

# The effects of a single high-dose injection of vitamin-D on healing of condylar fractures in osteoporotic rats

## Osteoporotik sıçanlarda tek yüksek doz D-vitamini enjeksiyonunun kondil kırığı iyileşmesine etkisi

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### SUMMARY

**Aim:** In this study we have tried to show the effects of a single high-dose injection of vitamin d on healing of condylar fractures in osteoporotic rats.

**Materials and method:** This study included sixteen female wistar rats. All rats were ovariectomized. Osteoporotic rats were randomly divided into two groups of eight animals/group. Group D animals received a single high dose of vitamin D (50.000 IU/kg) intraperitoneally and group C (control) animals received saline (0.05 mL) intraperitoneally. All injections were given immediately after condylar fracture. All rats were euthanized after 28 days, examined histopathological and immunohistochemical.

**Results:** Histopathological and immunohistochemical evaluations were done. Histopathological scores between group D and C were found statistically significant. TGFβ1 and osteopontin levels in group D were found higher than group C.

**Conclusion:** We have determined that a single high-dose injection of vitamin D given immediately after surgery increased TGFβ and osteopontin levels and enhanced new bone formation at condylar fractures of osteoporotic rats.

**Keywords:** Condylar fracture, osteoporosis, vitamin D, single dose

### ÖZET

**Amaç:** Bu çalışmada, osteoporotik sıçanlarda kondiler kırıkların iyileşmesinde tek yüksek doz verilen D vitamininin etkilerini göstermeye çalıştık.

**Gereç ve Yöntem:** Bu çalışmada 16 dişi wistar sıçanı kullanıldı. Tüm sıçanlara ovariectomi uygulandı. Osteoporotik sıçanlar rastgele her grupta sekiz hayvan olacak şekilde iki gruba ayrıldı. Grup D hayvanlarına intraperitoneal olarak tek doz D vitamini (50,000 IU / kg) verildi ve grup C (kontrol) hayvanlara intraperitoneal olarak serum fizyolojik (0,05 mL) verildi. Tüm enjeksiyonlar, kondiler kırıktan hemen sonra yapıldı. Bütün sıçanlar 28 gün sonra sakrifiye edildi, Histopatolojik ve immünohistokimyasal olarak incelendi.

**Bulgular:** Histopatolojik ve immünohistokimyasal değerlendirmeler yapıldı. D ve C grubu arasındaki histopatolojik veriler istatistiksel olarak anlamlı bulundu. Grup D' de TGFβ1 ve osteopontin düzeyleri C grubundan daha yüksek bulundu.

**Sonuç:** Cerrahi müdahaleden hemen sonra verilen tek yüksek doz D vitamini enjeksiyonunun, osteoporotik sıçanların kondiler kırıklarında TGFβ ve osteopontin seviyelerini ve yeni kemik oluşumunu arttırdığını tespit ettik.

**Anahtar kelimeler:** Kondil kırığı, osteoporoz, D vitamini, tek doz

### INTRODUCTION

Osteoporosis (literally: porous bone) is a systemic and metabolic disease of the skeletal system. The National Osteoporosis Foundation has identified some risk/predisposing factors for osteoporosis and related fractures. These include a history

of fractures in adulthood, smoking, estrogen deficiency at an early age, low body weight, poor health, low calcium intake, physical inactivity, little exposure to sunlight, alcohol abuse, and the use of oral glucocorticoid therapy for more than 3 months in postmenopausal females.<sup>1,2</sup> The disease is characterized by reduced bone density and quality, impairment of the bone microarchitecture, and reduced mechanical resistance. Such changes increase the risk of fracture.<sup>1</sup> Pisani et al. reported that almost 200 million subjects worldwide are affected by osteoporosis, suffering 8.9 million fractures annually; fracture associated with osteoporosis is thus a major public health problem.<sup>2</sup> The numbers of traumatic injuries in the elderly are increasing as advances in medicine are increasing human lifespan and populations feature ever-increasing proportions of older subjects, who seek to be as active as possible.<sup>3-5</sup> Chrcanovic et al. found that mandibular fracture was the most common type of facial fracture in those >60 years of age.<sup>4</sup> Fractures of the mandibular condyle are thought to account for about 25-35% of all mandibular fractures.<sup>6,7</sup> After direct mandibular trauma, indirect forces are transmitted to the condyle, and the weak condylar neck breaks easily to protect the brain from damage.<sup>8</sup> Many substances affect the time required for fracture healing. These include biphosphonates, hormones, and growth factors that exert different effects.<sup>9-11</sup> Earlier studies showed that vitamin D3 promoted fracture healing.<sup>12-15</sup>

