Effects of Vitamin D3 and Calcium on Fracture Healing in Rats

RATLARDA KEMİK İYİLEŞMESİNE VİTAMİN D3 VE KALSİYUMUN ETKİLERİ

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Abstract

Objective: We aimed in this study, to evaluate the effects and mechanism of action of single high-dose vitamin D_3 and calcium on fracture healing in rats.

Material and Methods: A total of 40 rats were divided into four groups; the first group (Group A) was treated with calcium, the second group (Group B) with vitamin D_3, the third group (Group C) with calcium and vitamin D_3 combination, and the fourth group was the control group (Group D). Tibiae of the rats were osteotomized by a Gigli saw. All rats were sacrificed 3 weeks after surgery. The results obtained were compared by mechanical testing, histological examination and radiographic evaluation.

Results: Mean radiographic scores were 1.6 ± 0.8 for group D; 1.7 ± 0.8 for group A; 2.3 ± 0.8 for group B; and 2.4 ± 0.7 for group C. There was a statistically significant difference between the mean radiographic scores of only groups C and D (p= 0.037). There was also a statistically significant difference between the corresponding fracture load values of group D and all the other groups (p= 0.000), but there was no such difference between the corresponding fracture load values of the groups A and B (p= 0.208). Mean histological scores were 5.6 ± 2.7 for group D; 6.0 ± 2.7 for group A; 7.0 ± 2.1 for group B; and 7.2 ± 1.9 for group C (p> 0.05).

Conclusion: These results suggested that the real effect of vitamin D on fracture healing was via calcium metabolism. We conclude that calcium and vitamin D given in the early stages of fracture healing gave opportunity for early weight bearing.

Key Words: Angiogenesis inducing agents, fracture healing, calcium, cholecalciferol

Özet

Amaç: Biz tek yüksek doz vitamin D_3 ve kalsiyumun ratlarda kemik iyileşmesine etkisini ve etki mekanizmasını değerlendirmeyi amaçladık.

Gereç ve Yöntemler: 40 rat 4 gruba bölündü ve bir deney grubu kalsiyumla (Grup A), 1'i vitamin D_3 ile (Grup B), 1'i kalsiyum ve vitamin D_3 kombinasyonuyla (Grup C) tedavi edildi ve 4. grup kontrol grubuydu (Grup D). Deneklerin tibiaları Gigli testere yardımıyla osteotomize edildi. Tüm ratlar ameliyattan 3 hafta sonra sakrifiye edildi. Mekanik test, histolojik muayene ve radyolojik değerlendirmeden elde edilen sonuçlar karşılaştırıldı.

Bulgular: Ortalama radyolojik skor grup D için 1.6 ± 0.8; grup A için 1.7 ± 0.8; grup B için 2.3 ± 0.8 ve grup C için 2.4 ± 0.7 hesaplandı. İstatistiksel açıdan yalnızca C ve D grubunun radyolojik skorları arasında fark vardı (p= 0.037). Grup D ve diğer gruplar arasında kırık yüklenme değeri arasına istatistiksel belirgin farklılık vardı (p= 0.000). Fakat A ve B gruplarının kırık yüklenme değeri arasında istatistiksel belirgin fark yoktu (p= 0.208). Ortalama histolojik skor D grubu için 5.6 ± 2.7; A grubu için 6.0 ± 2.7; B grubu için 7.0 ± 2.1 ve C grubu için 7.2 ± 1.9 olarak hesaplandı (p> 0.05).

Sonuç: Bu sonuçlar bize vitamin D'nin kırık iyileşmesine gerçek etkisinin kalsiyum metabolizması yoluyla olduğunu düşündürdü. Biz fraktür iyileşmesinin erken basamaklarında kalsiyum ve vitamin D verilmesinin erken ağırlık taşıma fırsatı vermiş olmasının yanlış bir fikir olmadığını düşünüyoruz.

Anahtar Kelimeler: Anjiyogenez, kalsiyum, kırık iyileşmesi, kolekalsiferol

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Many substances appear to influence fracture healing: Biphosphonates, hormones and growth factors and others. Their effects are due to different processes affecting bone healing such as calcium absorption, angiogenesis, collagen deposition, osteoblast stimula-

tion and bone remodelling.[1-5] The effects of most of the factors on fracture healing have not yet been absolutely verified.[6]

Vitamin D_3 (Cholecalciferol) is known to be one of the hormones promoting fracture healing.[6,7] In the past, many studies on the effects of vitamin D_3 on fracture healing were undertaken, frequently in vitamin D depleted animal models and with different protocols.[6,8-13] However, the mechanism of action of vitamin D_3 is not clearly defined.

