Joint Diseases and Related Surgery

Jt Dis Relat Surg
2026;37(1):248-260

ORIGINAL ARTICLE

Fracture healing potential of tetracalcium phosphate: An experimental study in a rat femur standardized diaphyseal osteotomy with open approach

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Bone is the principal structural element of the skeletal system, characterized by its metabolic activity, continuous remodeling capacity and biological vitality.[1] A fracture is defined as the loss of structural integrity and continuity of bone tissue resulting from endogenous or exogenous factors like N-acetylcysteine (NAC), which has antioxidant properties on healing in a rat femoral diaphysis fracture model.[2] Local irisin (IR) administration via injection into the hematoma of a closed fracture model, accelerated bone healing

Received: July 13, 2025
Accepted: September 16, 2025
Published online: November 24, 2025

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Citation: Karabak B, Durak YS, Karsan O, Tuncer K, Bayram C, Sezen S, et al. Fracture healing potential of tetracalcium phosphate: An experimental study in a rat femur standardized diaphyseal osteotomy with open approach. Jt Dis Relat Surg 2026;37(1):248-260. doi: 10.52312/jdrs.2026.2478.

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ABSTRACT

Objectives: This study aims to evaluate the impact of tetracalcium phosphate (TTCP) applied directly to the fracture line in a rat femur fracture model.

Materials and methods: Thirty-three male Sprague-Dawley rats weighing 350 to 400 g were randomly and equally divided into three groups: normal control (NC), fracture control (PC), and TTCP treatment group. Standardized femur fractures were created in the PC and TTCP groups and fixed using intramedullary Kirschner wires. In the TTCP group, TTCP dissolved in distilled water was applied directly to the fracture line. All rats were sacrificed on postoperative Day 28. Radiological, histopathological, and biochemical analyses were performed to assess fracture healing.

Results: Radiological scoring revealed significantly higher fracture healing in the TTCP group compared to the PC group on Days 14 and 28 ($p<0.05$). Histopathological analyses showed reduced inflammation and fibrosis, and increased chondrocyte activity and neovascularization in the TTCP group. On Day 28, serum tumor necrosis factor-alpha (TNF-α) levels were significantly higher in the PC group compared to TTCP and NC, while TTCP values were comparable to NC. No adverse tissue reactions were observed in any group.

Conclusion: The TTCP enhances fracture healing when applied directly to the fracture line, without causing soft tissue irritation or histologically detectable adverse reactions. Its biological activity and ease of application suggest that TTCP may be a promising adjunct in the treatment of complex fractures.

Keywords: Bioengineering, biomaterial, bone graft, fracture healing, tetracalcium phosphate.

through earlier callus transformation and superior biomechanical strength compared to platelet-rich plasma (PRP) and hyaluronic acid (HA). The superior efficacy of IR over PRP and HA may be explained by its ability to enhance osteoblast activity, promote vascularization, and reduce inflammation, creating an optimal environment for bone regeneration.[3]

In certain clinical scenarios, such as critical traumatic bone loss or defects resulting from primary tumor resection, the intrinsic healing capacity of bone is insufficient to ensure complete recovery.[2,3] In such cases, surgical intervention with additional materials is required to fill the defective areas. Autogenous bone grafting remains the gold standard for the treatment of these critical-sized defects. However, the use of autografts is associated with several drawbacks, including donor site morbidity, infection risk, postoperative pain, and limited graft availability.[4]

Calcium phosphates are recognized as a new generation of bone graft substitutes with significant potential for clinical application in orthopedic surgery. Due to their superior biocompatibility, osteoconductive properties, and ability to facilitate osseointegration, they are extensively employed in bone repair procedures within orthopedic practice.[5-7]

Calcium phosphates promote bone healing by releasing calcium and phosphate ions essential for osteogenesis, thereby stimulating cellular secretion of proteins involved in the regeneration of mineralized tissues.[5] Preclinical animal studies have shown that calcium phosphate materials can be resorbed by up to 95% over a period of 26 to 86 weeks, indicating their suitability as absorbable bone graft substitutes.[8]

In the light of these considerations, in the present study, we aimed to investigate the effects of tetracalcium phosphate (TTCP) a calcium phosphate compound with a high calcium-to-phosphorus ratio, excellent biocompatibility, osteoconductive properties, and strong bone remodeling capacity on the process of fracture healing in a rat model.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with institutional and international guidelines for the ethical use and care of laboratory animals. The study protocol was approved by the Atatürk University, Animal Experiments Ethics Committee (HADYEK) (Date: 31.08.2021, No: 201). All necessary measures were taken to minimize animal suffering, in line with the principles of the 3Rs (Replacement, Reduction, and Refinement).

Thirty-three healthy and physically active male Sprague-Dawley rats were included in this study. The animals had a mean age of nine (range, 8 to 10) weeks and an average body weight of 375 (range, 350 to 400) g. Group allocation was performed using a computer-generated random number sequence (simple randomization) to assign animals equally into three experimental groups (n=11). Allocation was concealed until the day of surgery (Table I). For intra-cage identification throughout the study, each rat was marked with henna in different colors (Figure 1).

Tetracalcium phosphate ($Ca_4(PO_4)_2O$) powder was obtained from (Permed Health Products, Çanakkale, Türkiye). The powder was used as supplied (particle size in the typical micrometre range; theoretical crystal density about 2.9 g/cm^3). For each fracture site, the powder was weighed, and approximately 200 mg was mixed intraoperatively with 1.0 mL sterile distilled water to obtain a cohesive paste that could be molded into the defect. The TTCP was used alone (not combined with DCPA/DCPD). In aqueous, near-neutral conditions, TTCP undergoes partial hydrolysis toward HA via dissolution-precipitation, which can lead to limited *in situ* hardening (not a full-strength CPC set). Our paste was intended to provide osteoconductive filling and local stability adjunct to Kirschner wire (K-wire) fixation rather than load-bearing strength. The ionic species released are calcium

TABLE I
Summary of the experimental groups (n=11 per group)

Groups	Procedures
Negative control	Untreated
Positive control	Bone fracture + K-wire
TTCP treatment group ($Ca_4(PO_4)_2O$)	Bone fracture + K-wire + TTCP

TTCP: Tetracalcium phosphate.



