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Original Article

Effects of single high-dose systemic vitamin D injection to dentin-grafted bone defects in osteoporotic rats

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Abstract

Aim: Osteoporosis is a metabolic bone disease posing a public health issue worldwide. Studies showed that dentine can be an alternative bone graft material in bone defects. The purpose of this study is to investigate the potential of the intraperitoneal administration of vitamin D on dentine grafted critical-sized cortical bone defects in osteoporotic rat model.

Material and Methods: Twenty-four rats were underwent bilateral ovariectomy and 6 weeks after ovariectomy osteoporotic rats model were obtained and divided into three groups: Group A; Dentine-D vit, Group B; Dentine and Group C (control). A 5-mm diameter critical- size defect was created in the calvarium of each animal. In Group C, untreated control defects. In Group Dentin, defects were filled with particulate human dentine. In Group Dentin-D vit, defects were filled with particulate human dentine and rats were injected intraperitoneally a single high dose (50.000 U.I./kg) vitamin D. All animals were euthanized at 28 days postoperative.

Results: New bone formation was evaluated by histologic and immunohistochemical analysis. Histologic analysis showed that Group Dentin-D vit and Dentin had significantly more new bone at 4 weeks compared with group C. According to immunohistochemical analyses, in group Dentin-D vit, BMP2 staining areas and density were statistically higher than Control group. TGF-β level differences between groups Dentin-D vit and Dentin were statistically significant. Group C had a significantly lower TGF-β grade than the other groups. The osteopontin staining areas in group Dentin-D vit were statistically higher grade than in group Dentin and group C. The difference between groups Dentin and C was also statistically significant.

Conclusion: Intraperitoneally injected single high dose vitamin D had positive effect on dentine graft and bone formation.

Keywords: Dentin; Osteoporosis; Vitamin D.

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass. and it has become very common in the world in recent years. Micro-impairments in bone tissue increase fragility and make the skeletal system vulnerable (1). One of the most common causes of osteoporosis is hormonal change in the postmenopausal period. A reduction of estrogen levels may cause postmenopausal osteoporosis. Diagnostic tests performed in postmenopausal women have revealed osteoporosis in 15-20% of patients. In the postmenopausal period. the bone formation and resorption balance changes and osteoclastic activity increases (2). In osteoporotic patients. surgical procedures are important because this imbalance has a negative effect. especially on intrabony defects and fracture healing (3).

Life quality has an important role in the prevention of osteoporosis. Inadequate vitamin D intake is one of the most important factors that significantly affects the quality of life. The most important vitamin D components in the human body are D2 (ergocalciferol) and D3 (cholecalciferol). Even though Vitamin D can be obtained with certain nutrients. the most important source is the skin. which is able to synthesize vitamin D from sun rays (4).

Bone defects due to tumor resection. segmental surgery or trauma are among the common problems in maxillofacial surgery. The human body can heal certain defects on its own; however. large defects may require surgery and different materials. The most commonly used method for defects requiring this type of intervention is bone grafting (5). Autogenous grafts have been shown as ideal graft material in the literature. They are accepted as the gold standard due their osteoinductive. osteoconductive. and osteogenic properties. In patients with diseases in

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which bone metabolism has been negatively affected. the healing process of the bone defect may lead to problems in the region where the autogenous graft is obtained. Therefore. different graft materials can be used in patients withosteoporosis (6).

Dentin covers most of the dental tissue and is yellowish in color. Dentin has a harder structure than compact bone with similar content. Similar to bone tissue. it contains organic and inorganic components with collagen and hydroxyapatite crystals. The dentin matrix also contains bone morphogenetic protein (BMP). many growth factors. and non-collagenous proteins such as transforming growth factor (TGF) and osteopontin (7). In literature. there are studies showing the positive effect of dentin as a graft material on bone formation (8-10).

The aim of this study was to investigate the potential of intraperitoneal administration of vitamin D on dentin-grafted critical-sized cortical bone defects in an osteoporotic rat model.

MATERIAL and METHODS

Animals and experimental design

The study was approved by the Animal Experimentation Committee of Bulent Ecevit University, Zonguldak, Turkey and Human Ethical Committee of Ondokuz Mayis University, Samsun, Turkey Twenty-four 6-to-8–week-old rats were used in this study. The cages of all animals were kept in rooms with a temperature of 22 ± 1°C and a humidity rate of 40-60%. Twelve-hour day-night standardization was configured using an illumination system. All rats underwent bilateral ovariectomy procedures. Six weeks after the ovariectomy procedures. the rats were divided into three groups; group A (n=8, dentin-vitamin D), group B (n=8, dentin), and group C (n=8, control).