In the present study, we aimed to evaluate the effects and mechanism of action of single high-dose vitamin D_3 and calcium on fracture healing in rats. We compared the results obtained by mechanical testing, histological examination and radiographic evaluation.

Material and Methods

General description of experimental protocols

Forty female, healthy (normocalcaemic and normophosphataemic) Sprague-Dawley rats, weighing between 250 and 300 gr were used. The study protocol was designed in accordance with Guide for the Care and Use of Laboratory Animals, and was reviewed and accepted by the institutional animal care and use committee. The right limbs were shaved and cleaned by betadine solution. The right tibia of each rat was exposed via an antero-medial skin incision under ketamine hydrochloride anaesthesia. Following the skin incision, the midshaft of the tibia was reached by blunt dissection. The shaft was then osteotomized transversly by a Gigli saw taking care to cause minimal soft tissue injury at the fracture site. An additional longitudinal parapatellar incision was made and a Kirschner wire was introduced to the intramedullary canal of the tibia for fixation. The largest size (1 mm) of Kirschner wires was used to fit the distal intramedullary space of the rat tibiae. Following haemostasis, the layers were closed with interrupted sutures. Perioperative antimicrobial prophylaxis, consisting of 50 mg/kg/day Cefazolin-sodium was administered. The animals were randomly divided into 4 groups of ten animals each. The first group (Group A) was treated with calcium, the second group (Group B) with vitamin D_3, the third group (Group C) with calcium and vitamin D_3, combination, and the 4th group was the control group (Group D). Animals in group A were injected intramuscularly with a total dose of 90 mg calcium in 10 days (9 mg/day); group B were injected intramuscularly with a single high-dose 50000 IU/kg of vitamin D_3; group C were injected intramuscularly with a single high-dose 50000 IU/kg of vitamin D3 and a total dose of 90 mg calcium in 10 days (9 mg/day), and the ones in group D were injected with 0.9% NaCl. The rats were allowed unrestricted weight bearing after recovery from anaesthesia. The rats in each group were offered isocaloric feedings and deionised water ad libitum. All diets consisted of a mixture of vitamins and salt that provided adequate concentrations of important elements such as magnesium, phosphorus, and zinc. Calcium and vitamin D were omitted from the diet. These animals were kept in individual cages; they were housed in a temperature of 24^{o}C, in a room with controlled air humidity (55%) and light (12 h lamp light, 12 h dark). Surgical wound infection did not occur. All rats were sacrificed 3 weeks after surgery. The fractured tibiae and fibulae were removed by careful dissection, and after the resection of the fibulae, intramedullary Kirschner wires were pulled out by applying small torsional movements.

Radiographs

Radiographs were performed in two planes after the animals were killed. All radiographs were randomized and were independently scored by 2 orthopaedists, who were unaware of the treatment the animal had received. Each fracture specimen was reviewed and classified for callus maturity according to the classification of Goldberg et al.[14] In that classification, stage 1 indicates non-union; stage 2, possible union; and stage 3, radiographic union. Median radiographic scores were calculated for each group. The differences between experimental and control groups were analyzed using Mann-Whitney U non-parametric test and balanced with the Kruskal-Wallis test if more then 2 groups were compared. $p \leq 0.05$ was considered statistically significant.

Mechanical testing

Twenty tibiae (five each from all groups) were prepared for the mechanical test. The control and experimental groups were numbered to blind the biomechanical measurements. The tibiae were kept at -20°C for further analysis. Prior to the tests, the tibiae were placed in a humid medium and were kept there for 4 h until they were thawed to room temperature. The distal and proximal parts of the tibiae were cut out to obtain a better adjustment to the three-point bending fixture.

The tibiae were placed on the three-point bending configuration on the Lloyd LS500 material testing device (Southampton, UK). The 500-N load cell was used for load detection, and the sampling rate was 4.0 Hz. The loading speed was 2 mm/min, and the load was applied at the mid-span in anteroposterior direction, with a span length of 40 mm. All the bones were kept in a humid medium during the tests. The load-deflection curves were stored in the computer to be processed later to obtain fracture load values.

Biomechanical data were compared by analysis of variance for parametric data (Tukey HSD test). $p \leq 0.05$ was used to define a significant difference between groups.

