Joint Diseases and Related Surgery

Jt Dis Relat Surg
2024;35(1):121-129

ORIGINAL ARTICLE

Vitamin D3 and omega-3 polyunsaturated fatty acids have beneficial effects on fracture union in an experimental rat model

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Fracture healing is one of the unique orthopedic problems that maintains its importance today. It is an important regenerative process including complex interactions between various anatomical, biomechanical, and biochemical processes that begins with inflammation after injury and ends with osteogenesis.[1,2] In this complex physiological process, many local and systemic factors play a crucial role. There are many studies on fracture healing and nonunion in the literature. The fact that there are many factors affecting fracture union has led orthopedic doctors to find treatment methods that will positively affect and accelerate fracture healing. Nutritional factors have a significant role in fracture union. For this purpose, experimental and clinical studies are continuing to determine various active substances to accelerate fracture healing in addition to surgical treatment.

Received: August 24, 2023
Accepted: October 04, 2023
Published online: November 02, 2023

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Doi: 10.52312/jdrs.2023.1397

Citation: Kafadar İH, Yalçın Y, Çakar B. Vitamin D3 and omega-3 polyunsaturated fatty acids have beneficial effects on fracture union in an experimental rat model. Jt Dis Relat Surg 2024;35(1):121-129. doi: 10.52312/jdrs.2023.1397.

ABSTRACT

Objectives: This study aimed to determine the influences of vitamin D3 and omega-3 polyunsaturated fatty acids (PUFAs) on fracture union in rats radiologically, histologically, and biomechanically.

Materials and methods: Forty-eight male Sprague-Dawley rats (mean weight: 435±31.15 g; range, 398 to 510 g) were indiscriminately separated into four groups, with 12 rats in each: Group 1 was the control group, Group 2 received vitamin D3, Group 3 received omega-3 PUFA, and Group 4 received both vitamin D3 and omega-3 PUFA. One day after surgery, only one intramuscular dose of 50,000 IU/kg vitamin D3 was administered to Group 2. From the first postoperative day until sacrification, 300 mg/kg omega-3 PUFA by oral feeding was administered to Group 3. In Group 4, both an intramuscular dose of 50,000 IU/kg vitamin D3 on the initial postoperative day and 300 mg/kg omega-3 PUFA were administered by oral feeding until sacrification. All rats were sacrificed by intracardiac potassium injection at the sixth postoperative week, and radiological, biomechanical, and histological studies were conducted.

Results: According to the radiological scores, the best scores were obtained in Group 4, and callus density and ossification were advanced in Groups 2 and 3 compared to Group 1. There was no statistically significant distinction between Groups 3 and 4, while a significant distinction was found between Group 4 and Groups 1 and 2. Biomechanically, the advanced values were attained in Groups 1 and 3. However, there was no statistically significant distinction among the groups. Histologically, although the advanced scores were attained in Groups 3 and 4, there was no statistically significant distinction among the groups.

Conclusion: The use of omega-3 PUFA together with vitamin D3 might have beneficial influences on fracture union. In the future, the combination of omega-3 PUFA and vitamin D3 might be used as an encouraging treatment choice that contributes to fracture healing.

Keywords: Animal experiment, fracture healing, omega 3 fatty acids, vitamin d3.

Not many researchers have focused on nutritional treatment for recovery of fractures; those include vitamin D and calcium due to their regulatory role in skeletal metabolism. However, the effect of vitamin D on fracture healing is a much less studied condition. Studies performed in animal models have shown promising effects that adequate dietary supplementation could enhance bone healing.[3-5]

In recent years, the regulatory role of fatty acids in bone restoration proceeding has been indicated.[6] As far as we know, only one animal study to date has reported the effect of omega-3 polyunsaturated fatty acids (PUFAs) on bone fracture healing.[7] Chen et al.[7] indicated significant acceleration in callus formation and fracture healing and that supplementation of omega-3 PUFAs was positively associated with fracture healing. However, it was also thought that omega-3 PUFA could increase the density of the mediators, which are efficacious in fracture union by promoting the bleeding around the fractured space, thus accelerating the fracture union.

