TETRACALCIUM PHOSPHATE TREATMENT ON EXPERIMENTAL FRACTURE MODEL IN RATS

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Objective:

Materials and methods: Our aim in this study is to investigate the effectiveness of tetracalcium phosphate on fracture healing in rat femur

Results:

Conclusions:

Keywords: Fracture, Tetracalcium phosphate, Fracture healing

1. Introduction

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. A problem that occurs in this process may result in non-union or late union. [1,2] Bone healing problems are common, especially in fractures with bone defects. In the literature, the effects of many minerals and drugs on fracture healing have been investigated. In the literature, 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, montelukast, boron have been used to stimulate and help heal fracture healing.

Tetracalcium phosphate is a component used in the formation of some hydroxyapatite calcium phosphate cements used to repair bone defects. [3] Our aim in this experimental study is to investigate the effect of tetracalcium phosphate, which is used as a filler in dentistry and is known to have remodeling potential. [4,5]

2. Materials and methods

2.1. Animal

Study approval was obtained from the Çanakkale Onsekiz Mart University (ÇOMU) Animal Care and Ethics Committee. The Sprague Dawley rats used were obtained from the ÇOMU Faculty of Medicine Surgical Research Center (ÇOMUSRC). Rats were housed and fed at the ÇOMUSRC throughout the study. All animal experiments were conducted in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (revised, 1985). Forty-two female Sprague–Dawley rats weighing approximately 250–350 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 7 days in the animal care laboratory to exclude any possibility of underlying disease.

2.2. Surgical Technique and Experimental Design

The rats were operated on following an intraperitoneal injection of ketamine HCl (30 mg/kg, Ketalar R, Eczacıbaşı, Istanbul, Turkey) and xylazine anesthesia (10 mg/kg, Xylasinbio, Bioveta, Ankara, Turkey), as described by Bonnarens. [6] 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 (Fig. 1). Then, the animals were randomized into two groups (groups 1 and 2, n= 21 for each). To constitute fracture after surgery, the osteotomy method was used. [7] 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 was 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 and 1b). Additional procedure was not applied to the rats in group 1. Rats in Group 2 were applied to the fracture line created by osteotomy and approximately 2 cc of Grandus® B-One, a Calcium Phosphate Cement developed by Permed Saglik Urunleri (Canakkale, Turkey). 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, İstanbul, Türkiye) followed by the skin with a nonabsorbable suture (Propilen 2/0, Doğsan, İstanbul, Türkiye). The same antibiotic used preoperatively was also administered subcutaneously 3 days postoperatively at 45 mg/kg. Each

animal was housed in its own cage and monitored daily for infection and mobility. Following surgery, 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) on the 2nd, 3rd and 4th weeks after the operation. This method has been recommended by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. [8] The broken femurs of the animals sacrificed at the 2nd, 3rd and 4th weeks 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 they belong were classified and numbered and placed in pathology containers containing 10% formalin solution for histopathological evaluation.