Bone histomorphometry

Twenty tibiae (five each from all groups) were prepared for the histological examination. The specimens were fixed in 10% neutral buffered formalin for one day and than decalcified with 6% aqueous HCl for 2 days. The tibia and fibula were separated and embedded in paraffin, which allows obtaining 5-micron transverse sections from each block and were stained with haematoxylin-eosin and Toluidin blue. The progression of fracture-healing in each specimen was quantified with the use of a scale that assigns a grade based on the relative percentages of fibrous tissue, cartilage, woven bone, and mature bone in the callus (Figure 1-3).[15,16] Grade 1 indicates fibrous tissue; grade 2, predominantly fibrous tissue with some cartilage; grade 3, equal amounts of fibrous tissue and cartilage; grade 4, all cartilage; grade 5, predominantly cartilage with some woven bone; grade 6, equal amounts of

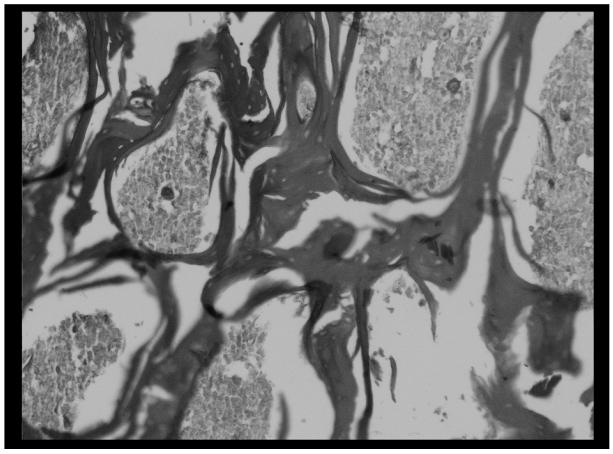

Figure 1. Lamellar and sclerosing bone tissue formation along mature bone tissue on rats which were treated with Vit D + calcium. Toluidin blue x 40.

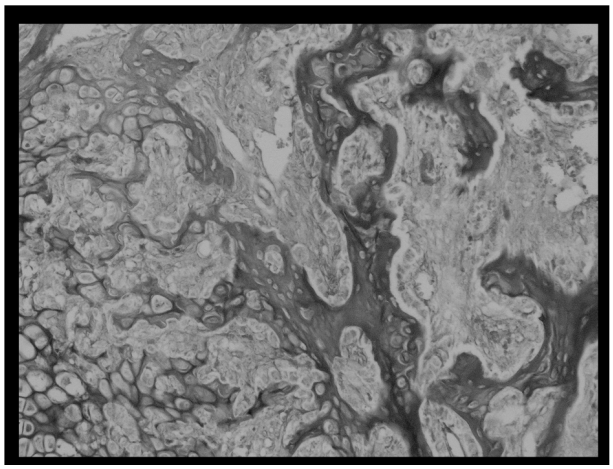

Figure 2. Osteoblastic activity combined with fibrous tissue and new bone formation in rats which were treated with Vit D + calcium. Toluidin blue x 100.

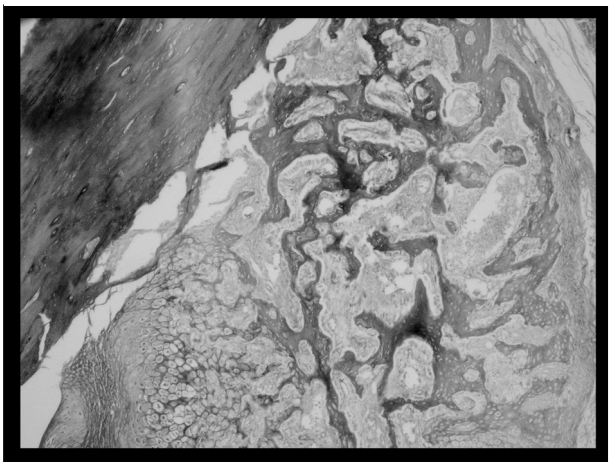

Figure 3. Formation of sclerosis of the bone lamels in rats that were with calcium. Toluidin blue x 100.

Table 1. Fracture healing: Mechanical, histological and radiological results.

	Radiographic Stage [&]			Biomechanical Test [§]			Histological Grade [£]		
Group	**n**	**mean ± SD**	**p***	**n**	**mean ± SD**	**p****	**n**	**mean ± SD**	**p***
D (Control)	10	1.60 ± 0.84		5	63 ± 13.50		5	5.60 ± 2.70	
A (Ca)	10	1.70 ± 0.82		5	214 ± 17.10		5	6.00 ± 2.73	
B (vit D)	10	2.30 ± 0.82	0.073	5	234 ± 16.35	0.000	5	7.00 ± 2.12	0.692
C (Ca + vit D)	10	2.40 ± 0.69		5	360 ± 14.14		5	7.20 ± 1.92	

