Histologic Evaluation of Bone Healing Capacity Following Application of Inorganic Bovine Bone and a New Allograft Material in Rabbit Calvaria

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Abstract

Objectives: Considering the importance of bone augmentation prior to implant placement in order to obtain adequate bone quality and quantity, many studies have been conducted to evaluate different techniques and materials regarding new bone formation. In this study, we investigated the bone healing capacity of two different materials deproteinized bovine bone mineral (DBBM with the trade name of Bio-Oss) and demineralized freeze-dried bone allograft (DFDBA with the trade name of DynaGraft).

Materials and Methods: This randomized blinded prospective study was conducted on twelve New Zealand white rabbits. Three cranial defects with an equal diameter were created on their calvarium. Subsequently, they were distributed into three groups: 1. The control group without any treatment; 2. The Bio-Oss group; 3. The DynaGraft group. After 30 days, the animals were sacrificed for histologic and histomorphometric analysis.

Results: Substantial new bone formation was observed in both groups. DynaGraft: 56/1 % ± 15/1 and Bio-Oss: 53/55 % ± 13/5 compared to the control group: 28/6 % ± 11/2. All groups showed slight inflammation and a small amount of residual biomaterial was observed.

Conclusion: Considerable new bone formation was demonstrated in both DynaGraft and Bio-Oss groups in comparison with the control group. Both materials are considered biocompatible regarding the negligible foreign body reaction.

Key Words: Animal Research; Bone Regeneration; Rabbit Calvaria; Allografts

INTRODUCTION

Alveolar bone defects are created by different factors such as accidental trauma, surgical trauma from tooth extraction, infections, developmental anomalies and periodontal disease [1, 2]. This can cause alveolar ridges with a reduced width and height leading to a great challenge in prosthetic reconstruction as well as during endosseous dental implant placement [2].

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A lot of techniques have been developed to achieve alveolar ridge augmentation before dental implant placement. Autogenous bone graft harvested from the patient’s skeleton is considered the gold standard, and it seems to be the most predictable technique used in alveolar defects, although there are some limitations with it, such as the second surgical site involvement, inadequate donor site and the morbidity and discomfort along with it [1-3].

Another method for reconstruction of bone defects is guided bone regeneration, which has been successful based on animal and clinical studies [4-6]. Clot contraction under the membrane; however, probably reduces its efficacy [7-10].

Graft materials such as allografts and xenografts are also proper alternatives for ridge augmentation, in order to overcome the autogenous bone restrictions [3]. Demineralized freeze-dried bone allograft (DFDBA) is a type of bone graft that has been extensively used, although lack of osteoinductivity, getting washed out in bleeding sites and difficulty in handling have questioned the effectiveness of the material.

To overcome this problem, DBM graft materials have been improved by using a carrier to maintain the particles together. It has some characteristics such as being hemostatic, expanding to fill the bony defect and having good stability. Materials such as glycerol, polymers, polymeric gels, and collagen have been utilized as a carrier to keep up the integrity of the particles. DynaGraft (GenSci Regeneration Sciences Inc., Irvine, CA) is a trade name for a type of DBM combined with a carrier that exists in different forms of gel, matrix and putty depending on the type of vehicle used. Poloxamer 407 is the carrier for the putty form of DynaGraft. At low temperatures, it is in liquid form and becomes firmer at body temperature. Based on histologic analysis, this material inhibits epithelial cell migration, playing the role of a barrier. In addition, it has been demonstrated to be guided tissue regenerative inherently, in human and animal studies [11, 12].

Bio-Oss (Geistlich Biomaterials, Wolhusen, Switzerland) is an inorganic osseous matrix that is produced after elimination of the organic components of medular bovine bone using a thermal treatment. After removal of the organic components, Bio-Oss preserves its trabecular architecture and porosity and it acts as an osteoconductive material. The physical properties permit clot stabilization and revascularization to let osteoblast migration, leading to osteogenesis [13]. The aim of this study was to evaluate the quality and quantity of bone healing after application of DynaGraft and Bio-Oss in experimentally induced bone defects in rabbit calvaria compared to natural healing.

