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EXPERIMENTAL / LABORATORY STUDIES

Effect of Demineralized Bone Matrix on Resorption of Autogenous Cortical Bone Graft in Rats

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Abstract: The purpose of this study was to evaluate the effect of demineralized bone matrix (DBM) on autogenous cortical bone resorption in rats.

Fifty-six male Wistar rats were used and divided into the baseline group (n = 8), the experimental group (n = 24) and the control group (n = 24). The experimental and contol groups were subdivided into three groups. Each subgroup was followed-up for 2, 4, and 12 weeks respectively. Initially, overnight fasting urine and blood samples were collected from the baseline group to obtain biochemical parameters in healthy rats. In the experimental group, bone defects 3 mm in diameter and 2 mm in depth were created in the right femur. Autogenous cortical bone graft 3 mm in diameter and 2 mm in depth was harvested with a standard trephine bur from the right femur 5 mm from the defect site. Bovine derived DBM gel was applied locally on the bone defect. Following this procedure, the graft was placed in the bone defect and stabilized by perifemoral wiring. In the control group, the same procedures were applied but the bone graft was placed without DBM. Serum calcium, phosphate, PTH and 25 dihydroxyvitamin D levels and urine pyridinoline, deoxypyridinoline, calcium and creatinine levels were measured during the study.

Urinary deoxypyridinoline and pyridinoline levels, which are the markers of bone resorption were significantly lower in the experimental group than in the control group at 2, 4, and 12 weeks. Significant reduction in the number of osteoclasts and resorptive lacunae revealed the pronounced suppression of the graft resorption in the experimental group.

The results of this study revealed that DBM is effective in decreasing the resorption of autogenous cortical bone graft.

Key Words: Demineralized bone matrix, autogenous graft, bone resorption.

Introduction

Bone-grafting techniques and materials are commonly used to fill defects and voids to restore form and function where bone is missing. Different types of graft materials accomplish this in different ways. It is known that acceptable clinical outcome of bone grafts depends on the choice of proper graft material and technique (1-3). In determining the type of graft to be used for a particular surgical procedure, a ranking of the desired properties should be considered before deciding on the specific graft (2-4). Where mechanical strength or resistance to resorption is required, autogenous monocortical or bicortical bone grafts are clearly preferred in bone grafting procedures (1,4). However, cortical bone grafts have a limited capacity to provide viable cells (3,4). Demineralized bone matrix (DBM) either alone (5-7), or in combination with bone marrow, autogenous bone graft (8), or other materials (9-10) are often used in clinical practice because of its osteoconductive and osteoinductive properties.

DBM has been shown to induce bone formation in animals as well as in human studies and utilized to promote bone regeneration (11-16). DBM contains active proteins such as bone morphogenetic protein (BMP), transforming growth factor-beta, osteogenin, insulin-like growth factor, and fibroblast growth factor, which are

indirectly involved in bone healing cascade (17-19). Previous studies have been focused on the efficacy of DBM on bone regeneration due to the presence of BMP. Active BMPs in DBM generate potent osteoinductive signals and induce bone formation in vivo and osteoblast differentiation from non-osseous cells in vitro by inducing ALP activity (10,19,20). DBM may act on bone resorption as well as bone formation, because bone resorption is an essential process in bone development and regeneration.

This experimental study was conducted to evaluate the effect of DBM on resorption of autogenous cortical bone graft.

Materials and Methods

Fifty-six male Wistar rats, weighing 480-510 ± 30.6 g were used in this study. The study was approved by the local Ethic Committee for Animal Research. The animals were divided into three groups; the baseline group (n = 8), the experimental group (n = 24) and the control group (n = 24). The experimental and the control groups were subdivided into three groups (n = 8 in each subgroup) which were observed at 2, 4, and 12 postoperative weeks respectively. Overnight fasting urine and blood samples were collected from the baseline group to obtain biochemical parameters in healthy rats.

All animals were anesthetized by intramuscular injections of ketamine hydrochloride 100 mg/kg and chlorpromazine 25 mg/kg. Surgical procedure was performed under sterile conditions. The right femurs of all rats were exposed by vertical skin incision and reflection of the periosteal flap. A bone defect of 3 mm in diameter and 2 mm in depth was created. Then a bone graft of 3 mm in diameter and 2 mm in depth was osteotomised 5 mm from the defect site with a standard trephine bur under continuous irrigation with saline. The bone graft was preserved in a moist gauze while preparing the recipient site. In the experimental group bovine derived DBM in gel form (75 mg) (Grafton, Osteotech, Shrewsbury, NJ, U.S.A.) was applied locally onto the bone defect. The graft was placed in the bone defect and stabilized by perifemoral wiring. The periosteum, muscle fascia and skin were then sutured in separate layers. Sefazolin was administered intramuscularly during the operation and for 3 days postoperatively. Healing progressed uneventfully in all animals and no postoperative complications were noticed during the study periods. All rats were sacrificed at the aformentioned post-operative weeks. Before sacrificing, overnight fasting urine and blood samples were collected to assay the biochemical markers of bone resorption and mineral homeostasis.