To our best knowledge, there are not enough experimental studies on the efficiency of omega-3 PUFA and vitamin D3 or their combinations on fracture healing in the literature. Hence, in this experimental study, we researched the influences of omega-3 PUFA and vitamin D3 on fracture union in rats radiologically, histologically, and biomechanically.

MATERIALS AND METHODS

The animal study was conducted on forty-eight 12-month-old male Sprague-Dawley rats (mean weight: 435±31.15 g; range, 398 to 510 g) obtained from our university's experimental animal research laboratory. They were kept in a 10- to 14-h day/night cycle at standard dampness and temperature. They were nourished with tap water and standard pellet feed. Rats were indiscriminately separated into four groups, with 12 rats in each: Group 1 was the control group, Group 2 received vitamin D3, Group 3 received omega-3 PUFA, and Group 4 received both vitamin D3 and omega-3 PUFA.

Surgical anesthesia was provided with 90 mg/kg ketamine hydrochloride (Ketalar®, Eczacıbaşı, Istanbul, Türkiye) and xylazine chloride (Rompun®, Bayer, Leverkusen, Germany). The left lower extremities of the rats were used. Arthrotomy with a midline incision through the knee was performed. The joint space and the middle/distal one-third of the femur were revealed. Periosteum was scraped and osteotomized from the mid-diaphyseal region of the left femur with the help of a mini electric saw (Triton, Micro sagittal saw; Medtronic, Minneapolis, MN, USA). Afterward, 1.5 mm K-wires were retrogradely sent from the knee joint for fixation. The length of the implanted K-wires was not uniform due to the K-wires being sent up towards the greater trochanter until they were palpated to protrude. They provided adequate stability of the fracture. After washing, the layers were closed with 3/0 sharp needle Ethicon® brand vicryl suture (Ethicon Inc., Raritan, NJ, USA). The rats were kept in separate environments during the recovery period after anesthesia and were placed in their cages after recovery with two subjects in each cage.

In the postoperative period, the control group (Group 1) was followed without any agent until the sacrification. The rats in Group 2 were administered of a single dose of 50,000 IU/kg intramuscular vitamin D3 (Devit-3® ampoule, 300,000 IU/mL; Deva Holding A Ş, Istanbul, Türkiye) was performed on the first postoperative day. Omega-3 PUFA (Solgar® capsule [504 mg eicosapentaenoic acid + 378 mg docosahexaenoic acid]; Solgar Inc., New Jersey, USA) 300 mg/kg/day was applied to the rats in Group 3 by oral feeding from the initial postoperative day until sacrification. Group 4 received both an intramuscular dose of 50,000 IU/kg vitamin D3 on the first postoperative day and 300 mg/kg omega-3 PUFA by oral feeding from the initial postoperative day until sacrification.

All rats were sacrificed at the sixth postoperative week. Ketamine-xylazine anesthesia was applied to all subjects. All rats were sacrificed by intracardiac injection of 75% potassium chloride (Galen İlaç Sanayi ve Ticaret, A.Ş., Istanbul, Türkiye). After sacrification, lateral and anteroposterior (AP) radiographs of the left femurs of all subjects were taken. In each group, six femurs (Subjects 1 to 6) were randomly dissected from each group of subjects and left for histological study in 10% formalin. In the remaining subjects (Subjects 7 to 12), six femurs were placed on moist gauze pads impregnated with saline and sent for biomechanical study.

After sacrification, all samples were numbered, and AP and lateral radiographs of femurs were taken on the same day. Radiographs were taken with a high-resolution digital radiography system (Multix Impact C; Siemens Healthcare GmbH, Erlangen, Germany). Imaging was standardized using 66 kV, 1.82 msec, 1.20 mAs,

TABLE I
Lane-Sandhu classification for evaluating radiological data

0	No callus
1	There is callus formation
2	Beginning of bony union
3	Absence of the fracture line
4	Complete osseous union

and 1X magnification from a distance of 110 cm. Assessment was performed according to the bone formation section of the Lane and Sandhu[8] scoring system (Table I). Anteroposterior and lateral radiographs were evaluated, and scores were given from 0 to 4 according to the scoring system (0 points: no evidence of bone formation (no callus); 1 point: bone formation occupying 25% defect (there is callus formation); 2 points: bone formation occupying 50% defect (beginning of bony union); 3 points: bone formation occupying 75% defect (absence of the fracture line); 4 points: full gap bone formation (complete osseous union).[9] Both AP and lateral radiographs were evaluated by a radiologist who was blinded to the study.