FIGURE 1. Henna labeling applied to distinguish individual rats within each experimental group.

and phosphate; no organic monomers are present. Key aspects of TTCP hydrolysis chemistry and HA formation in TTCP-based systems are summarized elsewhere.[9]

Surgical procedure

For postoperative analgesia, all rats received buprenorphine (0.05 mg/kg, subcutaneously, twice daily) for three consecutive days following surgery. This regimen was selected to minimize pain and distress while avoiding prolonged opioid exposure that might interfere with bone healing. Anesthesia was induced via intraperitoneal injection of ketamine (60 mg/kg; Ketalar, Pfizer, İstanbul, Türkiye) and xylazine (10 mg/kg; Rompun, Bayer, Germany).[10] For infection prophylaxis, all rats scheduled for surgery received a single intraperitoneal preoperative dose of cefazolin sodium (50 mg/kg; Eqizolin 1 g, All Team Medicine, İstanbul, Türkiye). The rats were positioned on adjustable heating pads (Paketherm, Vallgorguina, Barcelona, Spain) covered with sterile surgical drapes to maintain normothermia during the procedure. The surgical area over the right femur was then thoroughly shaved and disinfected using a standard antiseptic protocol.[11,12]

A 2-cm longitudinal incision was made over the lateral aspect of the right femur, passing through the skin and subcutaneous tissues. The fascia and muscle layers overlying the femoral shaft were separated by blunt dissection to expose the bone surface, and the periosteum was carefully stripped. We, then, performed a standardized mid-diaphyseal transverse osteotomy through both cortices to create a simple two-part fracture. Fracture stabilization was achieved using a 1.5-mm K-wire (Aysam Ortopedi ve Tıbbi Aletler San. Tic. Ltd. Şti., Samsun, Türkiye), which was initially inserted retrogradely through the distal femur using a motorized drill, until it reached the level of the greater trochanter. Intramedullary stabilization was performed using a 1.5-mm smooth stainless-steel K-wire. In our study, a 1.5 mm K-wire was used for intramedullary fixation; a similar use has been reported previously in adult Wistar rats (average weight ~392 g, six to nine months).[12] Thus, a 1.5-mm wire provided near endosteal contact without causing iatrogenic fracture propagation. Following anatomical reduction of the fracture, the wire was advanced anterogradely into the proximal fragment and left *in situ* without exiting the knee joint. The fascia and skin were, then, closed separately using appropriate suturing techniques.[13]

The rats in the negative control (NC, normal control) group did not undergo any surgical intervention. In the positive control (PC, fracture control) group, standardized mid-diaphyseal femoral fractures were stabilized using 1.5 mm K-wires. In the TTCP group, following fracture fixation with 1.5-mm K-wires, TTCP (Permed Health Products, Çanakkale, Türkiye) was applied directly to the fracture site. For application, 200 mg of TTCP powder was mixed with 1 mL of sterile distilled water to prepare an injectable paste.

At the end of the 28-day follow-up period, all rats were sacrificed under high-dose urethane anesthesia. Following euthanasia, intracardiac blood samples were collected, and the right femurs were harvested through disarticulation at the hip and knee joints for subsequent histological analyses.

Postoperative rat management

Following the surgical procedures, all rats were housed under standard laboratory conditions, including a controlled ambient temperature of 20±1°C, relative humidity of 50±5%, and a 12-h light/dark cycle for a period of 28 days. Postoperatively, all animals received intraperitoneal administration of Ringer's lactate solution at a dose of 8 mg/kg/day, pre-warmed to room temperature, to maintain fluid balance and hemodynamic stability.[14] Standard pellet feed and fresh water were made available *ad libitum* to all animals during the entire study period to ensure adequate nutritional support.

On the first postoperative day, all rats were able to stand on their hind limbs, exhibited normal exploratory behavior, and maintained a good general

condition. They were capable of self-grooming and independent feeding without difficulty. Within minutes following the surgical procedure, all animals were observed to move freely within their cages without the need for external support or splinting.

Throughout the postoperative follow-up, the incision sites in all rats remained clean, with no evidence of exudate, inflammation, or wound-related complications. No signs indicative of severe pain such as vocalization, restlessness, reduced mobility, impaired grooming behavior, abnormal posture, or lack of environmental interest were observed in any of the rats during the postoperative period.[15] All rats were weighed before the surgical procedure and on postoperative Days 7, 14, and 28 to assess changes in body weight as an indicator of overall health and well-being.

Predefined humane endpoint criteria for the early removal of animals from the experiment were established prior to study initiation. Throughout the experimental period, all animals were regularly monitored to assess whether any met these exclusion criteria. Animals were removed from the study if any of the following conditions were observed: *(i)* removal recommended by the attending veterinarian for humane reasons, *(ii)* weight loss exceeding 15% of initial body weight, *(iii)* inadequate food or water intake, *(iv)* markedly diminished response to external stimuli, or *(v)* signs of wound infection.

Radiographic examination

On postoperative Days 7, 14, and 28, all rats were sedated with intraperitoneal ketamine (30 mg/kg) and xylazine (5 mg/kg) for radiographic evaluation. In cases where movement occurred during imaging, inhaled sevoflurane anesthesia was additionally administered. Lateral single-plane radiographs of the right femur were obtained for all animals. Radiographs were evaluated using a modified Lane and Sandhu scoring system (0-10 points). Scores were defined as follows: 0-2, no callus formation or cortical bridging; 3-5, initial callus formation with partial cortical bridging; 6-8, abundant callus with near-complete cortical bridging; and 9-10, complete cortical bridging and remodeling. Scores were assigned independently by two blinded observers on postoperative Days 7, 14, and 28. Interobserver agreement was calculated using Cohen's kappa (κ) coefficient with 95% confidence intervals (CIs). All radiographs were independently evaluated by two blinded orthopedic surgeons, each with more than five years of experience in musculoskeletal imaging. Interobserver reliability was analyzed using Cohen's κ coefficient to assess the level of agreement between evaluators.[16]

Biochemical analyses

At the end of the 28-day study period, 5 mL of intracardiac blood was collected from each sacrificed rat and stored at -80°C until analyses. Serum levels of bone morphogenetic protein-2 (BMP-2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and nuclear factor kappa B (NF-κB) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (SinoGeneclon, Hangzhou, China), in accordance with the manufacturer's instructions.