Preparation of the dentin graft

Extracted human teeth were washed with water. cleaned. and all soft tissue including the periodontal ligament was removed from the root. The teeth were immersed in isopropanol for 2 hours to remove any remaining soft tissue. After isopropanol, the teeth were washed with distilled water several times to eliminate organic solvent and then boiled in water at 100°C. The teeth were fragmented into smaller pieces using a QUADRO COMIL (QUADRO, Ontario, CA) to pass through the special 1200-1500–µm sieve (11).

Surgical procedure and experimental models

General anesthesia was performed using ketamine hydrochloride (10% Ketasol; Richter Pharma AG, Wels, Austria) and a 3-mg/kg xylazine hydrochloride (Rompuns, Bayer, Leverkusen, Germany) injection. After aseptic preparations and under sterile conditions. a semilunar incision was made in the calvarial region of the rats. A full-thickness flap was reflected exposing the parietal and frontal bones. A 5-mm diameter critical-size calvarial defect was performed using a trephine bur under sterile saline irrigation. After the defects were performed. the rats were divided into three groups of eight rats each: group A (dentin-vitamin D): the defects were filled with a particulate dentin graft and the rats were injected with 50.000 mg/kg vitamin D. group B (Dentin): the defects were filled with a particulate dentin graft and the rats were injected with 50.000 mg/kg saline solution; and group C (control): defects were left empty and the rats were injected with 50.000 mg/kg saline solution.

After the surgical procedures, all flaps were sutured with resorbable 4/0 polyglactin 910 sutures (Vicryl; Ethicon, Somerville, NJ,USA). For postoperative infection control. 10 mg/kg cefazolin sodium (Sefazol; M Nevzat, Istanbul, Turkey) was injected into the animals, and metamizole sodium (Novalgin, Aventis, Turkey) was used as an analgesic for 5 days after the operation.

Four weeks after surgery, the animals were euthanized with a lethal injection of anesthetics. The skin was dissected, the calvaria was removed and immediately immersed in a 10% tempered solution of formaldehyde. Histopathologic and immunohistochemical analyses were performed.

Histopathologic analyses

The calvaria taken from all rats were dissected and immediately fixed in 10% neutral buffered formalin for 2 days. and decalcified in a formic acid–sodium citrate solution for 3 weeks. The tissues were dehydrated through graded alcohols. cleared with xylene. and embedded in paraffin. Coronal sections of the bones were cut at 5 µm. The sections were stained with hematoxylin-eosin (HE).

The bone formation level for each section was scored according to the method of Tatlı et al (12):

Fibrous tissue

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

Immunohistochemistry analyses

Five-micrometer sections were subjected to immunohistochemistry (IHC) using the streptavidin-biotin peroxidase complex (SABC) technique for the detection of bone morphogenetic protein (BMP)-2. tumor growth factor (TGF)β. and osteopontin antibodies (13).

Serial sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 15 min. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.2) and subsequently incubated in proteinase K for 15 min in 37°C. After washing with PBS. the

sections were preincubated in 10% rat nonimmune serum (Thermo Scientific, Fremont, CA) at room temperature for 10 minutes. Further. tissue sections were incubated with primary antibodies BMP-2 (NBP1 -19751. Novus Biologicals). TGFβ1 (sc-146, Santa Cruz Biotechnology), anti-osteopontin (ab8448, Abcam) for 1 hour and then rinsed with PBS at room temperature. The sections were then incubated with biotinylated goat anti-polyvalent antibody (Thermo Scientific, Fremont, CA) for 20 min and then rinsed in PBS. Streptavidin-peroxidase was applied for 10 min at room temperature. Labelling was visualized with 3-amino-9-ethylcarbazole (Thermo Scientific, Fremont, CA) as the chromogen. Sections were counterstained briefly with Mayer's hematoxylin. Negative controls were prepared by omitting the primary antibodies and replacing them with normal goat serum.

Results of immunohistochemistry were interpreted using a light microscope (Nikon Eclipse E600). Semi-quantitative evaluation of stained sections was performed only in the area of interest. Immunostaining results were evaluated semi-quantitatively for staining intensity (0, negative; 1, weak; 2, moderate; 3, strong staining) and by the ratio of positively stained area over the total area (0, negative; 1, <25%; 2, 26-75%; 3, >76%). All quantification was performed by the same pathologist.

