

# Histological evaluation of the effects of bioglass, hydroxyapatite, or demineralized freeze-dried bone, grafted alone or as composites, on the healing of tibial defects in rabbits

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## ABSTRACT

**الأهداف:** مقارنة فعالية الزجاج النشط (BG)، هيدروكسيباتايت الطبيعي (HA)، العظام المجففة بالتجميد المنزوعة المعادن (DFDB) في نقوصات العظام.

**الطريقة:** أُجريت الدراسة على جميع حيوانات التجربة في كلية الطب البيطري بجامعة سيلكوك - تركيا، تحت إشراف خطوط الإرشاد بجامعة سيلكوك للتجارب الحيوانية في عام 2005م. تم استخدام 18 أرنب نوع نيوزلاند في هذه التجربة. تم إعداد أربع تجويفات في عظم الساق الضنبوب الأيمن والأيسر. قمتنا بملاء التجويف في الضنبوب الأيمن إما باستعمال (BG) أو (HA) أو (DFDB). أبقى تجويف واحد كمجموعة تحكم. رُقع التجويف في عظمة الضنبوب الأخرى باستعمال مركبات (HA+BG, HA+DFDB, BG+DFDB) و (HA+BG+DFDB). أُجريت الفحوصات النسيجية بعد الشهر الأول، الثالث والسادس من العملية الجراحية.

**النتائج:** وفقاً للنتائج النسيجية، أعطى تكوين المركبات (HA+BG+DFDB) أفضل النتائج النسيجية ( $p < 0.05$ ).

**خاتمة:** تكون الزراعة بالمركبات (BG) (HA) و (DFDB) أكثر فعالية من استعمالها كعناصر مفردة.

**Objectives:** To compare the effectiveness of bioactive glass (BG), natural hydroxyapatite (HA), and demineralized freeze-dried bone (DFDB) in bone defects.

**Methods:** All animal experiments were conducted in Faculty of Veterinary Medicine, Selcuk University, Konya, Turkey, under the Selcuk University Guidelines for Animal Experimentation, in 2005. Eighteen New Zealand rabbits were used for the experiment. Four cavities were prepared on right and left tibias. The cavities on the right tibia were filled with either BG, HA or DFDB. One cavity was left empty as a control. The

cavities on the other tibial bone were grafted with HA+BG, HA+DFDB, BG+DFDB and HA+BG+DFDB composites. Histological examinations were performed at first, third, and sixth postoperative months.

**Results:** According to histomorphometric findings, the mixture containing HA+BG+DFDB obtained the best histological results ( $p < 0.05$ ).

**Conclusion:** The composite graft of HA, BG and DFDB is more effective than when used as individual agents.

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**B**one grafts are extensively used materials in the repair of oral and maxillofacial defects. Although most of the researchers agree that autogenous bone graft is the most suitable substance, however, it has some disadvantages such as donor site morbidity, harvesting difficulties, and inability to provide great amounts.<sup>1,2</sup> To overcome these problems, use of xenografts, allografts, and other biomaterials have been studied extensively.<sup>3-7</sup> However, they have some drawbacks, too. For example, allografts and xenografts should be prepared (demineralizing, freeze-drying, irradiating, or treating with ethylene oxide) to prevent the transmission of disease, and this decreases the material's integrity and osteogenic potential. In some cases, immunological

response to the graft material may diminish its incorporation into the recipient bone.<sup>8</sup> Alloplasts have no potential for disease transmission and they are available in large amounts. They are osteoconductive, offer various levels of structural support, and have no ability for osteoinduction.<sup>9</sup> Researchers have also investigated the use of different combinations of various graft materials.<sup>2,6,7,10</sup> The aim of this study was to compare, histologically and histomorphologically, the favorable effects of bio-active glass (BG), natural hydroxyapatite (HA), and demineralized freeze-dried bone (DFDB), either grafted alone, or as a combination, on the healing of artificially created bony defects.