Histopathological analyses

Following sacrifice on Day 28, the right femurs were harvested, and the K-wires were carefully removed. The specimens were then subjected to histopathological evaluation. Bone tissue samples were fixed in 10% neutral buffered formalin and decalcified in 10% nitric acid for 48 h. Subsequently, the specimens were processed through a graded ethanol-xylene series, embedded in paraffin, and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT). Histopathological evaluation was performed using a semi-quantitative approach to assess inflammatory cell infiltration, edema, neovascularization (graded on a 0-3 scale rather than vessel counts), fibrocyte/fibroblast activity, chondrocyte/chondroblast activity, fibrous tissue formation, and cartilage formation. Each parameter was scored on a four-point scale: none (0), mild (1), moderate (2), and severe (3). All histological assessments were performed independently by two blinded histopathologists, and interobserver agreement was evaluated using Cohen's κ coefficient to ensure scoring reliability.[17,18]

Histological evaluation was performed on decalcified femoral specimens sectioned through the callus region. The region of interest (ROI) was defined as the fracture gap and adjacent callus extending up to 1 mm into the native cortex. For each animal, three sections were analyzed at 200× magnification (H&E and Masson's trichrome). Fields were selected using a systematic random approach across the callus. Scores were averaged across sections to yield a single value per animal. Representative images are presented with scale bars (100 μm) and identical staining exposure settings.

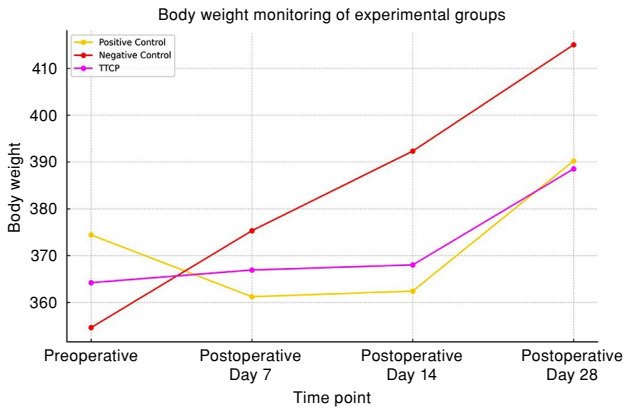


FIGURE 2. Body weight monitoring (g) of rats in the NC, PC, and TTCP groups throughout the 28-day experiment.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate.

Statistical analysis

Study power analysis and sample size calculation were performed using the G*Power version 3.1 software (Heinrich Heine University Düsseldorf, Düsseldorf, Germany). Sample size (n=11 per group) was determined *a priori* using an expected medium effect size (f=0.40, α=0.05, power =0.80) based on pilot data and previous studies. No animals were excluded from the analyses.

Statistical analysis was performed using the IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were presented in mean ± standard deviation (SD), median and interquartile range (IQR) or number and frequency, where applicable. Normality of biochemical variables was assessed with the Shapiro-Wilk test, and variance homogeneity with Levene's test. For normally distributed and homoscedastic data, one-way analysis of variance (ANOVA) with the Tukey's *post-hoc* was applied. For non-normal or heteroscedastic data, Kruskal-Wallis with Dunn-Bonferroni correction was used. Histopathological scores (ordinal) were analyzed with Kruskal-Wallis, followed by Mann-Whitney U tests with Bonferroni correction. Effect sizes for ordinal comparisons were reported as Cliff's delta. Radiographic scores on postoperative Days 7, 14, and 28 were obtained from the same animals at repeated timepoints. Therefore, data were analyzed using a Friedman repeated-measures test followed by Wilcoxon signed-rank tests with Bonferroni correction. A p value of <0.05 was considered statistically significant.

RESULTS

All experimental procedures were completed successfully, and no complications or attrition occurred during the study.

The rats in the NC and TTCP groups demonstrated continuous weight gain throughout the 28-day experimental period, with no episodes of weight loss observed. However, the rats in the PC group exhibited an initial decrease in body weight during the first postoperative week, followed by progressive weight gain thereafter (Figure 2).

No wire migration or breakage occurred in any animal. Distal femoral lysis at the wire tip was observed in four of 11 rats (36%) in the PC group and two of 11 rats (18%) in the TTCP group. No cases were recorded in the NC group. These radiographic changes were confined to the peri-implant region and did not affect fracture alignment.



FIGURE 3. Lateral radiographs of the NC group on postoperative **(a)** Day 7, **(b)** Day 14, and **(c)** Day 28.
NC: Normal control.

Interobserver agreement for radiographic scoring was high (κ=0.82, 95% CI: 0.70-0.94). All rats underwent uniaxial lateral radiographic imaging under anesthesia on postoperative Days 7, 14, and 28 (Figures 3-5). In the NC group, no radiographic abnormalities were observed at any time point, and all images were consistent with normal bone morphology. The TTCP group showed higher radiographic scores compared to the PC group, indicating radiological evidence of improved healing on Day 28. However, radiographic evidence of bone lysis-presumably due to K-wire micromotion was detected in the distal femoral region in both the TTCP and PC groups (Table II).