All samples were stored at –70 °C until assayed. Serum calcium, phosphate, PTH and 25 hydroxyvitamin D levels and urine deoxpyridinoline, pyridinoline, calcium and creatinine were measured. Serum and urine calcium and serum phosphate were measured by using a commercial kit (Sigma Diagnostics, St Luis, USA). PTH was measured by RIA, using a commercial kit (Diagnostic System Lab. Inc. Texas, USA). 25 hydroxyvitamin D was measured by RIA, using a commercial kit (IDS Chemical Systems, Milton, USA). Deoxpyridinoline and pyridinoline were measured by high performance liquid chromatography (HPLC), using a commercial kit (Chromsystems, München, Germany). Creatinine was measured by Jaffe methods, using a commercial kit (Roche Diagnostics, Basel, Switzerland).

The right femurs were dissected, and fixed in 10% buffered formalin solution. The specimens were decalcified in 15% formic acid for 3 weeks, then embedded in paraffin. 6mm thick sections were prepared longitudinally in the junctions between autogenous cortical bone graft and host bone. The sections were stained with hematoxylin and eosin (H&E) and evaluated at magnifications of 4X, 10X, and 40X under a light microscope (Olympus, Bx50) to characterize cellular reactions. The graft–host bone junction was selected for the histological measurements because it appeared to exhibit the highest bone remodeling. Four slides were used from each biopsy. The number of osteoclasts was counted and the number and size of resorptive lacunae per specimen were evaluated as an indication of bone resorption in a tissue area of 250 μm^2 at 40X magnification (eyepiece micrometer square 10mm/10, Olympus). Osteoclasts were defined as multinucleated eosinophilic cells on the border of grafted bone surface. The resorptive lacunae were defined as regions of grafted bone beneath large multinucleated cells.

Statistical Analysis

For the analysis of differences between the control and the experimental groups, the Kruskal-Wallis test was used, and in case of differences, the Mann-Whitney's U-

test was applied. Data for all parameters were compared within the groups using the Friedman test followed by Wilcoxon's test when significant differences were found. To compare the baseline group with the control and the experimental group, Wilcoxon's test was used.

Results

The comparison of mean values of serum and urinary biochemical parameters in the control and experimental group at 2, 4 and 12 weeks and baseline values are listed in Table 1, Table 2 and Table 3.

In the experimental group, there was a reduction in the serum calcium level at 2 ($P < 0.05$), 4 ($P < 0.05$) and 12 ($P > 0.05$) weeks in comparison with the control group. There was only a statistically significant increase in the experimental group at the 2nd week ($P < 0.01$) as compared with baseline values, while there was statistically significant increase in the control group at 2 ($P < 0.01$), 4 ($P < 0.01$) and 12 ($P < 0.01$) weeks.

There was also a distinguishable difference in serum phosphate level in both the control and the experimental group. There was a progressive increase in the experimental and the control group over 12 weeks, while values at the 12th week showed nearly identical scores with the baseline group. The serum PTH steadily decreased in the control group over 12 weeks. In the experimental group, serum PTH decreased for the first 4 weeks, then a prominent elevation was observed at the 12th week. The difference between the control and the experimental group was only significant at the 12th week ($P < 0.01$). The significant decrease in serum 25 hydroxyvitamin D were seen in both the control and the experimental groups at 2, 4 and 12 weeks as compared with the baseline values. However, the differences between the control and the experimental groups were not significant at 2, 4 and 12 weeks ($P > 0.05$). There was a significant increase in urinary calcium in the experimental group at 2 ($P < 0.01$), and 4 ($P < 0.01$) weeks, with an approximating baseline level at 12 weeks ($P < 0.01$). The control group demonstrated a higher increase in serum calcium over the time of the study periods, this difference being statistically significant (at the 2nd and the 4th weeks: $P < 0.01$, at the 12th weeks: $P < 0.05$).

There was a steady and significant increase in urinary pyridinoline and deoxypyridinoline over the course of treatment in both the control and the experimental groups as compared with baseline values. The experimental group demonstrated a statistically

Table 1. The comparison of mean values (±SD) of serum biochemical parameters in the control and experimental groups at 2, 4, and 12 weeks.

