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H. Ömeroğlu · Y. Ateş · O. Akkuş · F. Korkusuz
A. Biçimoğlu · N. Akkaş

Biomechanical analysis of the effects of single high-dose vitamin D_3 on fracture healing in a healthy rabbit model

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Abstract In a previous ultrastructural study, the benefit of a single high dose of vitamin D_3 on fracture healing in a healthy animal model was demonstrated. This study examined the biomechanical consequences of applying a single high dose of vitamin D_3 in a healthy rabbit model subsequent to femoral fracture. The fracture load, the values of energy absorbed until fracture and the flexural rigidity values of the vitamin D group were significantly higher than the corresponding ones of the control group in the case of fracture. On the other hand, for intact bones, those values did not differ significantly between the two groups. It was concluded that single high-dose vitamin D_3 application had positive effects on fracture healing in a healthy animal model, as far as the parameters related to mechanical strength are concerned.

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H. Ömeroğlu · A. Biçimoğlu
Ankara Numune Hospital,
3rd Department of Orthopaedic Surgery and Traumatology,
Ankara, Turkey

Y. Ateş
Ankara Hospital,
2nd Department of Orthopaedic Surgery and Traumatology,
Ankara, Turkey

O. Akkuş · N. Akkaş
Middle East Technical University, Faculty of Engineering,
Department of Engineering Sciences, Ankara, Turkey

F. Korkusuz
Middle East Technical University, Medical Center,
Ankara, Turkey

H. Ömeroğlu (✉)
Turgut Reis Caddesi 54/8, TR-06570 Ankara, Turkey

Introduction

Fracture healing and the factors affecting it have always attracted researchers' curiosity. Fracture healing is a complex process, and hormones, local and systemic growth factors all play a part at each stage [10, 28, 30]. Although the histopathological process of fracture healing is now almost completely understood, the effects of most of the factors on the fracture healing event have not yet been absolutely verified [5, 7, 13].

Vitamin D_3 (cholecalciferol) is known to be one of the hormones promoting fracture healing [5, 7, 13]. In the past, many studies on the effects of vitamin D_3 on fracture healing have been undertaken, frequently in vitamin D-depleted animal models and with different dose protocols. In previous work [25, 26] in which one of us (H.Ö.) took part, the positive effect of single high-dose vitamin D_3 application on fracture healing in a healthy animal model was pointed out for the first time. The present study was undertaken to see whether or not the results of that ultrastructural study could be biomechanically supported.

Materials and methods

Twenty male, healthy (normocalcaemic and normophosphataemic), 3-month-old New Zealand white rabbits, weighing between 1.6 and 1.9 kg, were used. They were randomly divided into two groups: 'control' and 'vitamin D'. The right femur of each rabbit was exposed via a lateral skin incision under ketamine hydrochloride and xylazin hydrochloride anaesthesia. Following the skin incision, a blunt dissection between the lateral vastus and hamstring muscles was carried out, and then the midshaft of the femur was osteotomized transversally by a Gigli saw. Steinmann pins (3 mm) were used for intramedullary fixation. Following haemostasis, the muscular and cutaneous layers were closed with interrupted sutures. The rabbits in the vitamin D group were injected intramuscularly with 50000 IU/kg of vitamin D_3, and the ones in the control group with 0.9% NaCl [25, 26]. The rabbits were allowed unrestricted weight-bearing after recovery from the anaesthesia. Each rabbit was caged individually and allowed free access to water and a standard pellet diet. Two animals in each group died of unknown reasons during follow-up. Moreover, two animals, one from the control group and one from the vitamin D group, were excluded

from the study because of an infection at the fracture site, leaving a total of seven animals in each group.

The remaining 14 rabbits were killed 6 weeks after surgery. The intact and fractured femurs were removed by careful dissection, and the intramedullary Steinmann pins were pulled out by applying small torsional movements. The control and vitamin D groups were numbered so as to keep the biomechanic measurements blind. The femurs were kept frozen at –20° C for further analysis. Prior to the tests, the femurs were placed in a humid medium and kept there for 4 h until they thawed to room temperature. The distal and proximal parts of the femurs were cut out to obtain a better adjustment to the three-point bending fixture.

The femurs were placed on the three-point bending configuration on the Lloyd LS500 material testing machine (Southampton, UK). The machine was controlled via a personal computer (486DX-50). 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 an anteroposterior direction, with a span length of 40 mm. Preloads of 1 N for fractured femurs and 5 N for intact femurs were applied before loading [1, 2]. All the bones were kept in a humid medium during the tests [31].