Statistical analyses

The IBM SPSS Statistics Ver. 22 (IBM SPSS. Turkey) program was used for analyses. The Kruskal-Wallis test was performed for the comparison of parameters between groups of A. B and C. The Bonferroni-corrected Mann-Whitney U test was used for determining which groups in the sample differ significantly. P<0.05 was considered as statistical significance.

RESULTS

Hematoxylin Eosin (HE)

Histologic analyses showed new bone formation all groups. In group C, new bone formation was observed only at the edges of the defect area. Between the edges. there was a thin connective tissue layer. In groups A and B, dentin grafts were surrounded by new bone. and in some areas. it reached the defect margins. In both dentin groups. connective tissue was superior and irregular between new bone areas (Figure 1). According to the histopathologic scores. the mean new bone formation in groups A, B and C were, 7±0.93, 4.75±1.04, and 1.75±0.71 respectively. The differences between the groups were statistically significant (Table 1).

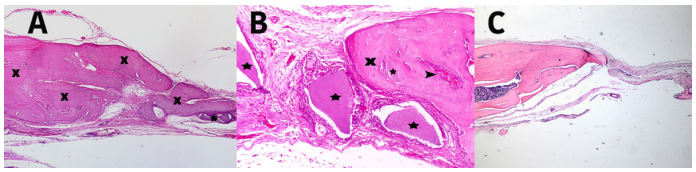

Figure 1. Histological sections showing the healing at the defect side by hematoxylin and eosin stain. Fig 1A: sample of group A. Fig 1B: sample of group B. Fig 1C: untreated defect. *;dentin graft. ➔ ;areolar space. **x**;woven bone

Table 1. Comparison of Histologic Data

	Mean±SD	Median (Min-Max)
Group A (Dentin-Vit D)	7±0.93	7 (6-8)
Group B (Dentin)	4.75±1.04	5 (3-6)
Group C (Control)	1.75±0.71	2 (1-3)
P	0.000**	

Kruskal Wallis Test *p<0.05

Immunochemistry

BMP-2

A mildly severe BMP-2 immuno-positive reaction was observed in the newly-formed young bone tissue. endosteal osteoblasts around the trabeculae. and periosteal region. BMP-2 antibody was similar in the dentin-treated groups (Figure 2).

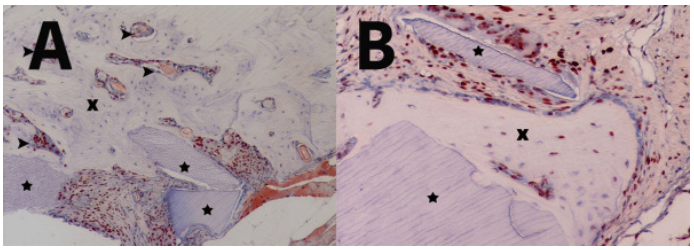

Figure 2. Histological sections showing bone healing at the fracture by BMP-2 immunopositive reaction. Fig 2A: sample of group A. Fig 2B: sample of group B. *; dentin graft. ➔; areolar space. **x**; woven bone

Groups were compared in terms of BMP-2 staining area and density. A statistically significant difference in BMP-2 staining areas was only found between group A and group C. BMP-2 staining density showed similar results for group A and B (Table 2 and 3).

Table 2. Comparison of Immunohistochemical Data

		Group A (Dentin-Dvit) Mean±SD	Group B (Dentin) Mean±SD	Group C (Control) Mean±SD	p
BMP-2	Stained Area	1.75±0.46	1.63±0.52	0.88±0.64	0.018*
	Staining Intensity	1.75±0.46	1.63±0.74	0.63±0.74	0.010*
TGFβ	Stained Area	2.25±0.46	1.5±0.53	0.25±0.46	0.000*
	Staining Intensity	1.88±0.35	1.38±0.74	0.25±0.46	0.001*
Osteopontin	Stained Area	2.63±0.52	1.75±0.46	0.88±0.64	0.000*
	Staining Intensity	2.13±0.64	1.38±0.74	0.63±0.52	0.002*