		Experimental group			Control group		
	Baseline n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8
Serum calcium (mg/dl)	6.24 ± 0.90	7.61 ± 0.61 [b]	6.74 ± 0.36 [b]	6.92 ± 0.66 [a]	9.24 ± 0.47	8.94 ± 0.84	8.24 ± 1.2
Serum phosphate (mg/dl)	4.72 ± 0.13	5.59 ± 1.14 [b]	6.60 ± 0.47 [b]	7.6 ± 0.94 [c]	7.34 ± 1.07	7.15 ± 0.41	4.31 ± 0.7
Serum PTH (ng/ml)	60.44 ± 5.44	38.57 ± 3.77 [a]	30.14 ± 3.62 [a]	58.77 ± 8.99 [c]	44 ± 7.52	35.14 ± 4.59	33.71 ± 4.57
Serum 25 hydroxyvitamin D (ng/ml)	56 ± 3.6	54.85 ± 4.98 [a]	54.85 ± 4.98 [a]	32 ± 1.15 [a]	51.14 ± 1.34	47.85 ± 2.67	24.71 ± 1.60

[a] $P > 0.05$, [b] $P < 0.05$, [c] $P < 0.01$; Mann-Whitney's U-test

Table 2. The comparison of mean values (±SD) of urinary biochemical parameters in the control and experimental groups at 2, 4, and 12 weeks.

	Experimental group				Control group		
	Baseline n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8
Urine calcium (mg/dl)	9.21 ± 0.42	12.54 ± 0.95	14.84 ± 0.45	10.64 ± 0.53	14.68 ± 0.45	18 ± 0.27	11.72 ± 0.44
Urinary pyridinoline (nM/mM)	38.42 ± 2.99	53.40 ± 2.69	61..08 ± 3.99	40.92 ± 6.60	60.82 ± 4.45	78.60 ± 2.13	54.12 ± 5.33
Urinary deoxypyridiniline (nM/mM)	5.81 ± 0.45	7.98 ± 0.5	11.94 ± 0.92	6.32 ± 0.55	10.92 ± 0.96	17.21 ± 0.56	14.34 ± 0.46

$^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$; Mann-Whitney's U-test

Table 3. The comparison of mean changes from baseline in serum and urinary biochemical parameters in the control and experimental groups at 2, 4, and 12 weeks.

	Experimental group				Control group		
	Baseline n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8
Serum calcium (mg/dl)	6.24 ± 0.90	7.61 ± 0.61 [c]	6.74 ± 0.36 [a]	6.92 ± 0.66 [a]	9.24 ± 0.47 [c]	8.94 ± 0.84 [c]	8.24 ± 1.2 [c]
Serum phosphate (mg/dl)	4.72 ± 0.13	5.59 ± 1.14 [b]	6.60 ± 0.47 [c]	7.6 ± 0.94 [c]	7.34 ± 1.07 [c]	7.15 ± 0.41 [c]	4.31 ± 0.7 [a]
Serum PTH (ng/ml)	60.44 ± 5.44	38.57 ± 3.77 [c]	30.14 ± 3.62 [c]	58.77 ± 8.99 [a]	44 ± 7.52 [b]	35.14 ± 4.59 [c]	33.71 ± 4.57 [c]
Serum 25 hydroxyvitamin D (ng/ml)	64.28 ± 5.34	56 ± 3.6 [b]	54.85 ± 4.98 [b]	32 ± 1.15 [c]	51.14 ± 1.34 [c]	47.85 ± 2.67 [c]	24.71 ± 1.60 [c]
Urine calcium (mg/dl)	9.21 ± 0.42	12.54 ± 0.95 [c]	14.84 ± 0.45 [c]	10.64 ± 0.53 [c]	14.68 ± 0.45 [c]	18 ± 0.27 [c]	11.72 ± 0.44 [c]
Urinary pyridinoline (nM/mM)	38.42 ± 2.99	53.40 ± 2.69 [c]	61.08 ± 3.99 [c]	40.92 ± 6.60 [a]	60.82 ± 4.45 [c]	78.60 ± 2.13 [c]	54.12 ± 5.33 [c]
Urinary deoxypyridiniline (nM/mM)	5.81 ± 0.43	7.98 ± 0.5 [c]	11.94 ± 0.92 [c]	6.32 ± 0.55 [a]	10.92 ± 0.96 [c]	17.21 ± 0.56[c]	14.34 ± 0.46 [c]

$^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$; Wilcoxon's test

significant decrease in the level of urinary pyridinoline and deoxypyridinoline at 2 ($P < 0.05$, $P < 0.01$), 4 ($P < 0.01$, $P < 0.01$) and 12 ($P < 0.05$, $P < 0.01$) weeks in comparison with the control group. In both groups, a decrease was observed in urinary pyridinoline and deoxypyridinoline from the 4th week to the 12th week, but the levels were significantly lower in the experimental group than in the control group ($P < 0.01$).