MATERIALS AND METHODS
It was a randomized, single-blinded, experimental study. The animal selection, management, and experimental protocol were approved by the Animal Care and Use Committee of Tehran University of Medical Sciences. The study was performed strictly according to the advices of Helsinki consensus for the use and care of animals.

Twelve New Zealand white male rabbits with 2.5 kg mean weight were included in the study. The animals were kept on standard laboratory chow with free access to water for 2 weeks preceding the day of surgery. An intramuscular injection of 2% (5mg/kg) xylazine and 10% (40mg/kg) ketamin (Alafason, WOEDEN, HOLLAND) was used to anesthetize the rabbits. All surgical procedures were performed under sterile conditions. Subsequent to shaving the surgical sites on the calvaria, 7% betadine was used to scrub the area for 5 minutes. A 10 cm anteroposterior incision was performed with NO. 15 surgical blade, and then the skin (dermal and subdermal tissues) and the periosteum were reflected using a fine periosteal elevator.
After that, a round bur was used to create three identical holes with an external diameter of 5 mm in the calvaria, under abundant irrigation with physiologic serum. Anatomic landmarks such as the occipital process and craniocaudal sutures were used to standardize the location of the defects. In the center of the calvaria, these two landmarks meet each other and make a plus sign (+).

Depending on the type of material, the defects were categorized into three groups:
1. The control group, in which the defect was left without any treatment
2. The Bio-Oss group, in which the defect was filled with Bio-Oss (particles sized 0/25 mm-1mm)
3. The DynaGraft group, in which DynaGraft was applied to fill the defect

The periosteum was closed using an absorbable 4/0 suture (Vicryl Johnson & Johnson Somerville, NJ), and the skin was sutured with a non-resorbable 4/0 suture (monofil Polylmid, Surgi Pro. Monofilament, PolyPropylene). Following the surgical procedure, the animals were transferred to a warm place, and they underwent medication with 3 days of narcotics (ketoprofen 0/1 mg/day) and 7 days of antibiotic therapy (enrofloxacin 0/6 mg/day) subcutaneously.

**Sample Preparation**

The animals were sacrificed via pentobarbital overdose (100mg/kg), injected intravenously after one month of healing. Then, the calvaria was removed and placed in 10% buffered formalin solution for 2 weeks and then it was decalcified in 10% formic acid for 14 days. Subsequently, they were dried out in graded alcohols and surrounded in paraffin. Before final preparation for sectioning, all samples were placed in formalin for 48 hours. Ten histologic sections with a thickness of 6 μm were prepared from each defect containing an intact border of the bone, and then the samples were routinely stained with hematoxylin and eosin.

The amount of inflammation, foreign body reaction, bone vitality, type of bone formation (sequence of collagen fibers) were evaluated by a light microscope (BX41, Olympus Co., Tokyo, Japan) at a magnification of ×40. A five-tiered grading system was utilized to scale the inflammation as follows: 0, without any inflammatory cells; I, slight inflammation; II, focal inflammation containing 5 to 10 inflammatory cells; III, focal inflammation with 10 to 50 inflammatory cells; and IV, focal inflammation with more than 50 inflammatory cells.

In case of observing multinucleated giant cells in granulomatous response, foreign body reaction was established. Bone vitality was confirmed by the presence of osteocytes in the trabecular lacunae.

The type of bone formation was characterized as woven bone alone (type I), both woven and lamellar bone (type II), and lamellar bone alone (type III).

Collagen bundles in concentric form in the bony trabecules were determined as lamellar bone; whereas, irregularly oriented collagen fibers in the trabeculae were considered as woven bone.

The amount of bone formation (percent) and remaining biomaterial were evaluated by means of graphic software (Photoshop 8.0 CS, Adobe Photoshop CS).

Statistical analysis for the amount of inflammation and type of bone formation was performed using Friedman test. Wilcoxon sign rank test was used to evaluate the intergroup differences (two by two) about the type of bone formation.