On postoperative Day 28, serum levels of TNF-α, IL-1β, BMP-2, and NF-κB were measured using ELISA. On Day 28, TNF-α levels were highest in PC (median [IQR]: 85 [79-90] pg/mL), significantly lower in TTCP (65 [59-70] pg/mL), and lowest in NC (63 [58-69] pg/mL). The Kruskal-Wallis test confirmed overall group differences ($p<0.01$). The Dunn-Bonferroni *post-hoc* tests showed PC significantly higher than both TTCP ($p=0.004$) and NC ($p=0.002$), while TTCP and NC did not

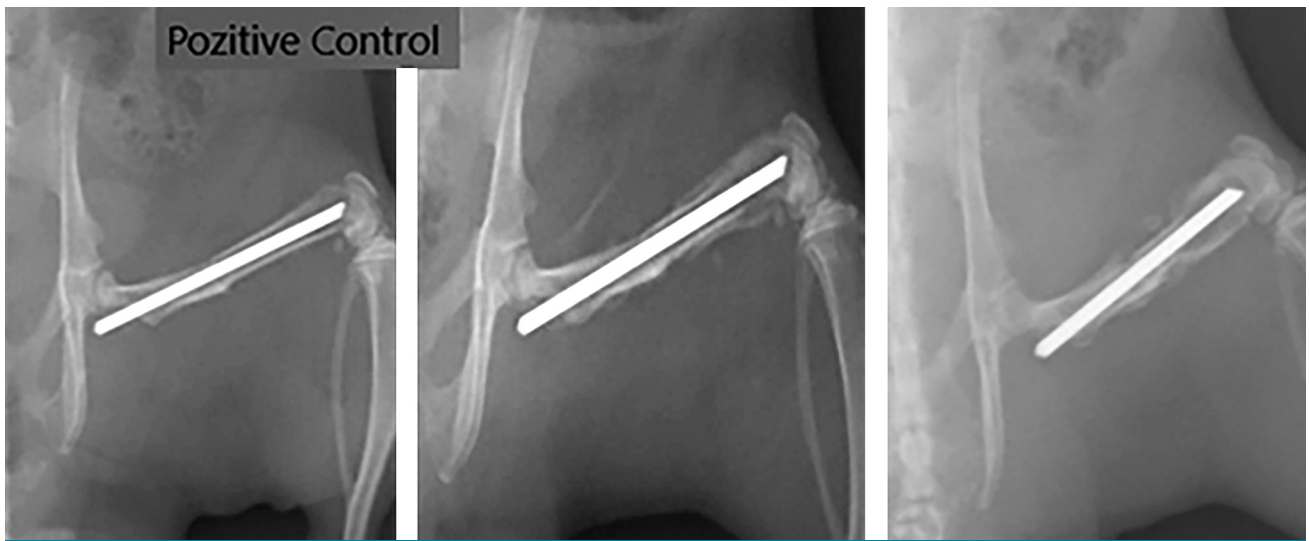


FIGURE 4. Lateral radiographs of the PC group on postoperative **(a)** Day 7, **(b)** Day 14, and **(c)** Day 28.
PC: Fracture control.

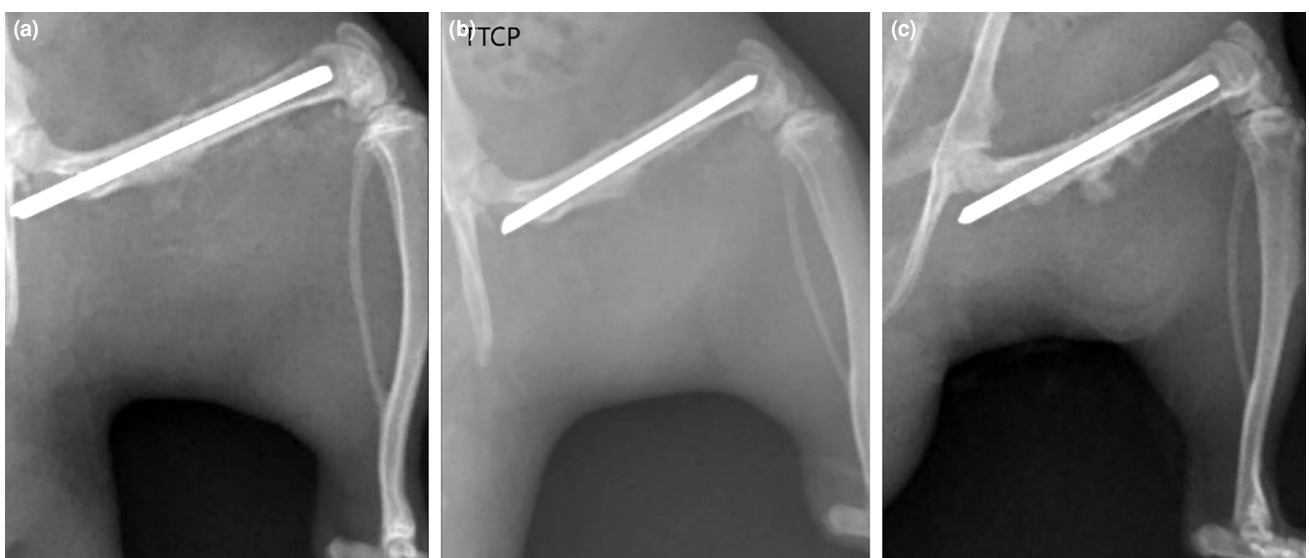


FIGURE 5. Lateral radiographs of the TTCP group on postoperative **(a)** Day 7, **(b)** Day 14, and **(c)** Day 28.
TTCP: Tetracalcium phosphate.

TABLE II
Comparison of radiological scores (Lane and Sandhu modified system, 0-10) among groups on postoperative Days 7, 14, and 28

	Time points					
	Day 7		Day 14	Day 14	Day 29	Day 29
TTCP	0	0-1	3	2-3	9	4-10
PC	0	0-1	2	1-3	6	3-8
p^1	0.569		0.008		0.027	
PC-TTCP p^2	-	-	0.403		0.302	

TTCP: Tetracalcium phosphate; PC: Fracture control; Interobserver agreement: Cohen's κ= 0.82, 95% CI: 0.70-0.94. p^1: comparison among all groups, p^2: PC *vs.* TTCP.