Positive effect of vitamin D on bone; it increases TGF synthesis and release, regulates osteoblast differentiation, stimulates tissue repair and osteocalcin and osteopontin production.<sup>16-21</sup> Although many recent studies have evaluated the effects of vitamin D on fracture healing in general, the effects of vitamin D injection on condylar fracture healing, particularly in osteoporotic patients, have been but rarely investigated. We aimed to evaluate the positive effect of vitamins on the condyle fractures treated with closed reduction of the patients with high risk of poor recovery such as advanced age, metabolic or primary bone diseases or malnourishment. In the present study we sought to accelerate healing of condylar fractures in osteoporotic rats. We used immunohistochemical and histological techniques to evaluate the effectiveness of the treatment.

## MATERIALS AND METHOD

The study was approved by the Animal Experimentation Committee of Bülent Ecevit University, Zonguldak, Turkey. The surgical procedures for this research were performed according to protocols approved by the animal care and ethics committee of Bülent Ecevit University (protocol number: 2016-33-02/06). This study included sixteen female Wistar rats 6-8 weeks of age (250-300 g) (n = 16) were housed in standard cages in rooms of relative humidity

40-60% at 22 ± 1°C under a 12-h:12-h light/dark cycle. All rats were ovariectomized. Osteoporotic rats were randomly divided into two groups of eight animals/group. Group D animals received a single high dose of vitamin D (50.000 IU/kg) intraperitoneally and group C (control) animals received saline (0.05 mL) intraperitoneally.

### Ovariectomy

Weights were digitally measured prior to ovariectomy. Each rat was anesthetized via intramuscular injection of 3 mg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany)/kg and 35 mg ketamine hydrochloride (10% [w/v] Ketazol; Richter Pharma AG, Wels, Austria)/kg. The dorsal part of the lumbar region was shaved and cleaned with 10% (v/v) povidone iodine. A 2-cm-long mid-dorsal skin incision was made caudal to the posterior border of the ribs. After dissection of the abdominal muscles (the m. obliques externus abdominis and the m. obliques internus abdominis) and the membrane, the omentum was displaced. The ovaries were sutured, commencing at the meso-ovarium proximally and extending to the cornu uteri distally, employing resorbable 4/0 polyglycolide-co-lactide (Vicryl; Ethicon, Somerville, NJ, USA). Both ovaries were removed and the muscles and deep tissues sutured with resorbable material. The skin was closed using 3/0 silk sutures (Medeks, Istanbul, Turkey). After ovariectomy, each animal was injected with 10 mg cefazolin sodium (Sefazol; M Nevzat, Istanbul, Turkey)/kg and 200 mg metamizol sodium (Novalgin; Aventis, Istanbul, Turkey)/kg on each of the next 5 days to control infection and provide analgesia. We monitored wound-healing, feeding behavior, systemic health, and activity levels. At the 6-week follow-up period, no rat had any local infection or obvious pathology, but all rats were now osteoporotic.<sup>22,23</sup>

### Experimental groups

Osteoporotic rats were randomly divided into two groups of eight animals/group. Group D animals received a single high dose of vitamin D (Devit D3 300,000 IU/1 mL from one ampule, Deva, Istanbul Turkey) (50.000 IU/kg) intraperitoneally and group C (control) animals received saline (0.05 mL) intraperitoneally. All injections were given immediately after condylar fracture.

### Surgical procedure

All surgery was performed under aseptic conditions in the operation room of an animal laboratory. All surgical procedures were performed under general anesthesia as in the ovariectomy procedure. A 10-mm-long pre-auricular incision was made directly below the zygomatic arch, followed by blunt dissection through the masseter muscle, and the condylar process was exposed. The condyle was fractured at the subcondylar neck using a mosquito forceps to deviate the condylar fragment medially (Fig. 1). The flaps were sutured with resorbable 4/0 polyglycolide-co-lactide (Vicryl; Ethicon, Somerville, NJ, USA). After

fracture, the animals were injected as described above. The same antimicrobial and analgesic drugs were used to allow of postoperative infection control and analgesia. Each animal was injected with 10 mg cefazolin sodium/kg and 200 mg metamizol sodium/kg on each of 5 days after operation.