Repeated measure ANOVA and paired LSD test were applied to analyze the amount of bone formation.

In order to compare the remaining bone material between groups, paired T test was performed accordingly.

Results were considered statistically significant at P<0.05.
RESULTS
All specimens showed vital bone formation. The results are shown in Table 1. Evaluating the amount of inflammation using Friedman test, all specimens showed slight infiltration (grade I and II) and there was no significant difference between groups (p=0.478). Seventy five percent of the control group, 66/66% of Bio-Oss group and 58/33% of DynaGraft group showed grade I inflammation; whereas, other specimens presented with grade II inflammation.

All specimens consisted of both lamellar and woven bone. Considering the type of bone formation, in the control group, 16/67 % and 83/33 % of the specimens displayed type I and II bone formation, respectively. In the DynaGraft group, 41/67% of the specimens showed type II bone formation, and bone type III was detected in 58/33% of the defects in this group. In the Bio-Oss group, type I, II and III bone formation was observed in 16/66%, 58/33%, and 25/01% of the specimens, respectively. According to Friedman test, the difference between groups was statistically significant (P value < 0.05).

Intergroup differences (two by two) were evaluated using Wilcoxon sign rank test, and a significant difference was detected comparing DynaGraft group with the control and Bio-Oss groups (P value < 0.017); whereas, there was no significant difference between Bio-Oss and the control group (P value > 0.017).

The mean amount of new bone formation was 28/6% ± 11/2 in the control group, and the DynaGraft and Bio-Oss groups demonstrated 56/1% ± 15/1 and 53/55% ± 13/5 mean amount of new bone formation, respectively. According to repeated measure ANOVA, the difference between groups was statistically significant (P value <0.001). For evaluation of the two by two differences, LSD test showed a significant difference comparing the control group with DynaGraft and Bio-Oss groups (P value < 0/05). Considering the remaining material, using paired T test, no significant difference was observed between Bio-Oss and DynaGraft groups (P value < 0/1).

Foreign body reaction was seen in two defects in the DynaGraft group, and four specimens in the Bio-Oss group, and the difference was not statistically significant (P value < 0/342).

DISCUSSION
Many experimental studies have been carried out to evaluate different techniques and materials in bone regeneration procedures [14-17]. Since the amount of bone formation and its quality are of significant importance, a large number of studies have compared the characteristics and treatment outcomes of a variety of materials and techniques [18, 19].

In the present study, we compared the effect of two types of bone grafts on the quality and quantity of bone healing in experimental defects in rabbit calvarium.

| Study Groups | Inflammation % | Bone Vitality | Bone Type % | Amount of Bone Formation % | Amount of Remaining Biomaterial % |
|--------------|----------------|---------------|-------------|-----------------------------|----------------------------------|
|              | I              | II            | I           | II            | III                |                                  |
| Control      | 9 (75%)        | 3 (25%)       | 100%        | 2             | 10                 | 28/6±11/2                       | 0                               |
| Bio-Oss      | 8 (66/66%)     | 4 (33/33%)    | 100%        | 2             | 7                  | 53/55±13/5                      | 30/65±20/2                      |
| DynaGraft    | 7 (58/33%)     | 5 (41/66%)    | 100%        | 0             | 5                  | 56/1±15/1                       | 29/9±15/3                       |
| P value      | 0/478          | 1             | P<0/05      | P<0/001       | P<0/1              |                                  |

Table 1. Comparison of Histologic Characteristics in Groups
The bone formation process of calvarium is intra membranous, similar to that of the alveolar bone. Thus, we investigated the calvarium of the rabbit in order to make an appropriate comparison [20].

Regarding the amount of new bone formation, both materials demonstrated favorable properties. Although not statistically significant, DynaGraft showed slightly more newly formed bone. This could be related to its good stability and its ability to act as a barrier membrane. Moreover, these characteristics interpret the osteoinductive and osteoconductive effect of DynaGraft in comparison to Bio-Oss with its osteoconductive properties. This is in accordance with the study of Callan et al. In their case series study, four to six months prior to implant placement, they carried out a bone augmentation procedure. Subsequent to material placement, a membrane barrier material was used in order to facilitate site closure. They established similar results about the new allograft material and they claimed it can be used in bone defects within a short time healing. The membrane they utilized, however could have influenced the outcome of their study [21].