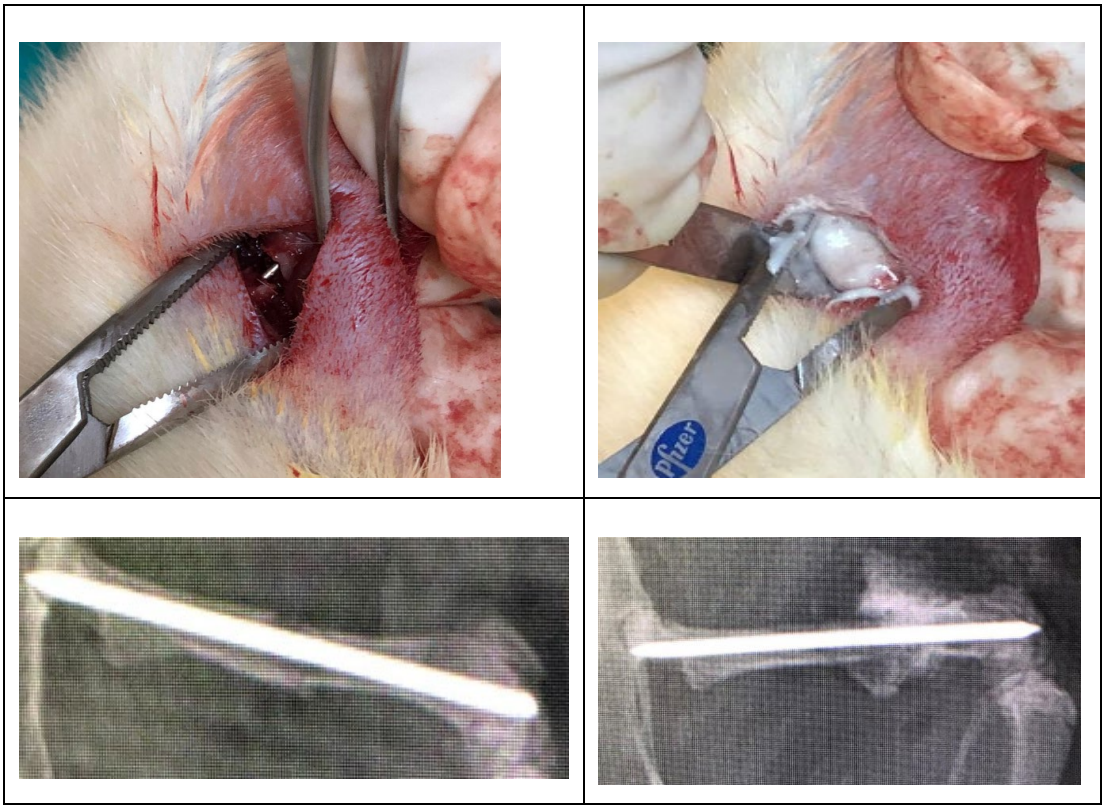

Fig. 1 Fracture formation in the femur (a) and application of Tetracalcium phosphate (b). Confirmation of the postoperative fracture formation by radiological imaging (c) and the radiological appearance of tetracalcium phosphate applied to the fracture site (d)

2.3. Radiographic Evaluation

Posteroanterior and lateral plain X-rays (X-ray system; FUJIFILM Corporation, Minato-ku, Tokyo, Japan) of the femur removed from the animals sacrificed on the 2nd, 3rd and 4th post-operative weeks were commented by two radiologists who were blinded to the content of the present study. They were asked to evaluate callus maturity according to classification of Goldberg et al. [9] In that 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 anterior–posterior (Fig. 2) and lateral radiographs (Fig. 3) of all groups were evaluated separately.