The samples containing the fracture healing area on the left femur of six rats (Subjects 1 to 6) from each group were fixed in a 10% formaldehyde solution. It was decalcified by keeping it in an acid solution for 24 h. They were then dehydrated with successive degrees of ethanol, rinsed with xylene, and embedded in paraffin. Four- to six-micron thick sections taken from paraffin blocks were stained with hematoxylin-eosin, nuclear fast red, and alcian blue. Afterward, four to six sections were examined by a specialist pathologist before the assessment of numerical grade for callus maturity under a light microscope (Nikon Optiphot-2; Nikon Instech Co., Ltd., Tokyo, Japan) using the Histological Scoring System, which was described by Huo et al.[10] (Table II). A grading system in which 10 phases of fracture repair were identified was used. Scoring was made from 1 to 10 points, with the least ossification receiving 1 point and the most ossification receiving 10 points.

The left femurs of the next six subjects (Subjects 7 to 12) from each group were randomly selected for biomechanical evaluation. The numbered samples were wrapped in a wet sponge impregnated with physiological saline solution and delivered to the Technology Research and Application Center Laboratory on the same day of sacrification. Soft tissues on the bone were cleaned.

TABLE II
The scoring system used by Huo et al.[10] to evaluate histological data

Grade	
1	Fibrous tissue
2	Predominantly fibrous tissue, little cartilage
3	Equal proportion of fibrous and cartilage tissue
4	Predominantly cartilage, little fibrous tissue
5	Cartilaginous tissue
6	Predominantly cartilage, little immature bone
7	Equal proportion of cartilage and immature bone tissue
8	Predominantly immature bone, little cartilage tissue
9	Immature bone and fracture healing
10	Fracture healing with mature bone

A three-point refractive test was applied for the biomechanical study (AG-XD 50 kN; Shimadzu Corp., Shimadzu, Kyoto, Japan). After the lower apparatus space was fixed at 20 mm, bone samples were placed so that the osteotomy area was centered on the upper apparatus. The device performed the breaking test by pressing at a speed of 1 mm/min). The experiment was terminated after the first break in the callus tissue and the moment when the force pressing was reduced. Obtained values were recorded in Newtons (N).

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U and Kruskal-Wallis tests were performed for intragroup and intergroup crosschecks, respectively. A p-value <0.05 was considered statistically significant.

RESULTS

Anteroposterior and lateral radiographs of the four groups are shown in Figures 1 to 4. Radiological union was observed in all groups. The mean values obtained according to the Lane and Sandhu[8] scoring were 2.67±0.88 in Group 1, 2.83±0.57 in Group 2, 3.50±0.52 in Group 3, and 3.83±0.38 in Group 4 (Table III). Although the best scores were obtained in Group 4, when each group was compared in pairs with the Kruskal-Wallis test, only the distinctions between Groups 1 and 4 and Group 2 and 4 were statistically meaningful ($p=0.001$ and $p=0.003$, respectively).