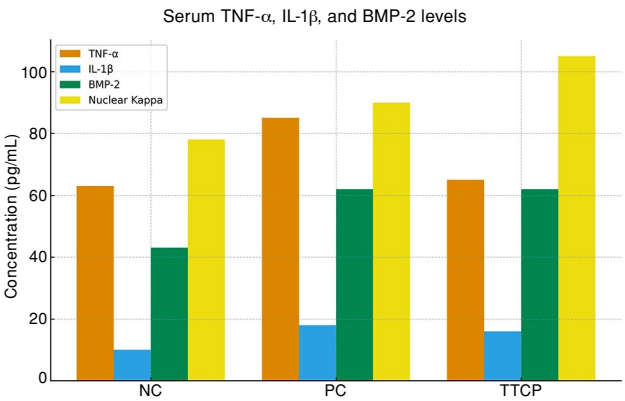


FIGURE 6. Serum TNF-α, IL-1β, and BMP-2 levels (pg/mL) measured by ELISA on postoperative Day 28 in the NC, PC, IL-1β, and TTCP groups.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; BMP-2: Bone morphogenetic protein-2; ELISA: Enzyme-linked immunosorbent assay.

significantly differ (p=0.72). In the IL-1β analyses, the PC group demonstrated significantly higher IL-1β levels than the NC group (p<0.05). However, no statistically significant differences were observed between the TTCP group and the other groups. For BMP-2, serum levels were significantly higher in the PC group compared to the NC group (p<0.05), while no significant differences were detected between the TTCP group and either of the other groups. Finally, NF-κB levels did not significantly differ among any of the experimental groups (p>0.05) (Figure 6).

Histopathological evaluation of H&E-stained sections focused on the analyses of inflammatory cell infiltration, edema, neovascularization, fibrocyte/fibroblast activity, and chondrocyte/chondroblast activity. These parameters were compared among the study groups to determine statistically significant differences (p<0.05) (Table III). When the TTCP and PC groups were compared, significant decreases in inflammatory cell infiltration, edema, neovascularization, and fibrocyte/fibroblast activity were observed in the TTCP group (all p<0.05). Furthermore, chondrocyte/chondroblast activity was significantly higher in the TTCP group compared to the PC group (p<0.05). The Masson's trichrome staining was used to assess fibrous and cartilaginous tissue formation. Statistically significant differences were observed between the groups for both parameters (p<0.05) (Table IV). Specifically, the TTCP group showed significantly more cartilage formation and less fibrous tissue

TABLE III
Histopathological findings of the groups from hematoxylin-eosin staining (semi-quantitative scoring, 0-3)

	Inflammatory cell infiltration	Edema	New blood vessel formation	Fibrocyte/fibroblast activity	Chondrocyte/chondroblast activity
Groups	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
PC	2.83±0.40^a	2.66±0.51^a	1.00±0.00^a	1.16±0.40^a	0.33±0.51^a
TTCP	1.16±0.40^c	1.00±0.00^c	2.66±0.51^b	2.83±0.40^c	2.66±0.51^b

SD: Standard deviation; PC: Fracture control; TTCP: Tetracalcium phosphate; a,b,c Different letters in the same column indicate significant differences between groups (p<0.05).

TABLE IV
Histopathological findings of the groups from Masson's trichrome staining (semi-quantitative scoring, 0-3)

	Fibrous tissue formation	Cartilage tissue formation
Groups	Mean±SD	Mean±SD
PC	1.16±0.40a	0.33±0.51a
TTCP	2.83±0.40c	1.83±0.40c

SD: Standard deviation; PC: Fracture control; TTCP: Tetracalcium phosphate; a,b,c Different letters in the same column indicate significant differences between groups ($p<0.05$).

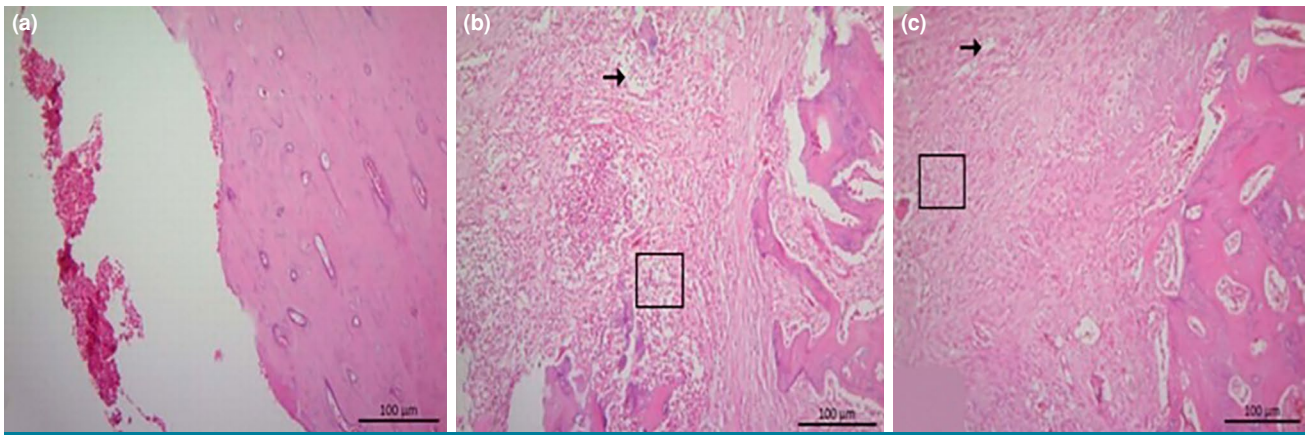


FIGURE 7. Representative histological images of the groups stained with hematoxylin-eosin (H&E). **(a)** NC group: normal histological appearance. **(b)** PC group: severe edema (arrow) and inflammatory cell infiltration (☐). **(c)** TTCP group: mild edema (arrow) and inflammatory cell infiltration (☐). Scale bar= 100 µm.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate.