#### Histopathology

All rats were euthanized by lethal anesthetic injection after 28 days. After skin dissection, the mandibles were harvested, fixed in 10% (v/v) neutral buffered formalin for 2 days, decalcified in a formic acid/sodium citrate solution for 3 weeks, dehydrated by passage through a series of graded alcohol bath, cleared with xylene, and embedded in paraffin. Coronal bone sections (5  $\mu$ m-thickness) were prepared and stained with hematoxylin-eosin (HE). The bone formation at the fracture gap was scored according to the method described by Tatli et al.<sup>24</sup> The details of the scoring method were shown in Table 1.

**Table 1: Scores of Stained Sections**

1- Fibrous tissue
2- Mainly fibrous tissue and small amount of cartilage tissue
3- Equal amount of fibrous and cartilage tissue
4- Completely cartilage tissue
5- Mainly cartilage tissue/ fibrous tissue and small amount of immature (woven) bone
6- Equal amount of immature bone and cartilage tissue/ fibrous tissue
7- Significantly immature (woven) bone and small amount of cartilage tissue/ fibrous tissue
8- Completely immature (woven) bone
9- Immature bone and small amount of mature bone
10- Mature (lamellar) bone

#### Immunohistochemistry (IHC)

Bone sections (5  $\mu$ m-thickness) were subjected to immunohistochemistry (IHC) using the streptavidin/ biotin peroxidase complex (SABC) technique to detect bone morphogenetic protein-2 (BMP-2), transforming growth factor beta (TGF $\beta$ ), and osteopontin. Serial sections were dewaxed in xylene and hydrated through a series of graded alcohol baths. Endogenous peroxidase activity was blocked by addition of 3% (v/v) H<sub>2</sub>O<sub>2</sub> in methanol for 15 min. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.2) and a proteinase solution added for 15 min at 37°C. After washing with PBS, the sections were pre-incubated in 10% (v/v) rat nonimmune serum (Thermo Scientific; Fremont, CA, USA) at room temperature for 10 min and then with primary antibodies against BMP2 (NBP1-19751, Novus Biologicals), TGF $\beta$ 1 (sc-146, Santa Cruz Biotechnology), or anti-osteopontin (ab8448, Abcam) for 1 h, and rinsed with PBS at room temperature. The sections were next incubated with a biotinylated goat anti-polyvalent antibody (Thermo Scientific) for 20 min and rinsed in PBS. Streptavidin peroxidase was added for 10 min at room temperature. Labelling was visualized

using 3-amino-9-ethylcarbazole (Thermo Scientific) as the chromogen. The sections were briefly counterstained with Meyer's haematoxylin. The negative controls were samples for which the primary antibodies were replaced with normal goat serum.

#### Immunohistochemical evaluation

The immunohistochemical results were interpreted using a light microscope (Nikon Eclipse E600, Nikon, Japan). Semi-quantitative evaluation of stained sections was performed on areas of interest only. Staining intensities were evaluated semi-quantitatively (0, negative; 1, weak; 2, moderate; 3, strong) and by calculating the ratios of stained to total areas (0, nil; 1, <25%; 2, 26-75%; 3, >76%). All quantification was performed by the same pathologist.

#### Statistical analysis

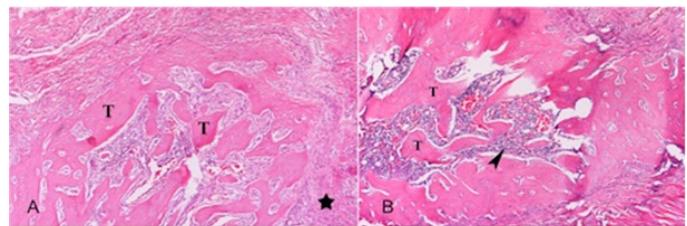
All statistical analysis was performed with the aid of IBM SPSS Statistics version 22 (IBM SPSS, Turkey); the Mann-Whitney U-test (a descriptive method) was used to compare all data. A p-value <0.05 was considered to reflect statistical significance.

## RESULTS

#### Histopathological evaluation

##### Control group (group C)

The fracture margins of the control group were not closed. Hematomas and inflammatory cells were evident between fracture margins, as were connective tissue bridges. The connective tissue contained primitive mesenchymal cells. Periosteal reactions were observed only at the fracture margins. New blood vessels were evident between trabeculae but myeloid cells were absent (Fig. 2 A).