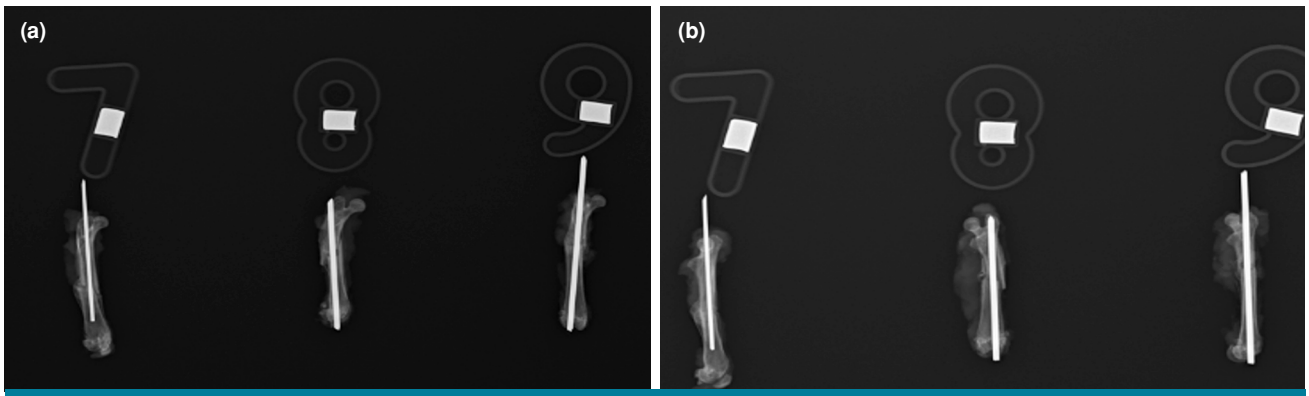

FIGURE 1. Radiographs of rats in Group 1. **(a)** Anteroposterior view, **(b)** lateral view.

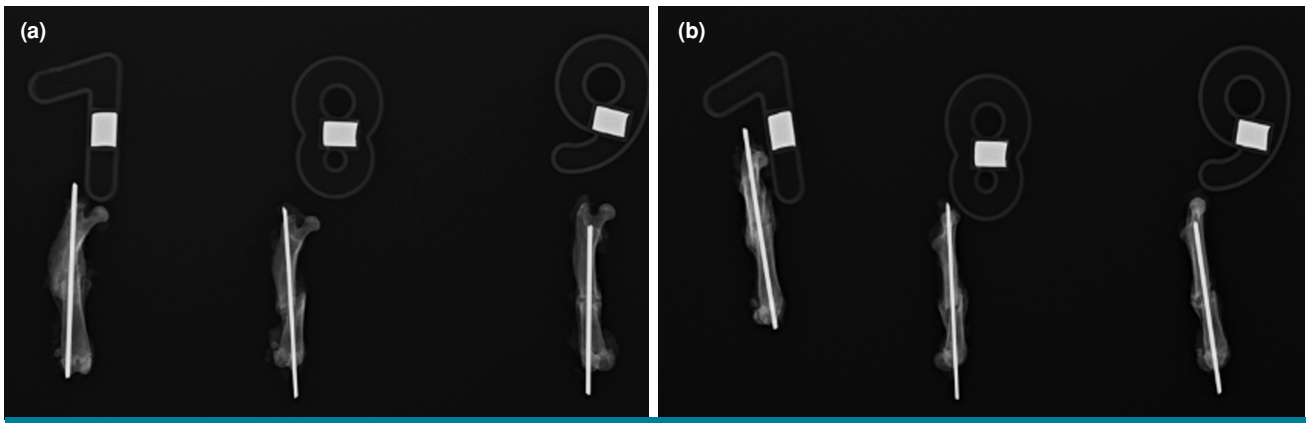

FIGURE 2. Radiographs of rats in Group 2. **(a)** anteroposterior view, **(b)** lateral view.