* Non-parametric Kruskal-Wallis test **Analysis of variance,
[&] stage 1= Indicates non-union, stage 2= Possible union, stage 3= Radiographic union, [§] Maximum load (Newton),
[£] grade 1= Indicates fibrous tissue, grade 2= Predominantly fibrous tissue with some cartilage, grade 3= Equal amounts of fibrous tissue and cartilage, grade 4= All cartilage, grade 5= Predominantly cartilage with some woven bone, grade 6= Equal amounts of cartilage and woven bone, grade 7= Predominantly woven bone with some cartilage, grade 8= Entirely woven bone, grade 9= Woven bone and some mature bone, grade 10= Lamellar (mature) bone.

cartilage and woven bone; grade 7, predominantly woven bone with some cartilage; grade 8, entirely woven bone; grade 9, woven bone and some mature bone; and grade 10, lamellar (mature) bone. The grading was done blindly without knowing which treatment had been given. Median fracture healing scores were calculated for each group. The differences between experimental and control groups were analysed using Mann-Whitney U non-parametric test and balanced with the Kruskal-Wallis test if more then two groups were compared. $p \leq 0.05$ was considered statistically significant.

Results

Radiographs

Mean radiographic scores were 1.6 ± 0.8 for group D, 1.7 ± 0.8 for group A; 2.3 ± 0.8 for group B, and 2.4 ± 0.7 for group C (Table 1). There was a statistically significant difference between the radiographic scores of only group C and group D (p= 0.037) (Table 2).

Mechanical tests

Our results showed that the application of stress in all groups resulted with an increase in the fracture load values of the fractured bone compared to those of the control group (Table 1). There was a statistically significant difference between the corresponding fracture load values of the control and other groups (p= 0.000). There was no statistically significant difference between the corresponding fracture load values of the group A and group B (p= 0.208) (Table 3).

Table 2. Statistically significant difference between the radiographic stages of the control and calcium + vitamin D group.

Group	n	Radiographic Stage mean ± SD	z	p*
D (Control)	10	1.60 ± 0.84	-2.084	0.037
C (Ca + vit D)	10	2.40 ± 0.69		

• Non-parametric Mann-Whitney U test.

Table 3. Statistical significance of the differences between the fracture load values of the control and other groups.

Group (I)	Group (II)	Mean Difference (I-II)	p*	95% CI
D (n= 5)				
	A (n= 5)	-151.00	0.000	-178.78 – -123.22
	B (n= 5)	-171.00	0.000	-198.78 – -143.22
	C (n= 5)	-297.00	0.000	-324.78 – -269.22
A (n= 5)				
	B (n= 5)	-20.00	0.208	-47.78 – 7.78
	C (n= 5)	-146.00	0.000	-173.78 – -118.22
B (n= 5)				
	C (n= 5)	-126.00	0.000	-153.78 – -98.22

* Tukey HSD.

Bone morphometry

Mean histological scores were 5.6 ± 2.7 for group D, 6.0 ± 2.7 for group A, 7.0 ± 2.1 for group B, and 7.2 ± 1.9 for group C (Table 1). There was no statistically significant difference between the groups (p> 0.05).

Discussion

It is known indeed that some patients have bone healing problems in clinical practice. Sometimes this problem is unpredictable and not only associated with technical failures but also with biological failures.[1,3,5,17] In these patients biological help could be supplied by adjuvant therapies affecting callus formation, remodelling and mineralization. We chose vitamin D_3 and calcium among the different alternative pharmacological treatments.

The hormonal or bioactive form of vitamin D is 1,25-$(OH)_2D_3$. It is generated from sequential hydroxylation of vitamin D_3, a secosteroid precursor that is obtained from the diet or produced in the skin upon exposure to UV light.[18] The first hydroxylation of vitamin D_3 occurs at the C-25 position and is catalysed by vitamin D-25-hydroxylase in the liver to produce 25-hydroxyvitamin D_3 [25 $(OH)D_3$], the major circulating form of vitamin D in mammals. 25 $(OH)D_3$ is the substrate for a second hydroxylase, the renal 25 $(OH)D_3$-1α-hydroxylase (1αOHase), resulting in the production of the most bioactive metabolite, 1.25-$(OH)_2D_3$.[18] Metabolites of vitamin D_3 have direct effects on bone and it is important in bone metabolism and fracture repair.[6,7]

Lindholm and Sevastikoglou reported increased healing rate, enhanced mineralization of fracture callus and cortical bone formation with treatment with small doses of 1 α $(OH)D_3$.[9] The voluminous callus, as such, could have contributed to increased fracture strength by forming a periosteal callus collar during the early healing.[19] The effect of 1.25 $(OH)_2D_3$ may have been an overall stimulation of callus bone turnover, resulting in a callus collar more disorganized and porous than normal, and with less strength.[8] Administration of 1.25 $(OH)_2D_3$ seemed to increase callus bone turnover with early mineralization, which gave temporary increased elastic stiffness over controls but did not increase tensile strength.[8]