The load-deflection curves were stored in the computer to be processed later to obtain fracture load, deflection at fracture, values of energy absorbed until fracture (EAUF) and flexural rigidity values. Flexural rigidity was calculated as the product of Young's Modulus (*E*, material property) and the moment of inertia (*I*, geometrical property) of the cross-section. It is known that both the cross-sectional area and the material properties change during the healing process. The flexural rigidity *(EI)* concept takes both of these parameters into account. Flexural rigidity can be obtained from the slope of the linear part of the load deflection curve. EAUF is defined as the energy absorbed by the bone until fracture and is equal to the area under the load-deflection curve.

Statistical analysis of the data was done with the Mann-Whitney U-test, and the level of significance was set at $P < 0.05$.

Results

Our results showed that the application of a single high dose of vitamin D_3 resulted in an increase in the fracture load, EAUF and flexural rigidity values of the fractured bone over those of the control group (Table 1, Fig. 1–5). There was a statistically significant difference between the corresponding fracture load, EAUF and flexural rigidity values of the control and vitamin D groups (Table 3). Deflection at fracture values did not differ significantly (Table 3). However, the vitamin D group had a slightly smaller deflection than the control, indicating a more mineralized callus (Fig. 3).

In intact bones, the average values of all parameters were similar in the control and in the vitamin D groups, and statistically there was no significant difference between these two groups (Tables 2, 3, Fig. 1–5).

Table 1 Mechanical parameters for fractured femurs of control and single high-dose vitamin D_3 groups

	Maximum load (N)	Maximum deflection (mm)	Energy absorbed until fracture (EAUF) (N. mm)	Flexural rigidity (EI; N. $mm^2 \times 10^{-3}$)
Vitamin D	208.13 ± 68.50	1.77 ± 0.54	190.69 ± 94.64	195 ± 80
Control	115.60 ± 26.05	1.96 ± 0.89	76.44 ± 18.06	69 ± 16

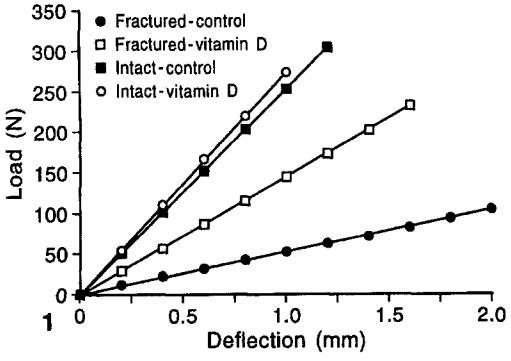

Fig. 1 Average load-deflection curves of all groups

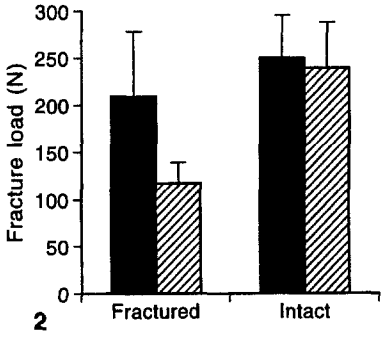

Fig. 2 Average fracture load values of all groups

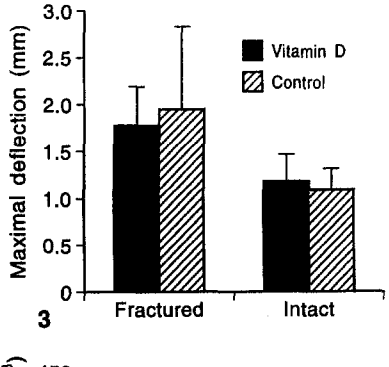

Fig. 3 Average maximum deflection values of all groups

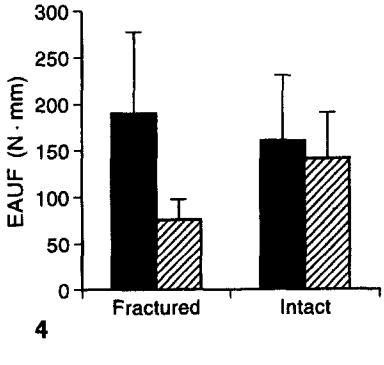

Fig. 4 Average energy absorbed until fracture *(EAUF)* values of all groups

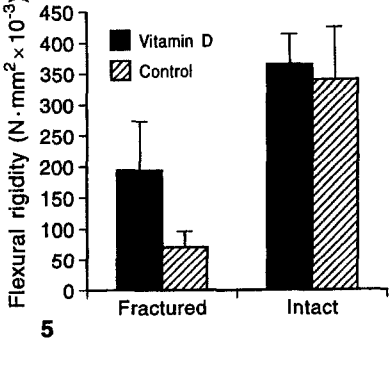

Fig. 5 Average flexural rigidity values of all groups

Table 2 Mechanical parameters for intact femurs of control and single high-dose vitamin D_3 groups