Babbush et al. also utilized the human glycoprotein containing DFDBA in their study. They used the material for osseous reconstruction associated with dental implants. In their case series, they performed fresh socket implantation in the maxillary anterior segment. Following extraction and fixture insertion, the residual defect was filled with the material. After approximately 6 months, at the second stage of implant surgery, a bone core was harvested from the distal aspect of the implant. Histological evaluation demonstrated new bone formation in the osseous defects [22].

In addition, in 2002 Clokie et al. evaluated bone regeneration of critical sized calvarial defects in rabbits utilizing demineralized bone matrix putty. They established a complete bone fill and closure of the defects with vital bone at 12 weeks [1]. Since a true critical-sized cranial defect in the rabbit model is 15 mm, in the present study, due to the small size of the cranium, three critical-sized defects could not be prepared in the rabbit calvarium. Moreover, many studies have shown that Bio-Oss is effective in bone regeneration procedures [23-26]. Khoshkhoonejad et al. observed substantial new bone formation in calvarial defects of rabbit containing Bio-Oss. The amount of new bone was comparable with Bio-Oss in conjunction with membrane [24]. In the present study, we found similar results regarding Bio-oss, but Dynagraft group was better in amount of bone formation, although not statistically significant. Zitzmann et al. investigated the healing of alveolar ridge defects augmented with cancellous bovine bone mineral and they found Bio-Oss an appropriate material for ridge reconstruction in humans [23]. On the contrary, Pinholt et al. implanted Bio-Oss subperiosteally for ridge construction purposes and heterotopically in the abdominal muscles of rats. In the microscopic evaluation, they found no osteoinduction or osteoconduction and a foreign body reaction was observed around the material [27].

In their previous investigation, the authors of this study concluded that using DBBM alone in the bone defects of rabbit calvaria did not increase bone regeneration compared to the control group. They attributed this result to their inadequate sample size. They also showed that osteogenesis in rabbit calvarium may improve by adding PRGF to DBBM. This can be related to osteoinductive properties of PRGF. It seems that PRGF accelerates the rate of degradation of the biomaterial [28]. In the current study, regarding the negligible inflammatory and foreign body reaction in histological analysis, we found both materials biocompatible. These characteristics were previously confirmed by Hammerle et al. [25], Slotte and Lundgren [26], and Khoshkhoone-
jad et al. [24] using Bio-Oss. While Callan et al., Babbush et al., and Clokie et al. displayed similar characteristics about DynaGraft [1, 21, 22]. In the present study, we did not use barrier membranes to cover the defects in order to eliminate its confounding effect; therefore, we could evaluate the role of DynaGraft as a barrier membrane in addition to its other properties. In the systematic review performed by Khojasteh et al., membranes did not influence the amount of bone formation in the osseous defects [29]. Moreover, Khoshkhoonejad et al. demonstrated that using a membrane did not increase the amount of bone regeneration in their study [24]. It is worth mentioning that membranes lack enough mechanical strength and they collapse into the defect, they separate the periosteum from the bone, and their early degradation and displacement may also contribute to their inefficacy [30, 31].

Considering the amount of remaining biomaterial, there was no significant difference between DynaGraft and Bio-Oss groups. The authors of this study, in their previous experiment showed a considerable amount of residual material in defects with Bio-Oss that reduced significantly when PRGF was added [28]. Bio-Oss is a hydroxyapatite compound and it is expected to have a slower rate of absorption. Since our histological sections were prepared 4 weeks after the grafting procedure, this result could be different between groups if the samples were prepared after a longer period.