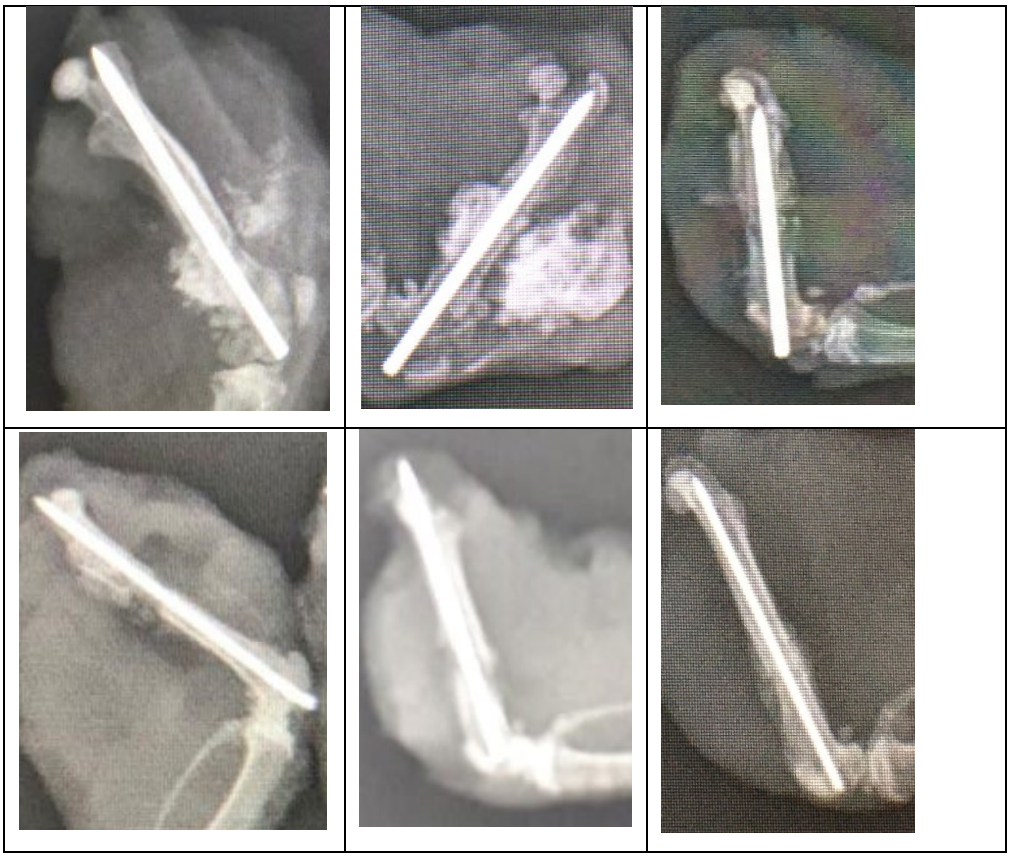

Fig. 2 Radiographic lateral assessment of rat fracture healing femoral radiograms of the rats given tetracalcium phosphate treatment groups ((A) Week 1, (B) week 2, (C) week 3) and untreated control groups ((D) Week 1, (E) week 2, (F) week 3). Callus maturation and bone union becomes more mature as weeks elapsed.