Kruskal Wallis Test * p<0.05

Table 3. The Multiple Comparison (Post-Hoc) of the Groups

	Groups	A-B	A-C	B-C
		p	p	p
HE		0.002*	0.001*	0.001*
TGFβ	Stained Area	0.602	0.011*	0.028
	Staining Intensity	0.890	0.007*	0.021
TGFβ	Stained Area	0.015*	0.000*	0.002*
	Staining Intensity	0.107	0.000*	0.007*
Osteopontin	Stained Area	0.007*	0.001*	0.011*
	Staining Intensity	0.054	0.001*	0.040

Bonferroni-corrected Mann-Whitney U test * p<0.017

GF-β

In groups A and B, primitive mesenchymal cells between trabeculae in newly-formed bone tissue. endosteal osteoblasts. and periosteal cells were positively stained by the TGFβ1 antibody. In the control group. TGFβ1 antibody was stained only in the periosteal region and endosteal osteoblasts at a mild level (Figure 3).

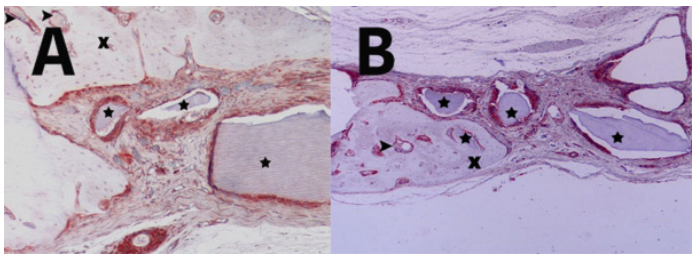

Figure 3. Histological sections showing bone healing at the fracture by TGF-β1 immunopositive reaction. Fig 3A: sample of group A. Fig 3B: sample of group B. *; dentin graft. →; areolar space. x; woven bone

According to the TGF-β staining area results. there was a statistically significant difference between group A and group B. Group B also had a statistically higher grade staining area than group C. TGF-β staining density results showed that group C had a significantly lower grade than the other groups (Table 2 and 3).

Osteopontin

Osteopontin antibody was immune-positive in primitive mesenchymal cells between the trabeculae. osteoblastic cytoplasm of newly-formed bone. and in the periosteal region in dentin-treated groups (Figure 4).

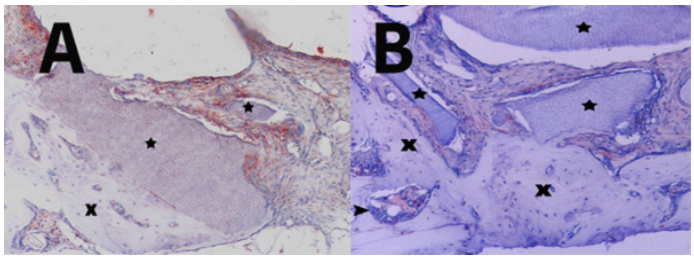

Figure 4. Histological sections showing bone healing at the fracture by osteopontin immunopositive reaction. Fig 4A: sample of group A. Fig 4B: sample of group B. *; dentin graft. →; areolar space. x; woven bone

Theosteopontin staining areas in group A were statistically higher grade than in group B and group C. The difference between groups B and C was also statistically significant. According to osteopontin staining density. a significant difference was only found between group A and group C (Table 2 and 3).

DISCUSSION

In our study, we hypothesized that vitamin D injection might have positive effects on defect treatment in osteoporotic rats. After bilateral ovariectomy operation, we applied a particulate dentin graft on 5-mm diameter critical-sized calvarial defects and administered a single high-dose vitamin D injection into osteoporotic rats. The data were analyzed through histopathologic and immunohistochemical methods.

Osteoporosis is an important disease that affects millions of people around the world and also may affect daily life negatively due to its effects on bone metabolism. The incidence of osteoporosis increases with hormonal changes in the postmenopausal period (2).It is difficult to heal intrabony defects in patients with osteoporosis who have increased fracture risk and imbalance in bone metabolism (3).

We chose rats as an experimental model in our study. Rats are inexpensive. easy to feed. and have a physiology similar to humans (14).The postmenopausal osteoporosis model was obtained using a bilateral ovariectomy procedure. To prevent routine healing and provide standardization. a critical-sized calvarial defect was performed; 5-mm diameter calvarial defects were accepted as a critical-sized defect in rats (15).