According to the type of bone formation, the majority of new bone consisted of type II in Bio-Oss and control groups; whereas, DynaGraft group mostly contained bone type III. The difference between groups was statistically significant. As we mentioned earlier in the present study, DynaGraft benefits from its osteoinductive and osteoconductive properties; it can accelerate bone regeneration while playing the role of a membrane. Our results were consistent with another study performed by Khoshkhoonejad et al., in which Bio-Oss group showed type II bone formation in all specimens after one month [24].

CONCLUSION
In the current study, considerable new bone formation was observed in both DBBM and DFDBA groups in comparison with the control group. The DynaGraft group showed slightly more bone formation compared to the Bio-Oss group. The presence of inflammation and residual biomaterial were negligible in all groups. Therefore, DynaGraft could be suggested for regeneration in bone defects.

REFERENCES
1- Clokie CM, Moghadam H, Jackson MT, Sandor GK. Closure of critical sized defects with allogenic and alloplastic bone substitutes. J Craniofac Surg. 2002 Jan;13(1):111-21; discussion 22-3.
2- Barboza EP, Duarte ME, Geolas L, Sorensen RG, Riedel GE, Wikesjo UM. Ridge augmentation following implantation of recombinant human bone morphogenetic protein-2 in the dog. J Periodontol. 2000 Mar;71(3):488-96.
3- Lee YM, Park YJ, Lee SJ, Ku Y, Han SB, Choi SM, et al. Tissue engineered bone formation using chitosan/tricalcium phosphate sponges. J Periodontol. 2000 Mar;71(3):410-7.
4- Shin SY, Park HN, Kim KH, Lee MH, Choi YS, Park YJ, et al. Biological evaluation of chitosan nanofiber membrane for guided bone regeneration. J Periodontol. 2005 Oct;76(10):1778-84.
5- Lundgren D, Nyman S, Mathisen T, Isaksson S, Klinge B. Guided bone regeneration of cranial defects, using biodegradable barriers: an experimental pilot study in the rabbit. J Craniomaxillofac Surg. 1992 Aug-Sep;20(6):257-60.
6- Retzepi M, Donos N. Guided Bone Regeneration: biological principle and therapeutic applications. Clin Oral Implants Res. 2010 Jun;21(6):567-76.
7- RA Hitti, DG Kerns. Guided Bone Regeneration in the Oral Cavity: A Review. Open Pathol J. 2011;5:33-45.
8- Buser D DK, Hirt HP, Schenk RK. Lateral ridge augmentation using autografts and barrier membranes: a clinical study with 40 partially edentulous patients. J Oral Maxillofac Surg. 1996;54(4):420-32.
9- Berglundh T, Lindhe J. Healing around implants placed in bone defects treated with Bio-Oss. An experimental study in the dog. Clin Oral Implants Res. 1997 Apr;8(2):117-24.
10- Hammerle CH, Schmid J, Olah AJ, Lang NP. A novel model system for the study of experimental guided bone formation in humans. Clin Oral Implants Res. 1996 Mar;7(1):38-47.
11- Babbush CA. The use of a new allograft material for osseous reconstruction associated with dental implants. Implant Dent. 1998;7(3):205-12.
12- Babbush CA. Histologic evaluation of human biopsies after dental augmentation with a demineralized bone matrix putty. Implant Dent. 2003;12(4):325-32.
13- Accorsi-Mendonca T, Conz MB, Barros TC, de Sena LA, Soares Gde A, Granjeiro JM. Physicochemical characterization of two deproteinized bovine xenografts. Braz Oral Res. 2008 Jan-Mar;22(1):5-10.
14- Requicha JF, Viegas CA, Munoz F, Azevedo JM, Leonor IB, Reis RL, et al. A Tissue Engineering Approach for Periodontal Regeneration Based on a Biodegradable Double-Layer Scaffold and Adipose-Derived Stem Cells. Tissue Eng Part A. 2014 Apr 22.