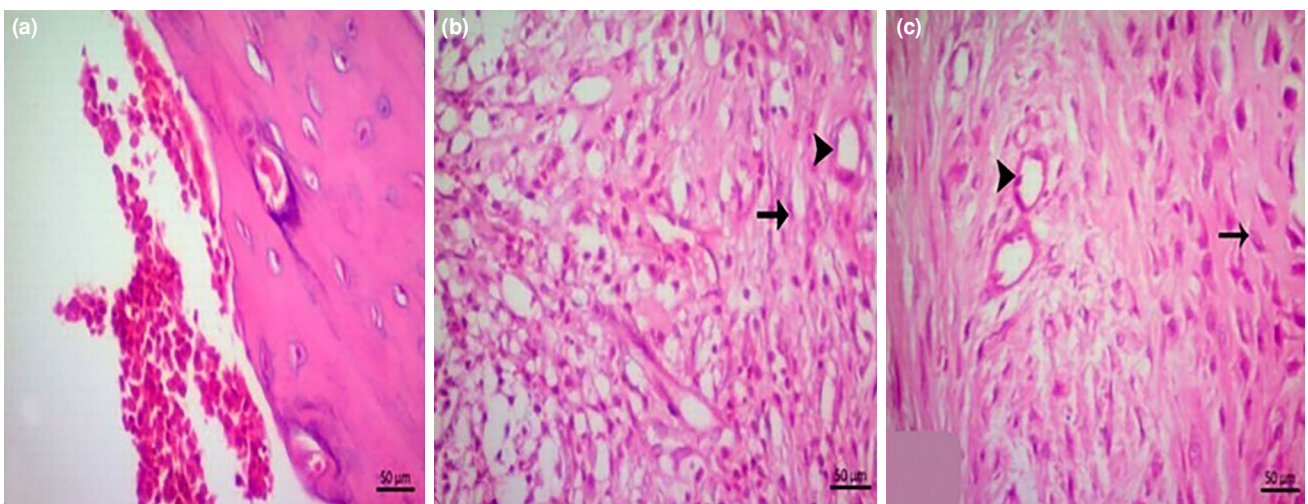


FIGURE 8. Representative histological images of the groups stained with hematoxylin-eosin (H&E). **(a)** NC group: normal histology. **(b)** PC group: mild neovascularization (arrowhead) and fibroblast/fibrocyte activity (arrow). **(c)** TTCP group: severe neovascularization (arrowhead) and chondrocyte/chondroblast activity (thin arrow). Scale bar = 100 µm.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate.

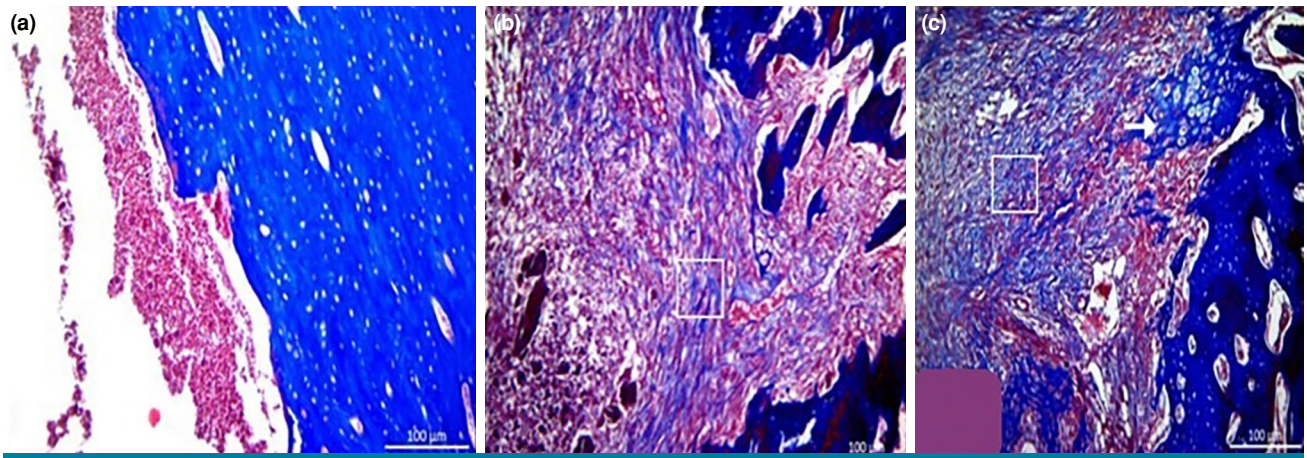


FIGURE 9. Representative histological images of the groups stained with Masson's trichrome (MT). **(a)** NC group: normal histology. **(b)** PC group: mild fibrous tissue formation (☐). **(c)** TTCP group: severe fibrous tissue formation (☐). Scale bar= 100 µm.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate.

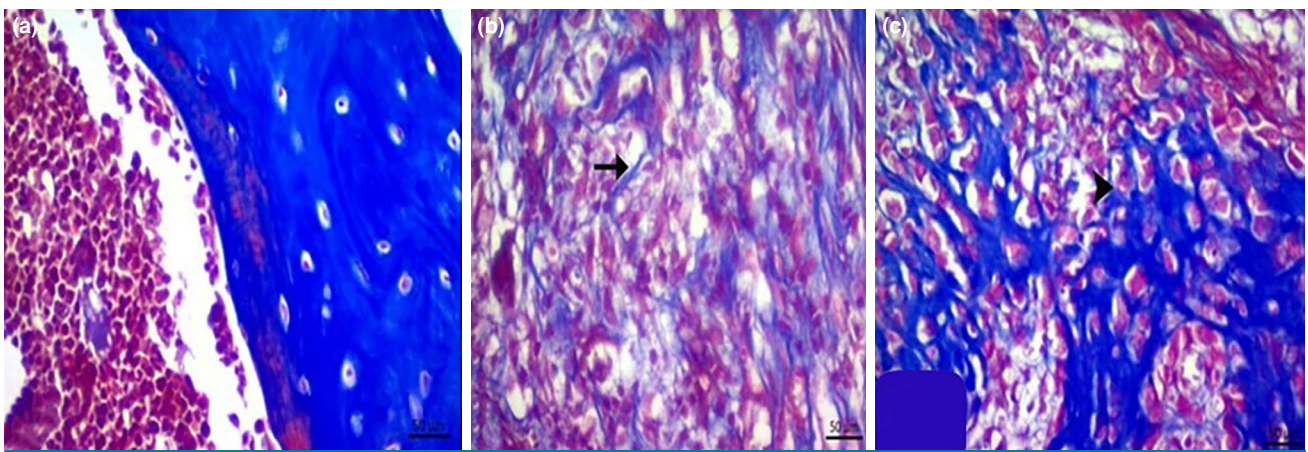


FIGURE 10. Representative histological images of the groups stained with Masson's trichrome (MT). **(a)** NC group: normal histology. **(b)** PC group: mild fibrous tissue formation (arrow). **(c)** TTCP group: moderate cartilage tissue formation (arrowhead). Scale bar= 100 µm.
NC: Normal control; PC: Fracture control; TTCP: Tetracalcium phosphate.

formation compared to the PC group ($p<0.05$) (Figures 7-10).