**Methods.** Eighteen New Zealand rabbits, 5-6 months old and equal numbers from both gender, weighing 3000-4000 g were used in this study. All animal experiments were conducted in Faculty of Veterinary Medicine, Selcuk University, Konya, Turkey under the Selcuk University Guidelines for Animal Experimentation, in 2005. All surgical procedures were performed under general anesthesia with intramuscular injection of 20 mg/kg Xylazine (Rompun® Bayer, Leverkusen, Germany) and 10 mg/kg ketamine hydrochloride (Ketanes®, Alke, Turkey) combination. The skin, over both right and left tibial regions, was shaved and disinfected with polivinylpyrrolidone-iodine solution (Batticon®, Adeka Ilac San., Istanbul, Turkey). Both left and right tibial bones were carefully exposed and the periosteum was dissected. Four cavities (3 mm in diameter) were prepared using a steel burr at slow speed (500 rpm) in the cortical bone of tibial bones. A profuse irrigation with physiological saline was maintained. After washing the bone cavities, the graft materials were tightly filled into the defects up to the level of the previous bone surface, and pressurized to prevent blood impregnation into the grafted material. All surgical wounds were closed in layers. One of the cavities in the right tibial bones was left empty as a control group, and the remaining 3 were filled with one of the graft materials: BG (Bioglass® American Biomaterials Corp., Baltimore, USA), HA (Osteograft N®, Ceramed, USA), and DFDB (DynaGraft®, Gen Sci, Toronto, Canada). The same procedures were performed in the left tibial bones, but instead of using graft material alone, they were filled with HA+BG, HA+DFDB, BG+DFDB, and HA+BG+DFDB composites. While preparing grafting composites, equal weights of biomaterials were blended in sterile Petri dishes. Six animals, from equal numbers of each gender, were killed by giving an overdose of pentobarbital sodium at first, third, and sixth month of the postoperative period. Tibia samples taken from grafted areas were fixed in 10% formaline, decalcified, and immersed in paraffin. Tissue sections were taken

in 5 µm and stained either with Masson's trichrome stain or with picro-thionine. Specimens were examined and photographed in x400 magnification with a light microscope (Leitz Ortholux II, Wetzlar, Germany). Histomorphometry was performed according to Izci et al.<sup>11</sup> Briefly, each image was printed on 18x24 cm photographic paper. Total areas of fibrocartilage, bone, grafted particles, fibrous connective tissue, and marrow tissue were measured with a digital planimeter (Sokhisha Digital Planimeter, Model KP 90, Kanagawa, Japan). Inflammatory, allergic, and foreign body reactions to the grafted materials, new bone formation and bio-absorption rates of the graft materials were examined.

The results were expressed as percentage of the total photographic area and analyzed. Statistical Package for Social Sciences Version 10.0 software, by using the one-way analysis of variance was used for statistical evaluation. Significance was determined at 0.05 level.

**Results.** No postoperative complications were observed in the healing period. None of the graft materials caused serious and long lasting allergic, toxic or graft rejection reactions. A weak and gradually declining mononuclear cell infiltration and giant cells were seen in DFDB (alone or combined with BG and HA) graft sites. Histomorphometric findings of the defect sites at different months after surgical operation are given in Tables 1-3. The mixture containing HA+BG+DFDB obtained the best histological results at the postoperative third month (Figure 1), and 6-months of the experiment as well.

**Discussion.** An ideal bone graft material should exhibit 4 main characteristics: i) osteointegration, the ability to bond chemically to the surface of bone without an intervening layer of fibrous tissue; ii) osteoconduction, the ability to support growth of bone over its surface; iii) osteoinduction, the ability to induce differentiation of pluripotent stem cells from surrounding tissue to an osteoblastic phenotype; and iv) osteogenesis, the formation of new bone by osteoblastic cells present within the graft materials.<sup>12</sup> Although only autogenous bone graft satisfies all these requirements, allografts have osteointegrative and osteoconductive properties, and may exhibit osteoinductive potential.<sup>13</sup> Demineralized freeze-dried bone has been used by some surgeons on the premise that it is an osteoinductive material;<sup>14</sup> however, inductive capacity in DFDB is limited as it has very little bone morphogenetic protein, which is one basic element of osteoinduction.<sup>15</sup> Since possible transmission of viral agents such as HIV and hepatitis through bone allografts has limited use, researchers have focused their efforts on developing synthetic bone graft substitutes. These materials at most

**Table 1** - Bone, fibrocartilage, connective and bone marrow tissue, and residual graft material in the area of the defects one month after surgery.