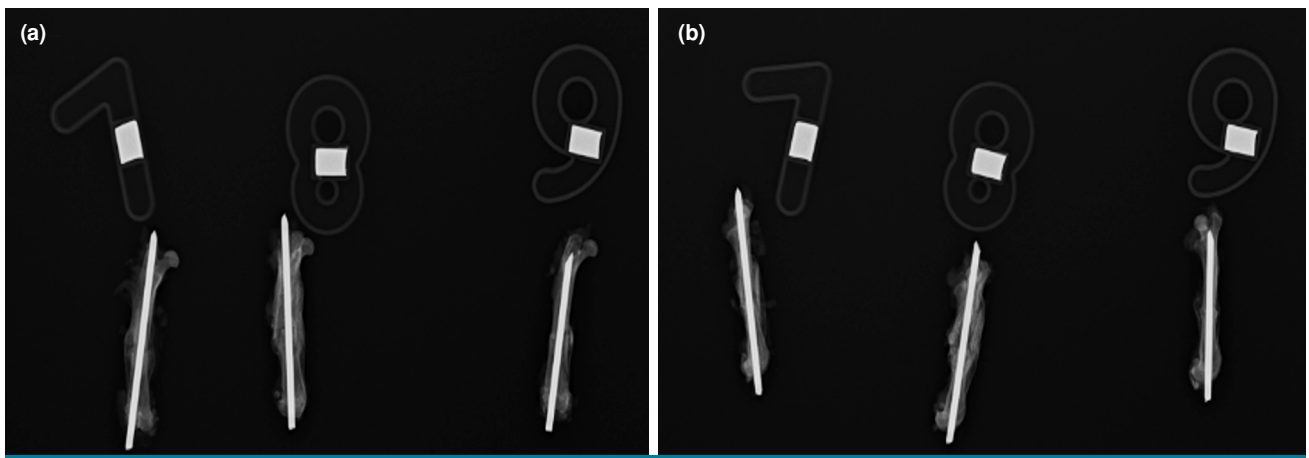

FIGURE 3. Radiographs of rats in Group 3. **(a)** anteroposterior view, **(b)** lateral view.

The histological image of the osteotomy line after sacrification is shown in Figure 5. According to the histological evaluation, all rats in all groups showed improvement with mature-immature bone tissue around the osteotomy line, which was consistent with the radiological evaluation. The mean values were 9±1.09 for Group 1, 9±1.09 for Group 2, 9.67±0.81 for Group 3, and 9.67±0.81 for Group 4 (Table IV).

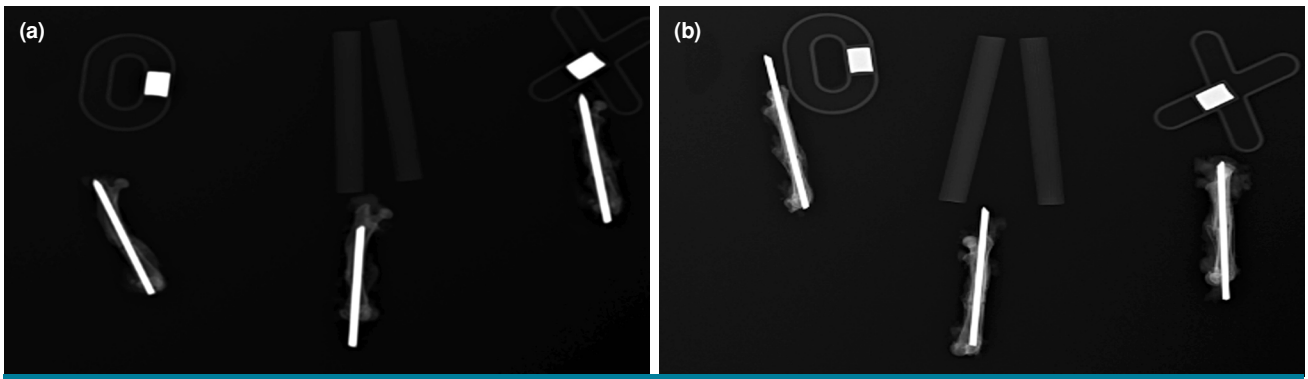

FIGURE 4. Radiographs of rats in Group 4. **(a)** anteroposterior view, **(b)** lateral view.

TABLE III
Radiological scores of the groups

Subject number	Group 1	Group 2	Group 3	Group 4
1	4	3	3	4
2	3	2	3	4
3	2	3	4	4
4	2	2	3	4
5	1	2	3	4
6	2	3	4	4
7	3	4	4	4
8	2	3	4	4
9	3	3	4	3
10	3	3	3	3
11	3	3	3	4
12	4	3	4	4
Total/mean	32/2.67	34/2.83	42/3.50	46/3.83

TABLE IV
Histological scores of the groups

	Histopathological scores			
Subject number	Group 1	Group 2	Group 3	Group 4
1	10	10	10	10
2	8	8	8	10
3	8	10	10	10
4	8	8	10	8
5	10	8	10	10
6	10	10	10	10
Total/mean	54/9	54/9	58/9.67	58/9.67