Although autogenous grafts are preferred as the gold standard in the treatment of large intrabony defects. it may lead to donor site problems in patients with osteoporosis. Therefore, physicians use different biomaterials to manage new bone formation in these patients (16). Dentin has similar properties to bone and can be used as a graft material in the treatment of bone defects. In the literature. studies have shown the effect of particulate dentin material in bone healing in different sites. Valdec et al. (8) augmented the extraction socket with autologous particulate dentin and placed an implant in the augmented area. After extraction. all vital structures. cementum. and the enamel of the teeth were removed and broken into particulesin a bone mill. According to the results. the authors stated that dentin could be used as a graft material in pre-implantologic operations. Kim et al. (9) studied the effect of a demineralized dentin matrix with recombinant human bone morphogenetic protein-2 (rhBMP-2) in an experimental animal model and clinical study. In the animal part, bone defects were made in beagles, filled with demineralized dentin matrix, and fixed using rhBMP-2. In the clinical part, demineralized dentin matrix and rh-BMP-2 was used with implant placement. Both study results showed that a demineralized dentin matrix with rhBMP-2 provided clinical efficacy and a

positive effect on bone formation.In another study, an experimental alveolar cleft model was prepared in rabbits and the efficacy of non-demineralized dentin with beta-tricalcium phosphate. It was found that dentin with beta tricalcium phosphate enhanced bone regeneration in the experimental rabbit alveolar cleft model (10). Similar to the literature. in our study. the dentin-treated groups showed greater bone formation than the control group.

Vitamin D is responsible for calcium, magnesium, and phosphate absorption in the body. Vitamin D plays an important role for bone tissue with the effect on calcium metabolism (17). Although vitamin D can be taken with certain nutrients. the main source is through our skin. High amount of vitamin D intake is only possible with medical preparations (4). Inadequate vitamin D intake is a risk factor for osteoporosis. and there are studies in the literature that investigated the effect of vitamin D on osteoporosis. Gaugris et al. (18) reviewed reports on the prevalence of vitamin D in postmenopausal women. They found that the prevalence of inadequate vitamin D levels were high in post-menopausal women, especially those with osteoporosis and fracture history. Fischer et al. (19) evaluated the effect of a calcium and vitamin D-deficient diet on femur fracture healing in an osteoporotic rat model. According to the study results, inadequate calcium and vitamin D intake during fracture healing causes posttraumatic bone loss and insufficient callus mineralization. In another study, the serum vitamin D levels of 118 patients were measured and compared using X-ray findings during fracture healing. Decreased serum vitamin D levels were found significantly associated with ossified bone (20). Our study results also showed that vitamin D had a positive effect on bone formation. New bone volumes were significantly higher in the vitamin D-injected group.

In the present study, histopathologic analyses were used to investigate the effect of vitamin D on new bone formation in dentin-grafted defects. Immunohistochemical analyses were used to examine BMP-2, TGF-β and osteopontin levels. Dentin contains BMP-2, TGF-β, and osteopontin-like bone tissue, and the effects of these on bone formation and vitamin D have been studied in the literature. Song et al. (21) investigated the effect of different doses of BMP-2 and vitamin D on the osteogenic differentiation of adipose stem cells. The authors found that BMP-2 and vitamin D promoted osteogenic differentiation of adipose stem cells. In another study. the effect of vitamin D was evaluated on TGF-β signaling in a hepatocellular carcinoma rat model. According to the study results. vitamin D administration showed better restoration of liver parenchyma (22). Lau et al. (23) tested different vitamin D receptor agonists on aortic calcification and osteopontin levels and found that vitamin D receptor agonist therapy upregulated osteopontin levels. HE staining scores were calculated for the histopathologic examination. Similar to the literature. HE scores showed that the vitamin D-injected group had greater bone formation. The immunohistochemical evaluation revealed that BMP-2 levels were higher grade in the dentin-grafted groups than in the control group. According to the TGF-β and osteopontin results, the vitamin D-injected group had significantly higher values.

CONCLUSION

In conclusion, a single-dose 50.000 mg/kg vitamin D injection had beneficial effects on histopathologicalnew bone formation,. and also increased TGF-β and osteopontin levels in our osteoporotic model. Further investigations aimed at determining optimal doses to maximize anabolic actions of vitamin D on bone formation are required.

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Ethical approval: The study was approved by the Animal Experimentation Committee of Bulent Ecevit University, Zonguldak, Turkey and Human Ethical Committee of Ondokuz Mayis University, Samsun, Turkey Twenty-four 6-to-8–week-old rats were used in this study.

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