Tissue types	Proportions (%) of the tissues in the defect sites of the groups (mean ± standard error)							
	Control	HA	BG	DFDB	HA+BG	DFDB+BG	HA+DFDB	HA+BG+DFDB
Bone	11.3±1.12 <sup>c</sup>	28.2±2.1 <sup>b</sup>	14.5±1.28 <sup>c</sup>	44.6±2.9 <sup>a</sup>	22.3±1.8 <sup>b</sup>	46.1±3.1 <sup>a</sup>	55.3±3.5 <sup>a</sup>	35.3±2.6 <sup>b</sup>
Fibro-cartilage	43.6±2.56 <sup>a</sup>	-	8.2±0.67 <sup>b</sup>	-	-	-	-	-
Connective and marrow tissue	35.1±2.11 <sup>a</sup>	9.5±0.95 <sup>c</sup>	24.33±1.3 <sup>b</sup>	13.1±1.1 <sup>c</sup>	27.5±1.6 <sup>b</sup>	10.5±0.9 <sup>c</sup>	11.8±0.8 <sup>c</sup>	18.9±1.3 <sup>c</sup>
Residual graft materials	-	62.3±3.8 <sup>a</sup>	53.0±3.2 <sup>a</sup>	42.3±2.6 <sup>b</sup>	50.2±2.6 <sup>c</sup>	43.4±2.8 <sup>b</sup>	32.9±2.4 <sup>c</sup>	45.8±3.4 <sup>b</sup>

BG - bioactive glass, HA - natural hydroxyapatite, DFDB - demineralized freeze-dried bone.  
a-c: the difference between mean values on the same line with different superscripts are statistically significant ( $p<0.05$ ).

**Table 2** - Bone, fibro-cartilage, connective tissue, bone marrow and residual graft material in the area of the defects in third month after surgery.

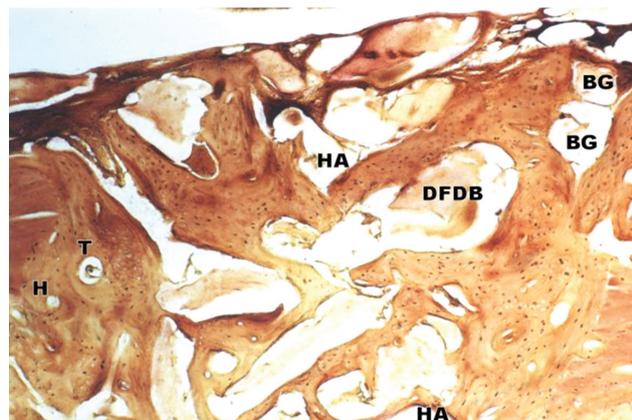
Observed tissue types	Proportions (%) of the tissues in the defect sites of the groups (mean ± standard error)							
	Control	HA	BG	DFDB	HA+BG	DFDB+BG	HA+DFDB	HA+BG+DFDB
Bone	31.78±2.90 <sup>c</sup>	44.60±3.12 <sup>b</sup>	36.63±1.80 <sup>c</sup>	56.45±3.86 <sup>a</sup>	48.93±3.25 <sup>b</sup>	54.67±3.42 <sup>a</sup>	56.34±3.78 <sup>a</sup>	52.35±2.87 <sup>a</sup>
Fibro-cartilage	-	-	-	-	-	-	-	-
Connective and marrow tissue	68.22±2.15 <sup>a</sup>	48.20±3.30 <sup>b</sup>	56.97±3.03 <sup>b</sup>	37.92±2.23 <sup>c</sup>	43.22±3.13 <sup>b</sup>	39.48±2.85 <sup>c</sup>	35.80±2.34 <sup>c</sup>	56.07±2.98 <sup>b</sup>
Residual graft materials	-	7.22±0.7 <sup>a</sup>	6.40±0.35 <sup>b</sup>	5.63±0.23 <sup>b</sup>	7.85±0.3 <sup>a</sup>	5.85±0.35 <sup>b</sup>	7.86±0.55 <sup>a</sup>	8.93±0.98 <sup>a</sup>

BG - bioactive glass, HA - natural hydroxyapatite, DFDB - demineralized freeze-dried bone.  
a-c: the difference between mean values at same line with different superscripts are statistically significant ( $p<0.05$ ).