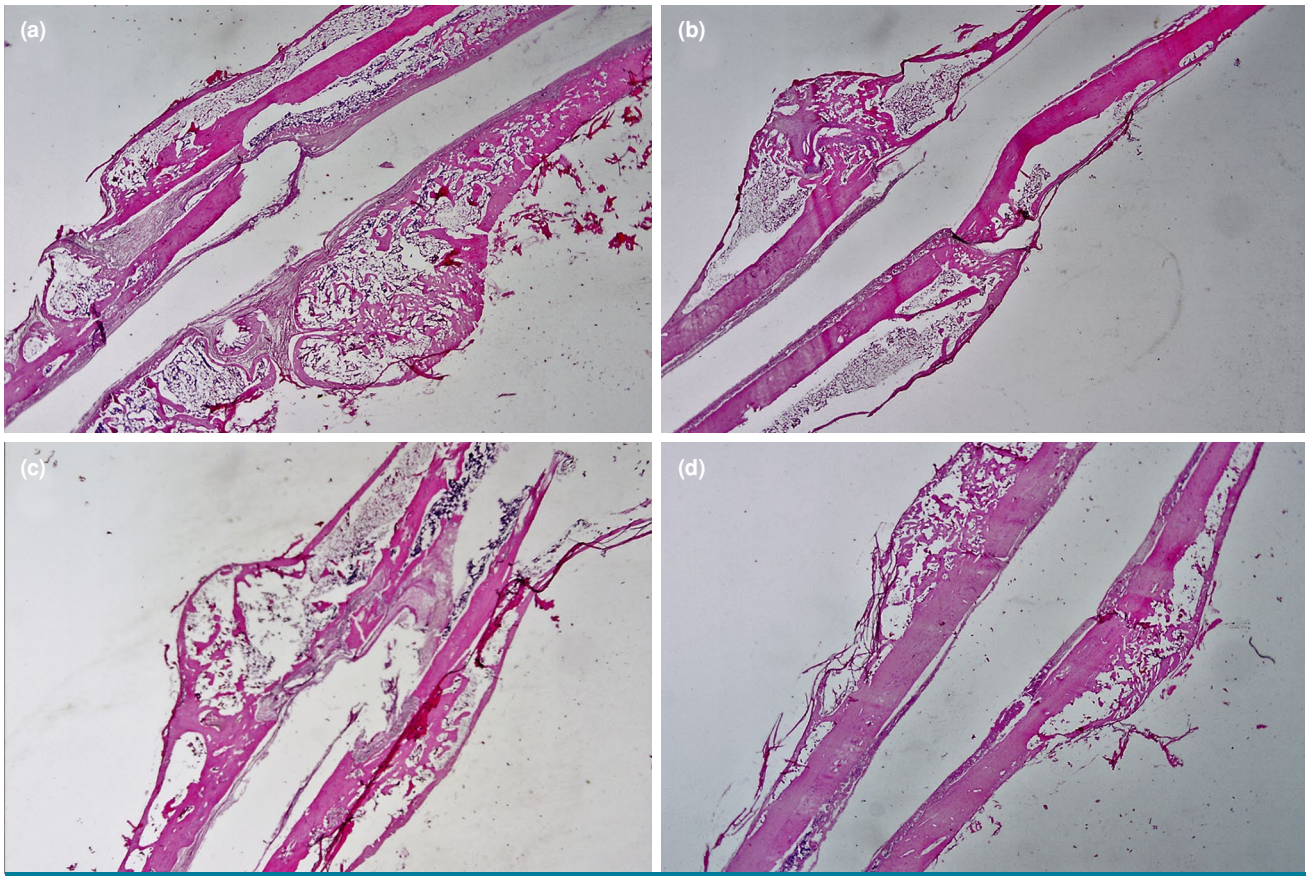

FIGURE 5. Histological images of groups (H&E, ×40). **(a)** Group 1, **(b)** Group 2, **(c)** Group 3, **(d)** Group 4.

The scores were higher in Groups 3 and 4. However, when the distinction between the groups was compared statistically, it was found that there was no statistically significant distinction among the groups (p=0.547).

In the three-point bending test performed in the biomechanical assessment, it was observed that the highest scores were in Groups 1 and 3 (Group 3 received omega-3 PUFA; Table V). The mean values were 400,575±99.43 N for Group 1, 46,910±26.08 N for Group 2, 396,159±392.92 N for Group 3, and 281,224±262.31 N for Group 4. The Kruskal-Wallis test was performed for statistical evaluation between groups, and the distinction was not found to be statistically significant (p=0.149).