DISCUSSION

In the present study, we investigated the biological performance of TTCP in the context of fracture healing, with the goal of assessing its clinical applicability as a synthetic bone graft material. The NC group, which did not undergo fracture or surgery, showed consistent weight gain, reflecting normal growth. However, the PC group, which received a femoral fracture and K-wire fixation without any graft material, exhibited early postoperative weight loss likely due to surgical stress, pain, and delayed functional recovery. Notably, the rats in the TTCP group, which received both fracture fixation and TTCP, maintained steady weight gain like the NC group. This finding suggests that TTCP may contribute to reduced systemic stress and improved early recovery, likely through enhanced local stabilization and accelerated healing.

The distal femoral lysis observed in some specimens likely reflects micromotion at the tip of the smooth intramedullary wire, a phenomenon previously noted in small-animal fixation models.[13] As mechanical stability strongly directs the callus

pathway,[19] part of the apparent benefit of TTCP may relate to mechanical space-filling. However, subgroup inspection of specimens without lysis still showed improved healing in TTCP, suggesting a genuine biological contribution.

Radiological evaluation revealed that bone union scores were significantly higher in the TTCP group compared to the PC group on Days 14 and 28, indicating a positive effect of TTCP on fracture healing. This finding supports the osteoconductive potential of TTCP when applied directly to the fracture site. Notably, radiographic signs of bone lysis were observed in both the TTCP and PC groups, particularly in the distal femur, which is likely attributable to micromotion of the intramedullary K-wire rather than the graft material itself. These findings suggest that while TTCP enhances bone healing, fixation-related mechanical factors may still influence the radiological appearance of the healing process.

On postoperative Day 28, TNF-α levels were significantly higher in the PC group compared with TTCP and NC, while TTCP and NC showed comparable values. This finding suggests that TTCP did not prolong systemic inflammatory signaling. However, interpretation of these systemic biomarkers should be made with caution, as they were measured only at a single 28-day timepoint and may not fully reflect their dynamic changes during fracture healing. Of note, IL-1β was significantly elevated in the PC group but remained unaffected by TTCP application, suggesting that TTCP did not amplify the inflammatory response beyond surgical trauma. Also, BMP-2 levels increased in the PC group, but were not further enhanced by TTCP, indicating limited systemic osteoinductive stimulation. The NF-κB levels were similar across all groups. These findings indicate that TTCP supports bone healing mainly through local osteoconductive effects, with minimal systemic modulation of inflammatory or osteogenic markers.

Comparable findings were reported by Tsai et al.,[20] who evaluated TTCP-derived calcium phosphate cement in a rat subcutaneous implantation model. Their study showed that, by Week 4, the material had largely transformed to apatite and was surrounded by a fibrous capsule, without evidence of osteoblasts or osteocytes, findings consistent with our observations at the same timepoint. More importantly, the aforementioned authors demonstrated that, while *in vitro* samples immersed in Hanks' solution exhibited progressive porosity and reduced mechanical strength, the implanted samples maintained structural integrity up to 24 weeks, with capsule thickness maturing after 12 weeks. These results highlight that TTCP cements exhibit stable *in vivo* mechanical behavior and favorable tissue responses, but the absence of osteogenesis at Week 4 underscores the need for longer-term evaluation to capture resorption kinetics and ossification dynamics.

Histopathological analyses revealed that TTCP application significantly attenuated local inflammatory responses, as evidenced by reduced inflammatory cell infiltration, edema, neovascularization, and fibroblast/fibrocyte activity compared to the PC group. This finding suggests that TTCP may help modulate the inflammatory phase of fracture healing, potentially facilitating a more favorable microenvironment for tissue regeneration. Additionally, the observed increase in chondrocyte/chondroblast activity in the TTCP group indicates enhanced cartilaginous callus formation, which is a key transitional stage in endochondral ossification. Masson's trichrome staining further supported these findings, showing significantly greater cartilage formation and reduced fibrous tissue development in the TTCP group. These results collectively suggest that TTCP promotes a more efficient and organized healing response, shifting the local tissue dynamics away from fibrosis and toward chondrogenesis and eventual bone regeneration.

Calcium phosphates are considered next-generation bone graft substitutes with promising clinical applicability in orthopedic surgery, primarily due to their biocompatibility, osseointegrative capacity, and osteoconductive properties.[5-7,21,22] In line with these characteristics, our findings demonstrated that TTCP exerts a positive effect on fracture healing. The results confirmed that TTCP is biocompatible and possesses both osteoconductive and osseointegrative potential. Histopathological evaluations at the time of sacrifice revealed that TTCP did not adhere to surrounding muscle tissues and did not induce necrosis or fibrosis, further supporting its biocompatibility. Moreover, TTCP was found to enhance and accelerate bone formation to a greater extent than the PC group, suggesting its efficacy in promoting bone regeneration.

A previous study showed that TTCP-based cement promoted bone formation and graft resorption without adverse tissue reactions by Week 24.[23] Similarly, our study found no necrosis, fibrosis, or infection around the TTCP graft and

demonstrated enhanced neovascularization and cellular activity. However, ossification and osteocytes were not observed at Week 4, likely due to the standardized diaphyseal osteotomy with open approach and short follow-up. Longer-term studies with stable fixation are needed to confirm TTCP's full osteogenic potential.

Graft healing follows a staged process requiring mechanical stability for proper vascularization and integration.[24] At Week 4, the PC group showed prominent inflammation and edema, indicating healing in our study. In contrast, the TTCP group exhibited advanced neovascularization, cellular activity, and cartilage formation, corresponding to a transition toward healing stages. These findings suggest that TTCP accelerates graft maturation compared to control.