**Table 3** - Bone, fibrocartilage, connective and bone marrow tissue, residual graft material in the area of the defects 6 months after surgery.

Observed tissue types	Proportions (%) of the tissues in the defect sites of the groups (mean ± standard error)							
	Control	HA	BG	DFDB	HA+BG	DFDB+BG	HA+DFDB	HA+BG+DFDB
Bone	33.43±2.02 <sup>c</sup>	64.67±2.97 <sup>a</sup>	62.78±3.35 <sup>a</sup>	52.45±5.34 <sup>b</sup>	57.56±4.23 <sup>b</sup>	73.32±5.67 <sup>a</sup>	72.67±6.32 <sup>a</sup>	66.98±5.87 <sup>a</sup>
Fibro-cartilage	-	-	-	-	-	-	-	-
Connective and marrow tissue	66.57±2.13 <sup>a</sup>	28.68±3.01 <sup>c</sup>	33.37±2.53 <sup>b</sup>	45.66±3.25 <sup>b</sup>	38.24±2.54 <sup>b</sup>	22.48±1.6 <sup>c</sup>	20.35±1.93 <sup>c</sup>	18.46±2.12 <sup>c</sup>
Residual graft materials	-	6.65±0.42 <sup>b</sup>	3.85±0.23 <sup>c</sup>	1.89±0.14 <sup>d</sup>	4.2±0.37 <sup>c</sup>	3.88±0.29 <sup>c</sup>	6.98±0.53 <sup>b</sup>	14.56±1.86 <sup>a</sup>

BG - bioactive glass, HA - natural hydroxyapatite, DFDB - demineralized freeze-dried bone.  
a-c: the difference between mean values at same line with different superscripts are statistically significant ( $p<0.05$ ).



**Figure 1** - Histology of the composite (HA+BG+DFDB) grafting site after 3 months. Remnants of the extensively resorbed particles are seen. The newly formed bone is highly organized with lamellar bone trabecules (T) and Haversian systems (H). BG - bioactive glass, HA - natural hydroxyapatite, DFDB - demineralized freeze-dried bone

posses only 2 of the 4 characteristics of an ideal bone graft material, osteointegration and osteoconduction.<sup>12</sup> Ideally, synthetic bone graft substitutes should be biocompatible, show minimal fibrotic reaction, undergo remodeling, and support new bone formation. Their strength should be similar to that of the bone being replaced.<sup>12</sup> The most common osteoconductive bone grafting materials used in implant dentistry are alloplasts and xenoplasts. Alloplastic materials are exclusively synthetic and may be ceramics, polymers, and composites. The most frequently used are ceramics, which may be characterized as bio inert (aluminum and titanium) or bioactive (calcium phosphate). Bioinert ceramics do not exhibit direct bonding with the host bone and are mechanically held in contact to the bone. The healing of bone around a bioinert osteointegrated implant is an osteoconductive process (AlO<sub>2</sub> and TiO). Bioactive ceramics include calcium phosphates such as synthetic hydroxyapatites. Calcium phosphate materials have no toxic effect. Hydroxyapatite, which is one of the calcium-phosphate ceramics, is a fundamental inorganic component of calcified human tissues and contains calcium and phosphate at a rate of 10:6. Since the HA materials include carbonates, their resorption rate is high.<sup>16</sup> They can bond with bone and that makes HA a preferable material characterized as osteoconductive and osteophilic, but not osteogenic. These materials are usually porous and/or amorphous forms of calcium phosphate ceramics. They are usually made up of HA, beta-tricalcium phosphate, or various combinations of both. All HAs are resorbable, depending on the surrounding pH, porosity, particle size volume, and crystallinity.<sup>17</sup> Differences in pH of the environment have an effect on the resorption as well. In an acidic environment, bone, dense HA, macroporous HA, crystalline HA, or amorphous HA are resorbed in 2 weeks.<sup>13</sup> It has been reported that an increase in bone volume from 16.4% after 4 months to 45.3% after 36 months in sinus augmented with HA alone.<sup>18</sup>