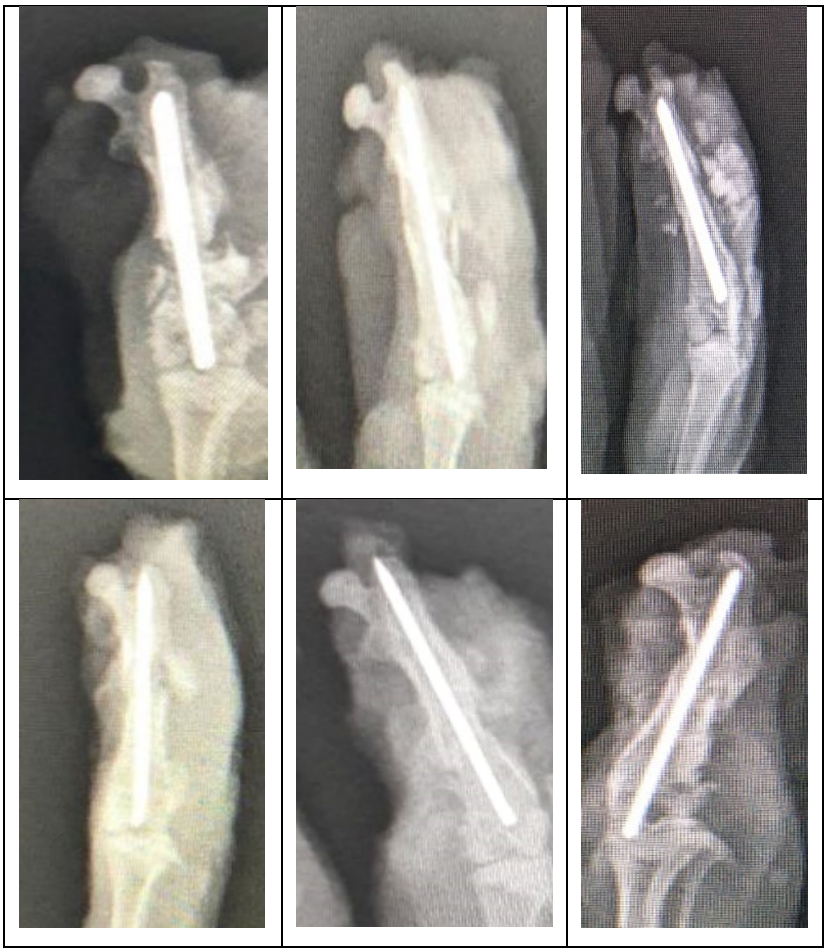

Fig. 3 Radiographic anterior–posterior assessment of rat fracture healing femoral radiograms of the rats given tetracalcium phosphate treatment groups ((A) Week 1, (B) week 2, (C) week 3) and untreated control groups ((D) Week 1, (E) week 2, (F) week 3). Callus maturation and bone union becomes more mature as weeks elapsed.

2.4. 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 % NaCl, 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 (Fig. 4). Fracture healing was graded (1–10), as per Huo et al. [10] (Table 1), based on the ratios of the fibrous tissue, fibrocartilage, cartilage, and bone areas in the fracture site.