Multinuclear giant cells were significantly reduced in the experimental group compared with the control group at 2 ($P < 0.001$), 4 ($P < 0.001$), and 12 ($P < 0.001$) weeks (Table 4). As shown in the histological sections, an increase in the number of osteoclasts was seen in the control group (Figure 1), whereas a lower number of multinuclear cells was observed in the experimental group (Figure 2 and Figure 3) at 2, 4, and 12 weeks. A significant reduction in the number and size of resorptive lacunae was noticed in the experimental group compared with the control group at 2, 4, and 12 weeks (Figure 2 and Figure 3).

Table 4. The comparison of mean values (±SD) of the number of osteoclasts in the control and experimental groups at 2, 4, and 12 weeks.

	Experimental group			Control group		
	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8	2 weeks n = 8	4 weeks n = 8	12 weeks n = 8
The number of osteoclasts	18..65 ± 1.34	12.45 ± 0.50	6.23 ± 0.65	40.14 ± 2.9	27.57 ± 3.3	16.00 ± 2.23

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[d] $P < 0.001$; Mann-Whitney's U-test

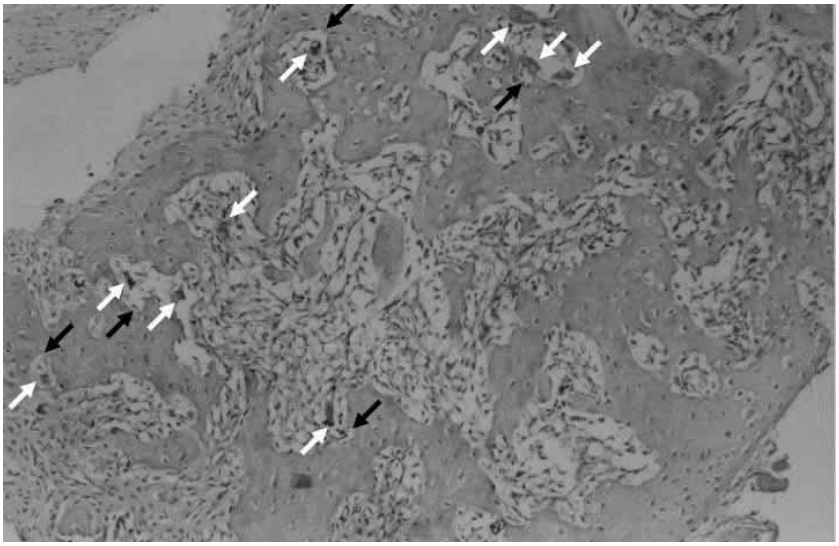

Figure 1. Increase in the number of osteoclasts (white arrows) and resorptive lacunae (black arrows) along with woven bone formation were observed in the control group at the 2nd week (H&Ex100).

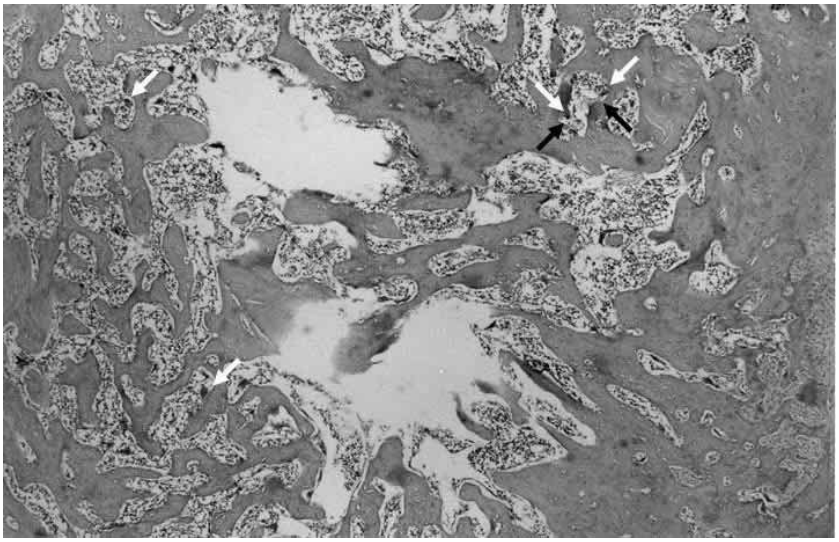

Figure 2. Decreased number of osteoclasts (white arrows) and resorptive lacunae (black arrows) and lamellar bone formation were noticed in the experimental group at the 4th week (H&Ex40).