Bio-active glass has largely been used in surgical procedures and also in dentistry for repairing hard tissues.<sup>19-21</sup> The advantages of using BG in reconstruction of hard tissues have been demonstrated in animal studies.<sup>22,23</sup> Histological analysis revealed that as a periodontal grafting material, BG has only limited regenerative process on the healing of intrabony defects around the teeth. Nevertheless, graft particles were found to be biocompatible, as evidenced by being embedded in stroma of dense connective tissue with minimal inflammatory infiltrate.<sup>17</sup> Bio-active glass causes no local or systemic toxicity, does not lead to foreign body reactions and particulate BG has also antibacterial features.<sup>1,3,24,25</sup> It has been reported that implants survived for up to 3 years in sites grafted with BG.<sup>4</sup> The repair of

bone defects results in the formation of new bone that can then undergo remodeling, optimizing its mechanical function. This constitutes the ideal goal of tissue engineering strategies to regenerate bone. Independent activities of osteoblastic and osteoclastic cells at an early stage are seen on alpha-tricalciumphosphate particles in an exquisite coordination and they couple at the later stage. At the early stage, osteoclasts impart space by recognizing the graft material as a kind of bone matrix, to permit osteoblastic cell lineages to migrate subsequently. Therefore, slow replacement of a given graft material allows a coupling of osteoclasts and osteoblasts at the later stage and this results in a regeneration of compact bone.<sup>5</sup> Without grafting material as seen in the control group, immature woven bone grew into the cavity so rapidly to fill the entire bone defect. However, the new bone remained as cancellous bone for a long period in a previous study.<sup>5</sup> The precise mechanism of bone remodeling in the biomaterial-to-cell interaction remains unknown. The surface of resorption lacunae may trap many organic components, such as bone matrix proteins, local factors, and secretory products by osteoclasts.<sup>5</sup> In an experimental study,<sup>25</sup> BG-grafted defects displayed more vital bone (59.5%) in 6-8 months healing biopsies than either DFDB or controls. Demineralized freeze-dried bone grafted (34.7%) and control sites (32.4%) had similar levels of vital bone. In another study,<sup>6</sup> 50.5% vital bone, 8.2% biomaterial remnants, and 41.31% marrow and fibrous tissue in DFDB grafted sites were reported. In HA grafted defects, vital bone was 46.8%, biomaterial 6.9%, and marrow and fibrous tissue 46.35%. Both HA and BG were found to be osteoconductive materials and histometrical results did not reveal any advantage for either of the materials. The materials were not totally resorbed after 10 months and their remnants integrated with the bone.<sup>6</sup> The use of autogenous bone in combination with BG is dictated by the fact that BG is designed to replace the inorganic components needed for bone formation.<sup>7</sup> Other authors used DFDB in combination with collagen membrane and suggested that the composite implant was better in integration.<sup>10</sup> They noted more rapid calcification and bone formation at the site of implantation of DFDB alone, or in combination either with BG or HA, the HA+BG+DFDB combination was found to be the most effective biomaterial combination in bone healing under the conditions of this study. Mixtures of biomaterials caused more extensive ossification than grafting the materials individually in earlier phases of the postoperative period. However, the calcification process started in relatively later stages of the postoperative period. At the sixth month following grafting, ossification was similar in all groups. The ossification process in the defect site grafted with HA+DFDB+BG mixture

was superior to the other groups, since homogeneous bony tissue replaced the resorbed particles concordant with resorption speed. The differences in percentage bone volume among the presented study and various previous research can be attributed to the bone healing pattern in different animal species and humans, length of the postoperative healing period, sites of bone biopsies, architecture, conformation and composition of the grafting material, ratio of autogenous bone in composite grafts, and potentially implant loading.<sup>6</sup> According to our results, the HA+BG+DFDB mixture showed better osseointegration when compared with the single use of these materials. Within the limits of the present study, we conclude that the HA, BG, and DFDB composite graft give better results than when used individually. We strongly stress that further studies are necessary to determine an efficient graft mixture by regarding resorption kinetics and osteo production mechanisms of the graft materials.

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