Table 1 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

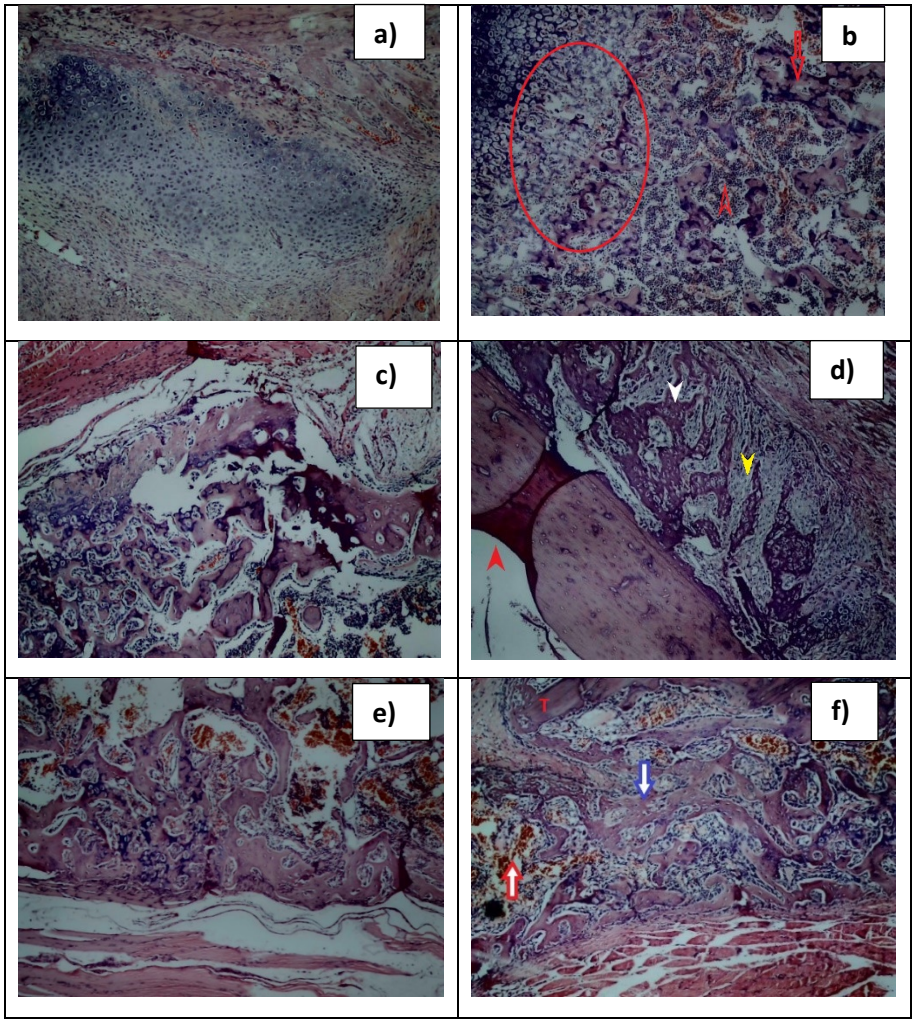

Figure 4. In comparison of the treatment and control groups within each week of the rats sacrificed at 1, 2 and 3 weeks, the treatment groups (T1, T2, T3) were found to be higher in histological scoring than the control groups (C1, C2, C3). There was no significant difference between the treatment and control groups except for the first week. **a)** Group 1, week 1, sections showing callus consisting entirely of cartilage in a grade 5 callus formation (H&E, ×40). **b)** Group 2, week 1, sections showing callus characterized by equal cartilage and immature bone in a grade 7 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 a grade 8 callus formation (H&E, ×40). **d)** Group 2, week 2, sections showing callus characterized by immature bone formation in a grade 9 callus formation (H&E, ×40). Red arrow shows healing area among minimum gap. White arrow shows new bone matrix and anastomosis of trabeculae. Yellow arrow shows active osteoblasts synthesizing bone matrix and fusiform progenitor cells **e)** Group 1, week 3, sections showing mature bone formation in a grade 10 callus formation (H&E, ×10). **f)** Group 2, week 3, sections showing mature bone formation in a grade 10 callus formation (H&E, ×40). It can be seen early stage intramembranous ossification. Red arrow shows defect area, hemorrhage and vanished bone matrix. Blue arrow shows new matrix and lamellar bone formation. T shows old trabeculae.

2.5. Statistical analyses

The data obtained in this study were analyzed with IBM Statistical Package for Social Sciences (SPSS) Statistics 22 software (SPSS Inc., Chicago, IL, USA). The normality of the distribution for the variables was tested with the Shapiro Wilk Normality test. Nonparametric tests were used for variables without normal distribution. Continuous data without normal distribution were analyzed by Mann-Whitney U test. Quantitative data were expressed as mean,

standard deviation, median, quarter scale, minimum and maximum values. Confidence interval was 95%, p value less than 0.05 was considered statistically significant.

Cohen's kappa statistical analysis was used to evaluate inter-observed agreement, and the results were interpreted according to Landis and Koch's guidelines[11] 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.

3. Results

3.1. Radiographic Analysis

Callus formation, remodeling and bridging bone formation were evaluated on the radiograms of the femur removed at the 1st, 2nd and 3rd post-operative weeks. The two orthopedists demonstrated adequate inter-observer agreement for anterior-posterior and lateral radiographs (kappa= 0.627 and 0.656, respectively). Callus maturity and bone union increased every week according to Goldberg criteria but the comparisons of the weekly values of both groups were insignificant (Table 1). Radiological examination failed to show any beneficial or harmful effects of TTCP on fracture healing.

	Radiologic stage of AP			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 1. The mean Radiological stages of both groups. Radiological fracture healing can be seen as weeks advanced. No significant differences were found between the groups.

3.2. 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 the 1st, 2nd, and 3rd post-operative weeks that the fractures healed in a remarkably steady fashion in both groups. Histopathological scores of the treatment group were statistically significantly higher in the evaluation made for the 1st week callus progression and fracture healing (p:0.024). In the

evaluation made in terms of callus progression at the 2nd and 3rd weeks, there was no statistically significant difference between the groups although the treatment group had higher scores than the average C group (p: 0,104, p: 0.462). The distribution of the histological scores of the groups by weeks can be seen in Figure 4. Histopathological examination was not able to exert any stimulating or inhibitory effects of TTCP treatment on fracture healing except week 1. The minimum-maximum values, averages and p values of all histopathological results in the 1st, 2nd and 3rd weeks postoperatively are summarized in Table 2.