Calcium phosphate grafts are widely used as resorbable bone fillers, though concerns have been raised regarding potential soft tissue irritation.[8] In our study, TTCP was easily applied to the fracture line and did not cause ossification, necrosis, or infection in adjacent muscle or soft tissue. No TTCP residue remained at the sacrifice point. These findings align with those of Tsai et al.,[23] who reported no inflammation, necrosis, or fibrous encapsulation around TTCP implants in rabbits, along with progressive vascularization, osteoblast activity, and near-complete resorption by Week 24. Similarly, we observed fibroblasts, chondroblasts, and neovascularization in the TTCP group. However, full remodeling was not evident at Week 4, still highlighting the need for longer-term studies.

Although various bioactive cements have been investigated for their effects on fracture healing, experimental studies specifically focusing on TTCP remain limited. Addressing this gap, the present study contributes valuable preliminary data on TTCP's biological behavior and its potential role in bone regeneration and may serve as a reference for future research in this area.

Following fracture, TNF-α and IL-1β levels typically exhibit a bimodal increase peaking during the inflammatory phase and again during the remodeling phase.[25,26] In our study, TNF-α levels were significantly elevated in the PC group, consistent with an ongoing inflammatory response. On the other hand, TNF-α levels in the TTCP group were comparable to those in the NC group, suggesting that the inflammatory phase had largely resolved, although the remodeling phase had not yet commenced.

It has been well established that TNF-α contributes to this phase by promoting receptor activator of nuclear factor kappa beta ligand (RANKL) secretion from chondrocytes and enhancing monocyte migration, thereby increasing osteoclast activity and bone resorption. These cytokines are also involved in early intramembranous ossification and trabecular bone remodeling.[27] In our study, by Day 28, TNF-α remained elevated in the PC group, whereas TTCP values were significantly reduced and comparable to NC. This suggests that TTCP application may suppress prolonged pro-inflammatory signaling, consistent with the histological evidence of advanced healing. Conversely, TNF-α levels in the TTCP group were lower and comparable to those in the NC group, indicating resolution of inflammation and a potentially more regulated transition toward the remodeling phase IL-1β and TNF-α are known to increase again during the remodeling phase, typically beginning between Days 21 and 28 post-fracture.[28,29]

Bone morphogenetic proteins, particularly BMP-2, are key regulators of bone formation and repair, promoting chemotaxis, proliferation, and osteogenic differentiation.[30,31] Previous studies have shown that BMP-2 levels typically peak earlier in normal union and later in cases of delayed or nonunion. In our study, elevated BMP-2 levels were observed on Day 28 in both fracture groups, which may indicate a delayed healing process. This finding supports the notion that prolonged BMP-2 elevation could be associated with slower progression through the fracture repair phases.

In the literature, experimental studies examining the effects of TTCP on fracture healing remain scarce. However, based on our findings, TTCP appears to significantly support fracture union when injected into the fracture line, as demonstrated by superior histological and radiological outcomes compared to the PC group. Owing to its injectability and osteoconductive properties, TTCP may be particularly beneficial as an adjunct to fixation in cases involving multiple, closely located fractures that are difficult to stabilize with implants alone. These results are consistent with the existing literature and suggest that TTCP-based formulations have potential as an active component of fracture treatment strategies.

This study has several methodological strengths. First, the use of a standardized diaphyseal osteotomy with open approach allowed for standardized fracture morphology and precise graft

application, facilitating consistent callus formation and more accurate intergroup comparisons. Second, the selection of intramedullary K-wire fixation created a semi-stable biomechanical environment, enabling the assessment of the biological effects of TTCP under conditions that more closely resemble clinical reality. Additionally, the multidisciplinary collaboration among veterinarians, pharmacologists, and orthopedic specialists ensured rigorous experimental design, animal care, and data interpretation.

Nonetheless, there are certain limitations that must be acknowledged. First, biomechanical testing was not performed, and animals were sacrificed only at the end of the experiment, preventing evaluation of progressive healing. Second, histopathological assessments were semiquantitative, radiographic imaging was limited to uniaxial views, and computed tomography was not utilized. Third, only adolescent male rats were included to eliminate hormonal variability, which enhances internal validity but limits generalizability. Additionally, the relatively small sample size (n=11 per group) may restrict the statistical power and generalizability of the findings. Also, the limited number of studies investigating TTCP alone also restricted our ability to compare findings within a broader research context. Longer-term studies incorporating advanced imaging modalities and biomechanical analyses are warranted to fully evaluate the therapeutic potential of TTCP in fracture healing. Another limitation to this study is the absence of a vehicle-only control group (distilled water without TTCP applied to the fracture site). As local fluid application can potentially influence initial clot organization and early callus formation, we cannot entirely exclude a contribution of the vehicle effect. However, the consistent differences observed between TTCP and PC group suggest that the main effect was material related. Taken together, future studies should employ longitudinal sampling at multiple timepoints to capture the dynamic sequence of inflammation, cartilage formation, and ossification during fracture healing. Stability-controlled fracture models and integration of biomechanical assessments would provide deeper insight into the relationship between mechanical environment and TTCP-mediated repair. Studies on release kinetics could clarify whether TTCP modulates local ion concentrations or growth factor activity during the healing process. Furthermore, composite systems combining TTCP with dicalcium phosphate anhydrous (DCPA) or collagen may improve handling characteristics and resorption profiles, potentially broadening clinical applicability.

In conclusion, our study results demonstrate that TTCP, when applied directly to the fracture line, provided radiographic and histological evidence of improved healing on Day 28 without adverse reactions. Owing to its biocompatibility, osteoconductive properties, and ease of application, TTCP shows promise as a synthetic bone graft adjunct in complex fractures. Further long-term and biomechanical studies are still needed to confirm its clinical utility.

Acknowledgments: The authors would like to thank Atatürk University Scientific Research Projects Unit for their valuable support (Project code: TTU-2022-10243). The knowledge and technical support provided during this research were greatly appreciated.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Idea/concept, B.K., K.T.; Design, writing the article, references and fundings: B.K.; Control/supervision, critical review: B.K., O.K.; Data collection and/or processing: B.K., S.S.; Analysis and/or interpretation: B.K., C.B.; Literature review: B.K., Y.S.D.; Materials: B.K., U.O.

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

Funding: The authors received no financial support for the research and/or authorship of this article.

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