Lindgren et al reported that in healthy adult rats fracture healing was slightly more advanced by the application of 1.25 $(OH)_2D_3$.[10] Dekel et al demonstrated in vitamin D-depleted chicks that the mechanical strength of the callus was increased by the application of 1.25 $(OH)_2D_3$ plus 24.25 $(OH)_2D_3$, cholecalciferol, 1.25 $(OH)_2D_3$ and 24.25 $(OH)_2D_3$ in decreasing order.[11] Lidor et al studied the increase of the levels of vitamin D metabolites in the calluses of chicks, and stated that the 1.25 $(OH)_2D_3$ found in the callus coincided with remodelling and bone formation, and the 24.25 $(OH)_2D_3$ with formation of cartilaginous callus.[12,20] 24.25 $(OH)_2D_3$ was also found to promote the maturation and mineralization of osteoid. Brumbaugh et al noted that 1.25 $(OH)_2D_3$ apparently promoted normal fracture healing in chicks.[21]

Vit D_3 increases Ca absorption from intestine during calcification and callus formation. Increased use of active metabolites of Vit D_3 in intestinal mucosa and bone tissue and/or stimulation of synthesis in these tissues may be responsible for Ca metabolism and fracture remodelling.[22-24]

Both 1.25 $(OH)_2$ D_3 and 24.25 $(OH)_2$ D_3 are effective directly in enchondral bone formation. 24.25 $(OH)_2$ D_3 increases volume and length of callus and regulates Ca. Locally applied 24.25 $(OH)_2$ D_3 has an accelerating effect on fracture healing.[22,25] There are some publications suggesting that 1α-(OH) D_3 causes an elevation in total bone mineral density with aging. Fracture risk is 1/3 in those receiving 1 α (OH) D_3 than those in the control group.[6,26,27]

High doses of vit D (1.25 $(OH)_2$ cholecalciferol) induces fracture healing. It has a positive effect on adequate blood circulation, synthesis and arrangement of collagen fibers, proliferation and differentiation of osteoprogenitor cells, and the calcification of the matrix. It is well-established that vit D increases EGF receptors in bone cells and especially the amount of Type 1 collagen in human bone cell cultures. Biomechanical studies reported that callus was stronger and postfracture osteopenia was less frequently observed in adult rats that received vit D.[8,26,28]

Ömeroğlu et al also concluded that a single high dose of vitamin D_3 given intramuscularly accelerated fracture healing in a healthy animal

model by four mechanisms: Advancing the blood supply at the fracture site: Accelerating the proliferation and differentiation of osteoprogenitor cells in the callus; increasing the amount of collagen present in the callus and stimulating the organization of collagen fibres; and activating mineralization of the matrix. In another study Omeroglu et al reported that the application of single high-dose vitamin D_3 positively affected the mechanical strength at the fracture site.[13] This seems to support the ultrastructural findings of the previously mentioned study.

The question of whether the mineralizing effect of vitamin D_3 and its metabolites were due to the maintenance of serum calcium and phosphate levels or to a direct action of vitamin D or a specific metabolite was not clearly answered.[11,29] The outcome of our study is that, the application of calcium, single high-dose vitamin D_3 and combined calcium and vitamin D_3 positively effects the mechanical strength at the fracture site in the early stages of fracture healing. However, no statistically significant radiographic and histological differences were found. In our study, we performed the mechanical assessment in the early stage of fracture healing. Looking from this point of view, we may suggest that calcium and vit D given in the early stages of fracture healing gives opportunity of early weight bearing. Under the influence of this study with a further assessment after the remodelling phase of fracture healing, the ultimate effects of calcium and vit D on fracture healing may be better understood.

It is known that in addition to its effect on calcium metabolism, Vit D acts on fracture healing via other mechanisms. Vitamin D_3 has positive effects on some systemic and local growth factors that are both mitogenic and tissue-specific differentiating.[18,30-32] Single high-dose vitamin D_3 was thought to act on these angiogenic growth factors during the early phases of fracture healing in a healthy animal model.[13] The blood supply was found to be one of the most important factors influencing fracture healing, probably mediated by various angiogenic growth factors.[17,33] An increase in receptors for epidermal growth factor was also shown in a bone cell line after application of 1.25 $(OH)_2D_3$.[34] No statistically significant difference was found between high-dose calcium and vit D groups in our study, which led us to think that the real effect of vit D on fracture healing was via calcium metabolism.

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