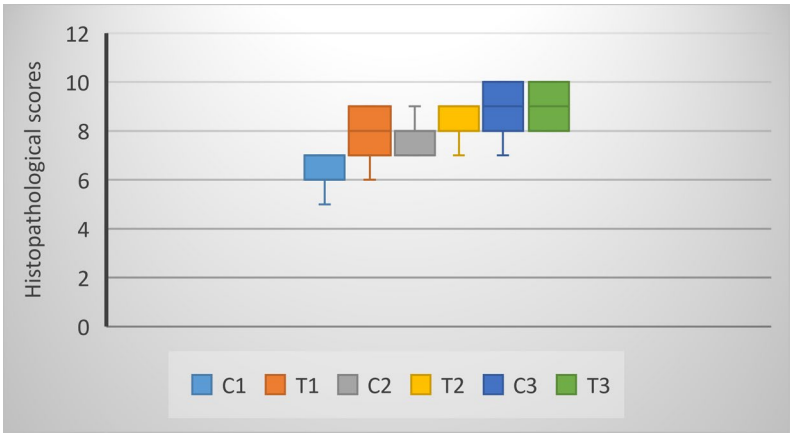

Figure 4. Comparison of histological assessment between groups or rats

	Histopathologic grade					
Week	Groups	Minimum	Maximum	Mean	Standard deviation	P value
Week 1	Groups 1	5	7	6,28	0,76	0,024
	Groups 2	6	9	7,86	1,21	
Week 2	Groups 1	7	9	7,71	0,76	0,104
	Groups 2	7	9	8,43	0,78	
Week 3	Groups 1	7	10	8,71	1,23	0,462
	Groups 2	8	10	9,12	0,89	

Table 2. Grade of histological assessment between groups in rats

4. Discussion

The clinical effect of fractures is important as it can lead to permanent deformity disability. If union cannot be achieved in the fracture, patients may suffer from long-term

disability, and as a result, conditions may result in loss of labor force, quality of life, and morbidity that can lead to loss of limb. Examples of complications that may occur in fracture healing and union are malunion, nonunion, reflex sympathetic dystrophy and degenerative joint disease in fracture distal. [12]

The fracture healing consists of three stages, which are roughly the inflammatory phase, the repair phase, and the remodelization phase, which follow each other and can be intertwined in places. The negativities that may occur in each of these stages will delay or prevent the healing of the fracture and the formation of the mature bone desired as a result. 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 hours and continues until the 7th day. [13] 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. [14] It then continues to crystallize and harden in vivo, reaching 50% of the ultimate compressive strength in one hour and 80% of the ultimate compressive strength in four hours. Therefore, it provides mechanical support when applied to areas with bone defects. Because 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. [14,15] 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. [15]

Preclinical animal studies have shown that Grandus® B-One is osteoconductive and gradually reshapes over time. In the study of the fracture with the defect in the proximal tibia in dogs, it was reported that the bone defect filled with Grandus® B-One showed significant bone placement in a short period of time, such as 2 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. [16] In histological study in humans, TCF reported that the bone tissue completely settled in the biopsy sample 1 year later, cement resorption is prominent near osteoclasts and is usually accompanied by new bone formation that looks qualitatively similar to normal bone remodeling. [17] In this study, he 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 Grandus® B-One can produce better stability, stiffness and strength than using implant fixation only. Clinical studies have shown that TCF 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. [15]

Although many bioactive cements' fracture healing has been studied in the current literature, as far as we know, there are limited number of experimental studies on the effect of tetracalcium phosphate, 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 the comparative radiological evaluation of groups 1 and 2, there was no significant difference in callus formation and bone union. Histopathologically, the fractures healed normally as the weeks progressed in both groups. In the comparison of both groups, no significant difference was found outside the 1st week, although the histological scores of group 1, who were treated for all weeks, were higher in terms of fracture healing. Histological scores of group 2 who received treatment in rats sacrificed in the first week were significantly higher. In this study, we think that TTCP may be useful on fracture healing especially 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.

As a result, we think that it has a positive effect on fracture healing by replacing the defective bone in the bone defects in a short time by showing the mechanical support feature of the Grandus® B-One , and replacing it with the host bone with cell-mediated remodeling.

This study is the first experimental study to examine the effect of Grandus® B-One on fracture healing. In addition to histopathological studies, it should be supported by experimental studies that will be carried out in a larger animal group, including biomechanical

studies. Although we did not observe any harmful effects on the healing process of Grandus® B-One in our study, more comprehensive additional studies on this subject should be done.

5. References

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