15-von Arx T, Buser D. Horizontal ridge augmentation using autogenous block grafts and the guided bone regeneration technique with collagen membranes: a clinical study with 42 patients. Clin Oral Implants Res. 2006 Aug;17(4):359-66.
16-Urban IA, Lozada JL, Jovanovic SA, Nagursky H, Nagy K. Vertical ridge augmentation with titanium-reinforced, dense-PTFE membranes and a combination of particulated autogenous bone and anorganic bovine bone-derived mineral: a prospective case series in 19 patients. Int J Oral Maxillofac Implants. 2014 Jan-Feb;29(1):185-93.
17- Shahriari S, Housemand B, Razavian H, Khazaei S, Abbas FM. Effect of the combination of enamel matrix derivatives and deproteinized bovine bone materials on bone formation in rabbits' calvarial defects. Dent Res J (Isfahan). 2012 Jul;9(4):422-6.
18- Tolstunov L, Chi J. Alveolar ridge augmentation: comparison of two socket graft materials in implant cases. Compend Contin Educ Dent. 2011 Nov-Dec;32(9):E146-55.
19- Hernandez M, Pette GA, Grenier A, Villanueva C, Lask E, Parker W. A clinical and histological comparison of two different bone augmentation materials in the atrophic pre-maxilla. Compend Contin Educ Dent. 2012 Feb;33(2):e26-32.
20- Rokn A, Moslemi N, Eslami B, Abadi HK, Paknejad M. Histologic Evaluation of Bone Healing Following Application of Anorganic Bovine Bone and beta-tricalcium Phosphate in Rabbit Calvaria. J Dent (Tehran). 2012 Winter;9(1):35-40.
21- Callan DP, Salkeld SL, Scarborough N. Histologic analysis of implant sites after grafting with demineralized bone matrix putty and sheets. Implant Dent. 2000;9(1):36-44.
22- Babbush CA. The use of a new allograft material for osseous reconstruction associated with dental implants. Implant Dent. 1998;7(3):205-12.
23-Zitzmann NU, Scharer P, Marinello CP, Schupbach P, Berglundh T. Alveolar ridge augmentation with Bio-Oss: a histologic study in humans. Int J Periodontics Restorative Dent. 2001 Jun;21(3):288-95.
24- Khoshkhoonejad AA, Miremadi AR, Rokn AR, Eslami B, Dehghan M, Kalbassi H. Effect of GBR in Combination with Deproteinized Bovine Bone Mineral on the
Healing of Calvarial Defects in Rabbits. J Dent (Tehran). 2006;3(2):77-86.
25- Hammerle CH, Olah AJ, Schmid J, Fluckiger L, Gogolewski S, Winkler JR, et al. The biological effect of natural bone mineral on bone neoformation on the rabbit skull. Clin Oral Implants Res. 1997 Jun;8(3):198-207.
26- Slotte C, Lundgren D. Augmentation of calvarial tissue using non-permeable silicone domes and bovine bone mineral. An experimental study in the rat. Clin Oral Implants Res. 1999 Dec;10(6):468-76.
27- Pinholt EM, Bang G, Haanaes HR. Alveolar ridge augmentation in rats by Bio-Oss. Scand J Dent Res. 1991 Apr;99(2):154-61.
28- Paknejad M, Shayesteh YS, Yaghobee S, Shariat S, Dehghan M, Motahari P. Evaluation of the Effect of Plasma Rich in Growth Factors (PRGF) on Bone Regeneration. J Dent (Tehran). 2012 Winter;9(1):59-67.
29- Khojasteh A, Soheilifar S, Mohajerani H, Nowzari H. The effectiveness of barrier membranes on bone regeneration in localized bony defects: a systematic review. Int J Oral Maxillofac Implants. 2013 Jul-Aug;28(4):1076-89.
30- Schliephake H, Tavassol F, Gelinsky M, Dard M, Sewing A, Pompe W. Use of a mineralized collagen membrane to enhance repair of calvarial defects in rats. Clin Oral Implants Res. 2004 Feb;15(1):112-8.
31- Fugazzotto PA. GBR using bovine bone matrix and resorbable and nonresorbable membranes. Part 1: histologic results. Int J Periodontics Restorative Dent. 2003 Aug;23(4):361-9.