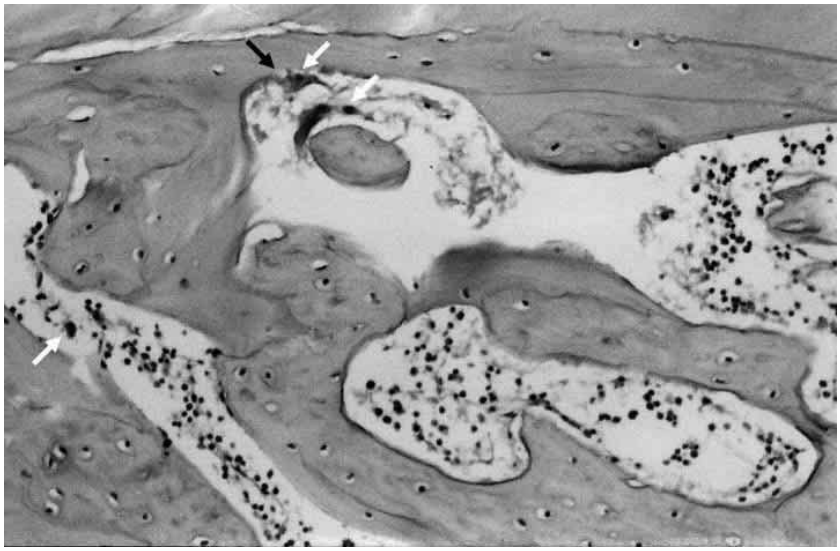

Figure 3. Histopathological sections of the experimental group with smaller number of osteoclasts (white arrows) and resorptive lacunae (black arrow) and new lamellar bone formation at the 12th week (H&Ex200).

Discussion

Bone grafts are frequently used in the fields of periodontology, oral-maxillofacial surgery and orthopedics to repair bone defects and promote new bone formation. These materials are osteoinductive/osteoconductive and accelerate healing by formation of new bone tissue. DBM has been studied extensively as an osteoinductive agent due to the presence of BMP (10,12,18,19,21-24). The efficacy of DBM was previously tested in animal models and found to promote new bone formation at orthotopic and heterotopic sites, such as muscle tissue, with no adverse reaction (13,15,25). However, the effectiveness of DBM is still controversial (26). Many studies demonstrated a beneficial effect of its use (7,18,26), whereas others reported that DBM has no significant effect on new bone formation of lamellar bone (5,6,22,27-29). The conflicting results are attributed to the age of the donor and host animals, methods of preparation, embryologic origin of DBM, size of the DBM particles, amount of the implanted material, and the period and storage conditions of the material (6).

It was demonstrated that following bone grafting, remodeling takes place both in the recipient bone and in the graft (4,30). During the remodeling periods, both bone formation and resorption occur simultaneously. The changes in the rate of new bone formation would probably affect the rate of bone resorption during graft remodeling. The effect of DBM on bone resorption is not clear. To our knowledge, no report exists about the direct effect of DBM on bone resorption of autogenous bone graft. Previous studies have focused on the efficacy of DBM on bone regeneration. DBM was compared with autogenous bone graft in most of the researches (6,27,29,31). In the present study, the effect of DBM on bone resorption of autogenous cortical bone graft was examined by biochemical and histological means. Groeneveld et al. (8) compared the bone resorption in human sinus floor elevations grafted with DBM and autogenous bone. They reported that the number of osteoclasts were fewer in DBM than in autogenous bone. Babbush (18) histologically evaluated human biopsies after dental augmentation with demineralized bone matrix putty. He did not directly examine bone resorption, but noticed only a few osteoclasts in the specimens.

In the present study, the suppression of urinary excretion of deoxypyridinoline was significantly greater in the experimental group than in the control group at 2, 4, and 12 weeks, showing significant inhibition of bone resorption. The decreased number of osteoclasts and resorption lacunae at 2, 4, and 12 weeks also confirmed

the reduction of bone resorption in the experimental group.

During the remodeling phase of the bone graft, the degree of bone resorption is very important to stimulate new bone formation and maintain graft volume (14,32). Less bone resorption may provide the volumetric maintenance of the bone grafts (32,33) and contraction of the remodeling space. In the present study, the volume of the graft was not evaluated during remodeling. Further histomorphometric studies are needed to determine changes in the graft volume after application of DBM.

In conclusion, both biochemical and histological results of the present study consistently showed that DBM is effective in decreasing bone resorption of autogenous cortical bone grafts.

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