclinical animal studies have shown that TTCP is osteoconductive and gradually reshapes over time.[25,26] In the study of the fracture with the defect in the proximal tibia in dogs, the bone defect filled with TTCP showed significant bone placement in a short period of time, such as two weeks. In the following months, evidence of remodeling similar to normal bone and evidence of cement's osteoclastic absorption, vascular ingrowth and bone formation were reported.[25] In histological study in humans, tricalcium phosphate (TCP) was reported to be settled to the bone tissue completely one year later.[27] In this study, the authors also reported no vascularization into the cement and no fibrous tissue formation.

Cadaver studies have shown that, in some fractures of the radius distal, tibia plateau, proximal femur and calcaneus, in addition to traditional internal fixation, the use of TTCP cement can produce better stability, stiffness and strength than using implant fixation alone.[25,28] Clinical studies have demonstrated that TCP augmentation in tibial plateau and calcaneus fractures provides a reduction in the time required to achieve full postoperative load time, faster power and range of motion when used in distal radius fractures, and better stability in some hip fractures.[17]

It is emphasized that calcium phosphate cements can be used in addition to fixation in the treatment of metaphyseal fractures such as distal radius, tibial plateau, calcaneus, hip and possibly spine.[17] Kiakojoori et al.[29] reported, in their meta-analysis studies, that calcium phosphate cements had poor to moderate mechanical performance and their compressive strength was comparable to cancellous bones. Although the importance of the histological difference in the first week we obtained in our study is controversial, we believe that the use of TTCP in addition to the detection of metaphyseal fractures can prevent compression fractures. On the other hand, we believe that TTCP has a positive effect on fracture healing with the mechanical support effect on the fracture site and the replacing defective bone in bone defects with host bone with cell-mediated remodeling effect. In addition to histopathological studies, it should be supported by experimental studies that would be carried out in a larger animal group, including biomechanical studies. Another important finding we obtained in this study is that TTCP did not have a negative effect on fracture union. Although we did not observe any harmful effects on the healing process of TTCP in our study, more comprehensive additional studies on this subject should be done.

This study has several limitations. The faster and better bone and soft tissue healing in rats than in humans may have prevented significant differences between groups. On the other hand, the small number of subjects may have prevented us from reaching statistical significance in this study. This experimental study should be supported by studies using different experimental protocols such as nonunion fracture model, osteoporosis and pseudoarthrosis.

In conclusion, this study is the first experimental study to examine the effect of TTCP on diaphyseal fracture healing. The histopathological results of this study show us that the use of TTCP in addition to the intramedullary implant applied in diaphyseal fractures contributes to the fracture healing in the early stages of fracture healing. We believe that TTCP may be used in the metaphyseal and diaphyseal fractures owing to its positive effects on fracture union.

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**Declaration of conflicting interests**

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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