DISCUSSION

Vitamin D and its metabolites play a crucial role in bone metabolism and fracture healing.[11] There are studies reporting a decrease in vitamin D levels in fracture union, and it has been shown in the literature that vitamin D has positive effects on fracture healing.[12] In an editorial that was written by Atik,[13] it was stated that vitamin D3 supplementation is effective. Therefore, we wanted to evaluate the influences of vitamin D3 on fracture healing in this study.

Omega-3 PUFAs are a group of indispensable fatty acids that cannot be adequately synthesized, thus they should be taken with nutrition.[14] In literature reviews, influences of omega-3 PUFA on fracture healing in the field of orthopedics and traumatology are insufficient, although scientific studies have been conducted and even used in many branches. Although previous studies have provided new information on the impacts of omega-3 PUFA on bone metabolism, a few studies have drawn attention to the impacts of omega-3 PUFA on fracture union. From this point of view, we planned an experimental study to reveal the impacts of vitamin D3 and omega-3 PUFA on experimental fracture union and wanted to add new information to the literature.

TABLE V
Biomechanical results and mean values of the groups

	Subject number	Maximum power (Newton)	Mean
Group 1	7	469,462	400,575
	8	448,738	
	9	537,732	
	10	302,690	
	11	351,972	
	12	292,809	
Group 2	7	82,749	46,910
	8	16,347	
	9	21,084	
	10	39,748	
	11	67,120	
	12	54,413	
Group 3	7	154,660	396,159
	8	64,287	
	9	463,846	
	10	1144,82	
		341,434	
	12	207,908	
Group 4	17	418,678	281,224
	8	67,510	
	9	704,538	
	10	25,204	
	11	135,297	
	12	36,864	

In experimental studies, it was viewed that vitamin D3 was applied as a daily dose or a single high dose. In these studies, vitamin D3 was administered as a single high dose of 50,000 IU/kg.[15-17] However, when studies using omega-3 PUFA were examined, it was observed that the daily dose applied by the authors to search the impacts of omega-3 PUFA was 300 mg/kg.[18-20] In light of this information, we applied omega-3 PUFA to the rats at a daily dose of 300 mg/kg, alone or in combination with vitamin D3.

Aydoğan et al.[21] evaluated the effects of vitamin D and bisphosphonate on fracture union and evaluated radiological and histological scores. Radiologically, the advanced scores were observed in the group given a bisphosphonate and a combination of bisphosphonate and vitamin D, but it was not found statistically significant. In the histological assessment, the advanced scores were observed in the same groups, and they evaluated this histological improvement as significant compared to the control group. In another study conducted by Aslan et al.[22] with 40 rats divided into four groups, the effects of vitamin D3 and calcium on fracture union were evaluated. The rats were evaluated radiologically, histologically, and biomechanically, as in our study. They found that there was a significant improvement in the group that received the combination of vitamin D3 and calcium in terms of fracture union compared to the control group. Although radiological scores were advanced in the group that was given only calcium and only vitamin D3 compared to the control group, they could not find a significant distinction. Similarly, histological scores were highest in the same group, but it was not statistically meaningful. In the biomechanical evaluation, they found statistically significant distinction in the groups given only vitamin D3, calcium, and the combination compared to the control group. However, they found that there was no distinction between the groups given only calcium and only vitamin D3. In the study of Fu et al.,[23] 40 rats were divided into two groups. Medium-chain triglyceride was given to one group, and vitamin D3 was administered to the other group by gastric lavage. In the radiological evaluation performed six weeks later, they found that the fracture line was less pronounced in the vitamin D3 group, and at the end of the 16th week, the fracture mark was not evident in both groups. In histological assessment, it was shown that the calf tissue was better remodeled in the vitamin D3 group. In addition, the biomechanical evaluation revealed that the biomechanical values of the group given vitamin D3 were approximately one-fold higher than the other group at the end of the sixth week ($p=0.001$), and the biomechanical values were better in the group given vitamin D3 at the 16th week.