	Maximum load (N)	Maximum deflection (mm)	Energy absorbed until fracture (EAUF) (N. mm)	Flexural rigidity (EI; N. $mm^2 \times 10^{-3}$)
Vitamin D	248.25 ± 46.64	1.18 ± 0.25	162.76 ± 71.01	367 ± 52
Control	238.11 ± 49.89	1.09 ± 0.25	141.17 ± 46.81	341 ± 86

Table 3 Statistical analysis (Mann-Whitney U-test) of the mechanical parameters for fractured femurs of vitamin D_3 and control groups

	Maximum load	Maximum deflection	Energy absorbed until fracture (EAUF)	Flexural rigidity *(EI)*
Fractured	$P = 0.034$*	$P = 0.714$	$P = 0.015$*	$P = 0.004$*
Intact	$P = 0.948$	$P = 0.477$	$P = 0.561$	$P = 0.434$

* Statistically significant difference

Discussion

Previous studies demonstrated that vitamin D_3, its major metabolite 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) and another of its metabolites 24,25–dihydroxycholecalciferol (24,25-$(OH)_2D_3$) were important in bone metabolism and fracture repair.

Lindgren and associates [22] reported that in healthy adult rats fracture healing was slightly more advanced by the application of 1,25-$(OH)_2D_3$. Dekel and co-workers [11] 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. Lidor and colleagues [20, 21] 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. 24,25-$(OH)_2D_3$ was also found to promote the maturation and mineralization of osteoid [15]. Brumbaugh and associates [6] noted that 1,25-$(OH)_2D_3$ apparently promoted normal fracture healing in chicks.

Clinical studies were also performed to understand the correlation between vitamin D_3 and the fracture process. There was no agreement on the correlation between the risk of fracture and serum levels of vitamin D metabolites at the time of fracture [14]. Alkalay and associates [3] found that the serum level of 1,25-$(OH)_2D_3$ was significantly reduced after fracture, and levels of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were significantly raised in the bone around the fracture site. Meller and co-workers recorded a significant increase in serum level of 24,25-$(OH)_2D_3$ during fracture healing in normal young adults [23], but this increase was not observed in geriatric patients [24].

In an ultrastructural study, Ömeroğlu and colleagues [26] 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; activating mineralization of the matrix. The outcome of our study, that the application of single high-dose vitamin D_3 positively affects the mechanical strength at the fracture site, seems to support the ultrastructural findings of the previously mentioned study.

The present study also showed that, in intact bones, single high-dose vitamin D_3 application produced a slight increase in EAUF, fracture load and flexural rigidity values; however, the differences were not statistically significant.

Another noteworthy finding was that the fracture load and EAUF values after single high-dose vitamin D_3 was applied to fractured bone were nearly as high as the corresponding ones of the intact contralateral. Moreover, the EAUF value of the vitamin D_3-treated fractured bone was on average higher than that of the intact contralateral. This is probably due to the fact that the former is more flexible. The presence of interfragmentary callus made the deflection capacity of fractured bones higher compared with the intact contralateral. However, the flexural rigidity values of intact bones were comparably higher than those of fractured bones.

Systemic and local factors affect fracture healing by acting directly or, more frequently, by activating mediator mechanisms. Fracture healing failure often coincides with malfunctions of these mediator mechanisms [16, 17]. The mechanism by which fracture healing is promoted by vitamin D_3 is uncertain. There have been conflicting reports concerning the effects of vitamin D_3 and its metabolites on alkaline phosphatase activity and collagen synthesis, but usually a stimulating effect was accepted [4, 15, 27, 29]. It has been reported that 1,25-$(OH)_2D_3$ is an important modulator of the growth and differentiation of human bone cells [4, 32]. The question of whether the mineralization effect of vitamin D_3 and its metabolites was due to maintenance of serum calcium and phosphate or to a direct action of vitamin D or a specific metabolite has not been clearly answered [6, 9, 18, 27].

Systemic and local growth factors are both mitogenic and tissue-specific differentiating [8, 10, 16, 28, 30]. Vitamin D_3 has positive effects on some of them. The blood supply was found to be one of the most important factors influencing fracture healing, probably mediated by various angiogenic growth factors [16, 19]. 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 [26]. Positive effects of 1,25-$(OH)_2D_3$ on the production of osteocalcin (bone GLA protein), have already been demonstrated, which appeared in the developing bone after the deposition of bone mineral and which may have an effect on bone mineralization [10, 12, 15, 27, 29]. An increase in receptors for epidermal growth factor was also shown in a bone cell line after application of 1,25-$(OH)_2D_3$ [29].

Although the benefits of single high-dose vitamin D_3 on fracture healing have been demonstrated both ultrastructurally and biomechanically in healthy animal models, more research must be done in higher animal models before its clinical application. It is uncertain whether the same results will be obtained in highly evolved animals and humans.

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