**Figure 2: Histological sections showing bone healing at the fracture by hematoxylin and eosin stain A: Sample from the control group. B: Sample from the vitamin D group. T; trabecular, P; periosteum, ★; primitive mesenchymal cells, ▶; myeloid cell.**

##### Experimental group (group D)

The fracture margins were almost closed and intramembranous ossification was apparent. The extent of periosteal cell proliferation at the fracture margins and around new bones was higher than that of the control. Blood vessels, myeloid cells, and primitive mesenchymal cells were evident between the trabeculae (Fig. 2 B). The histopathological scores of groups D and C were  $7.25 \pm 1.58$  and  $4.13 \pm 1.96$  respectively; the difference was statistically significant ( $p=0.003$ ;  $p<0.01$ ) (Table 2).

Table 2: Comparison of Histologic Data

	Mean±SD	Medyan (Min-Max)
<b>Group D (Vitamin D)</b>	7,25±1,58	7 (5-9)
<b>Group C (Control)</b>	4,13±1,96	5 (1-6)
<b>P</b>	<b>0,003**</b>	
<i>Mann Whitney U Test</i>	<b>** p&lt;0.01</b>	

**Immunohistochemical evaluation**

**BMP-2**

New bone, and the endosteal osteoblasts around the trabeculae and periosteal regions, were weakly immunopositive for BMP-2 in both groups. Neither the BMP-2 intensity nor area stained differed between the two groups (p=0.264; p>0.05) (Fig. 3, Table 3).

Table 3: Comparison of Immunohistochemical Data

		Vitamin D	Control	p
		Mean±SD Median(Min-Max)	Mean±SD Median(Min-Max)	
<b>BMP 2</b>	<b>Stained Area</b>	1,38±0,52 1 (1-2)	1,13±0,35 1 (1-2)	<b>0,264</b>
	<b>Staining Intensity</b>	1,25±0,46 1 (1-2)	1,13±0,35 1 (1-2)	
<b>TGF-β</b>	<b>Stained Area</b>	2,5±0,53 2,5 (2-3)	0,25±0,46 0 (0-1)	<b>0,001**</b>
	<b>Staining Intensity</b>	2±0 2 (2-2)	0,25±0,46 0 (0-1)	
<b>Osteopontin</b>	<b>Stained Area</b>	2,88±0,35 3 (2-3)	1,25±1,04 2 (0-2)	<b>0,001**</b>
	<b>Staining Intensity</b>	2,5±0,53 2,5 (2-3)	0,63±0,52 1 (0-1)	

*Mann Whitney U Test*      **\*\* p<0.01**

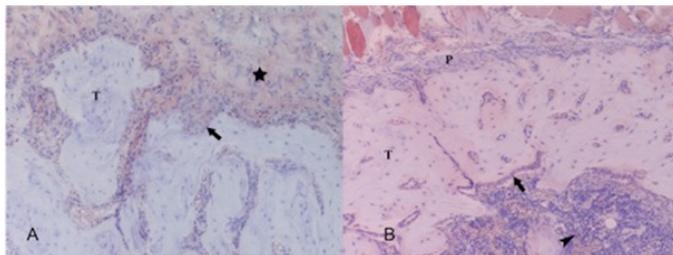


Figure 3: Histological sections showing bone healing at the fracture by BMP-2 immunopositive reaction. A: Sample from the control group. B: Sample from the vitamin D group. ; osteoblast, T; trabecular, P; periosteum, ★; primitive mesenchymal cells, ▶; myeloid cell.

**TGFβ1**

Primitive cells between the trabeculae, endosteal osteoblasts, and periosteal cells reacted positively with the anti-TGFβ1 antibody in group D. However, in the control group, only one sample was positive. The TGFβ1 staining density, and the area stained, were significantly higher in group D than group C (p=0.001; p<0.01) (Fig. 4, Table 3).

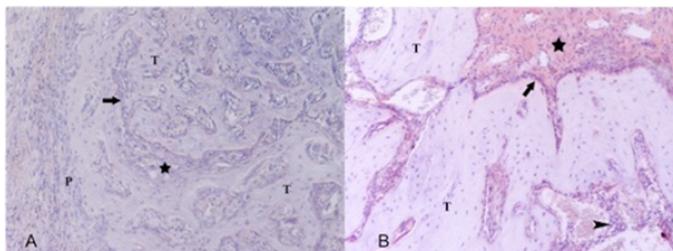


Figure 4: Histological sections showing bone healing at the fracture by TGF-β1 immunopositive reaction. A: Sample from the control group. B: Sample from the vitamin D group. ; osteoblast, T; trabecular, P; periosteum, ★; primitive mesenchymal cells, ▶; myeloid cell.