In this study, when we examined the radiological point of view according to the Lane and Sandhu[8] scoring system, it was found that the best values were in Group 3, which received omega-3 PUFA, and Group 4, which received vitamin D3 + omega-3 PUFA. Accordingly, in terms of radiological union findings, it was seen that the combination of vitamin D3 and omega-3 PUFA had positive effects. When the scores of the groups in the histological evaluation were investigated, it was seen that there was no significant distinction between the groups, but Groups 3 and 4, which were given omega-3 PUFA, had the highest scores.

Ömeroğlu et al.,[4] in the experimental fracture healing model in which they applied high-dose

vitamin D3 as a single dose, displayed that one dose of vitamin D3 increased the load endurance and rigidity at the fracture location. In another study by Hussain et al.,[15] in which one dose of high-dose vitamin D3 was administered, they compared the tibia of rats with the three-point bending test in terms of biomechanics. The values of the control group were found to be minimally higher, and no statistically significant result could be obtained. After the experimental femoral fracture in another study, rats were separated into two groups.[5] Vitamin D3 was given to the first group, and no active substance was given to the other group. The biomechanical study was performed at the end of five weeks, and they showed that there were positive results in the group that was given vitamin D3. It has been suggested that if similar results are obtained in a study conducted on humans, it might be an alternative way to contribute positively to fracture healing in the elderly.[5] In another study, Lindgren et al.[24] divided the tibia of 16 rabbits into two groups after fixation with K-wire after a closed fracture. No substance was given to the control group until they were sacrificed, and they administered 75 ng subcutaneous vitamin D3 daily to the study group until they were sacrificed. As a result of the biomechanical test in the study, the endurance value was found to be 101±21 N in the control group and 57±8 N in the study group. Therefore, contrary to other studies, it was determined that vitamin D3 had a negative effect on biomechanics. Again, in the study of Lidor et al.[25] on chicks, one group formed the control group without any detection after open tibia fracture, and the other group was locally administered 1.8 µg of vitamin D3. In the biomechanical comparison made after sacrification two weeks later, it was found that the group given vitamin D3 had less torsion resistance.

In our study, from the biomechanical point of view, it was seen that Group 1 (control group) and Group 3 (omega-3 PUFA group) had the highest mean scores. However, the difference was not statistically significant. In biomechanical studies in the literature on vitamin D3, some demonstrate positive effects, whereas others reveal negative effects of vitamin D3.[23-25] In our study, similar to some studies in the literature, it was determined that vitamin D3 had a negative effect on biomechanics. Group 2 had the lowest mean score (46,910±26.08 N). Therefore, lower resistances of the callus tissues against bending might depend on vitamin D3.

The current study is one of the unique experimental studies investigating fracture healing in which omega-3 PUFA was applied exogenously. Nevertheless, there are limitations of the current study. One limitation is the lack of biochemical assessment. Fracture healing was evaluated radiologically, histologically, and biomechanically in the current study. However, biochemical studies might have contributed to the study in terms of supporting the fracture healing findings. The other limitation of the study is the effect of vitamin D3 on the biomechanical results. High doses of vitamin D3 might have negative effects on fracture healing. Therefore, more detailed results could be achieved by using low doses of vitamin D3 alone or in combination with omega-3 PUFA. Further experimental studies could include more groups for this purpose.

In conclusion, vitamin D3 and omega-3 PUFA consumption might have beneficial impacts on fracture union radiologically and histologically. In light of our findings, it was determined that omega-3 PUFA may have beneficial impacts on fracture union. Although it is not used much in orthopedic surgery practice, we think that the use of omega-3 PUFA in clinical studies should increase.

Ethics Committee Approval: The study protocol was approved by the Erciyes University Animal Experiments Local Ethics Committee (date: 13.01.2016, no: 16/020). The study was conducted in accordance with the principles of the Declaration of Helsinki.

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

Author Contributions: The study control/supervision, conception and design were performed: I.H.K.; Material preparation, data collection and analysis were performed, the first draft of the manuscript was written: I.H.K., Y.Y., B.Ç.; The final checks of the article were performed: I.H.K., B.Ç.; All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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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