**Osteopontin**

In group D, the cytoplasm of osteoblasts around new trabeculae, that of primitive mesenchymal cells, and that of cells in the periosteal regions, were immunopositive for

osteopontin. In group C, the primitive cells between fracture margins and the periosteal cells stained only weakly; the differences were significant (p=0.001; p<0.01) (Fig. 5, Table 3).

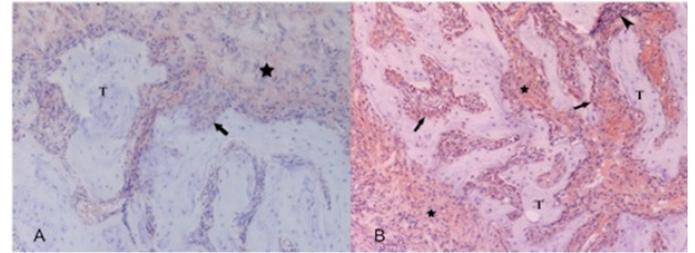


Figure 5: Histological sections showing bone healing at the fracture by osteopontin immunopositive reaction. A: Sample from the control group. B: Sample from the vitamin D group. ; osteoblast, T; trabecular, P; periosteum, ★; primitive mesenchymal cells, ▶; myeloid cell.

**DISCUSSION**

In this study, we aimed to evaluate the positive effect of vitamins on the condyle fractures treated with closed reduction of the patients with high risk of poor recovery such as advanced age, metabolic or primary bone diseases or malnourishment.

Fractures associated with osteoporosis are a major public health problem associated with morbidity, impairment, a decrease in the quality-of-life, and mortality, in both females and males. Approximately 50% of females and 25% of males ≥50 years of age will experience an osteoporotic fracture in their lifetimes. Given this global problem, we studied fracture healing in ovariectomized (osteoporotic) rats. Pisani et al. reviewed factors associated with osteoporotic fracture risk; these included a low body mass index, a history of fragility fractures, environmental risks, early menopause, smoking, lack of vitamin D, endocrine disorders, use of glucocorticoids, and excessive alcohol intake.<sup>2</sup> As lifespan has increased, the quality-of-life has also improved, allowing the elderly to be more physically active but, thus, increasing the risk of injury. Gerbino et al. described 2,235 patients hospitalized over a 10-year interval to treat fractures in the maxillofacial region.<sup>3</sup> Of these, 222 were aged 60-91 years. Over time, the prevalence of maxillofacial trauma in elderly patients increased from 7 to 12%.<sup>3</sup>

Mandibular fractures are the most common fractures of the maxillofacial region that constitute 24-78% of all maxillofacial fractures,<sup>25,26</sup> whereas condylar fractures constitute 25-35% of all mandibular fractures.<sup>6,7</sup> Yamamoto et al. retrospectively analyzed trends in, and the characteristic features of maxillofacial fractures in older patients.<sup>5</sup> The cited authors collected data from 247 patients aged ≥65 years treated for maxillofacial fractures from 1981 to 2010. Of all maxillofacial fractures, 56.7% were in the mandible. Condylar fractures were the most common (64.6%) mandibular fractures in elderly patients.<sup>5</sup>

No area of the mandible is as controversial as the condylar region in terms of appropriate choice of treatment. The

treatment should include a non-chew diet alone or as an additional treatment following the maxillomandibular fixation, in patients whom open reduction of the condyle is not indicated.<sup>27</sup> Patients without significant displacement of the fracture segments are best treated with closed reduction and a short period of immobilization. Patients at high risk for poor healing such as advanced age, metabolic or primary bone diseases or malnourishment have also a greater risk of ankylosis, malunion or nonunion.<sup>27</sup> Studies related with osteoporosis have shown that bone healing to be prolonged with decreased levels of mesenchymal stem cells and decreased levels of angiogenesis.<sup>28</sup>

Because of the negative effects of the osteoporosis on the bone healing we preferred osteoporotic rat model for this study. In this study, we aimed to evaluate the positive effect of vitamins on the condyle fractures treated with closed reduction of the patients with high risk of poor recovery such as advanced age, metabolic or primary bone diseases or malnourishment. In the present study we sought to accelerate healing of condylar fractures in osteoporotic rats. Studies showed that adult female rats underwent bilateral ovariectomy and became osteoporotic 6 weeks later.<sup>22,23</sup>

In the literature it was already shown the effect of experimental ovariectomy on osteoporosis. The duration to see influence of bone loss after ovariectomy should be 2 weeks at least.<sup>29,30</sup>

Subsequently, unilateral condylar fractures were created using the method of Cavalcanti et al.<sup>31</sup> The time required for fracture healing<sup>32</sup> is affected by the levels of growth factors and hormones, osteogenic cells and osteoconductive scaffolds, the mechanical environment (the stability of fixation), and other parameters including the levels of anti-dickkopf-1 and anti-sclerostin antibodies, prostaglandin E2 receptor agonists, vitamins C, D and E, and thrombin-related peptide (TP508) (which enhances bone healing)<sup>11</sup> The various factors influence bone healing differently, modulating calcium absorption, angiogenesis, collagen deposition, osteoblast stimulation, and bone remodeling.<sup>9,10</sup> Some studies found that vitamin D promoted fracture healing by playing a major role in calcium metabolism; adequate vitamin D is essential for skeletal health.<sup>33</sup> Guidelines for the management of postmenopausal osteoporosis suggest that vitamin D sufficiency is important to ensure an optimal response to treatment.<sup>34,35,36</sup> Lips et al. studied 2,606 postmenopausal females with osteoporosis (from 18 countries) and found that 64% had inadequate levels of vitamin D.<sup>33</sup> Lack of vitamin D was a major risk factor for postmenopausal osteoporosis. A multidisciplinary clinical study found that vitamin D deficiency was common, being evident in 57-82% of postmenopausal osteoporotic females.<sup>33</sup> Mezquita-Raya et al.

showed that vitamin D insufficiency was a common risk factor for osteoporosis associated with increased bone remodeling and low bone mass in postmenopausal populations.<sup>37</sup> Pisani et al. found that vitamin D, calcium, and protein insufficiencies were very common in the elderly.<sup>2</sup> In adults, vitamin D deficiency exacerbates osteopenia and osteoporosis. Insufficient vitamin D reduces bone mineral density and bone strength, increasing the risk of instability when mobile.<sup>38</sup> Vitamin D insufficiency negatively affects calcium metabolism, osteoblast activity, matrix ossification, bone mineral density (BMD), and bone remodeling.<sup>39,40</sup> The need for increased vitamin D intake in females with postmenopausal osteoporosis has been emphasized.<sup>33,41</sup> Vitamin D was required for hard callus formation and bone remodeling; Gorter et al. showed that future research should focus on vitamin D supplementation to aid fracture healing.<sup>15</sup> The role of vitamin D in fracture healing has been studied in various animal models and with different application of vitamin D.<sup>13,42,43</sup> In this study we preferred to intraperitoneally injected a single high dose of vitamin D application, as did Omeroglu et al.<sup>13</sup>

Vitamin D increased the synthesis and release of TGF $\beta$ 20 and upregulated the expression levels of TGF $\beta$  receptors I and II by osteoblasts.<sup>16,19,21</sup> TGF $\beta$ 1 regulates osteoblast differentiation and plays a key role in tissue repair.<sup>17,18</sup> We found that TGF $\beta$ 1 expression was significantly higher in the group D than the control group, consistent with earlier results. Van Leeuwen et al. showed that production of osteocalcin and osteopontin by osteoblasts was stimulated by vitamin D.<sup>19</sup> We found that osteopontin expression was significantly higher in the group D than control group, again consistent with earlier results. Also, we found that the proliferation of all of periosteal cells, blood vessels, myeloid cells, and primitive mesenchymal cells lying between trabeculae, were significantly higher in the group D, in line with the results of prior studies.<sup>13,14,43</sup> Positive effect of vitamin D on bone; It increases TGF synthesis and release, regulates osteoblast differentiation, stimulates tissue repair and osteocalcin and osteopontin production.<sup>16-21</sup>

The use of vitamin D to accelerate fracture healing will allow an early return to daily activity by, and reduce the morbidity of, patients with condylar fractures associated with osteoporosis. We explored the effects of a single high-dose systemic injection of vitamin D on condylar fracture healing in osteoporotic rats.

The use of a single dose of vitamin D in the clinic may be beneficial because of the favorable effect of vitamin D on condyle fractures treated with closed reduction and reduce complication of the patients with a high risk of poor recovery, such as advanced age, metabolic or primary bone diseases or malnourishment.

## CONCLUSION

We found that a single high-dose injection of vitamin D given immediately after surgery increased TGF $\beta$  and osteopontin levels and enhanced new bone formation at condylar fractures of osteoporotic rats. The exact role played by vitamin D in these contexts remains unclear; more clinical study should research positive effect of vitamin D supplementation on fracture healing.

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