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# Evaluation of regeneration after the application of 2 types of deproteinized bovine bone mineral to alveolar bone defects in adult dogs

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## ABSTRACT

**Purpose:** The aim of this study was to evaluate the preclinical results of 2 types of commercially available deproteinized bovine bone mineral (DBBM) when applied to alveolar bone defects in dogs.
**Methods:** This study was conducted using 6 beagles. Alveolar defects in the mandible were formed and filled with 2 DBBMs produced by a similar procedure. Defects were randomly assigned to be filled using DBBM 1 or 2. All defects were covered with a collagen membrane and had a healing period of 12 weeks. After the dogs were sacrificed, histological, histomorphometric, and linear/volumetric analyses were performed.
**Results:** Both DBBM groups showed similar histological findings, demonstrating that bone remodeling had occurred and new bone had formed. The residual bone particles were surrounded by newly formed vital bone. In the histomorphometric analysis, the ratio of the area of vital bone and residual bone substitute in DBBM 2 (38.18% and 3.47%, respectively) was higher than that of DBBM 1 (33.74% and 3.41%, respectively), although the difference was not statistically significant. There were also no statistically significant differences between both groups in linear and volumetric analyses using micro-computed tomography scans and digitized images of dental casts.
**Conclusions:** In the present study, DBBM 1and 2, which were produced by similar processes, showed similar results in histological, histomorphometric, and volumetric analyses. Further studies are needed to identify more specific differences between the 2 DBBMs.

**Keywords:** Animal model; Biocompatible materials; Bone regeneration; Bone resorption; Bone substitute; Xenograft

## INTRODUCTION

Following tooth extraction, resorption of the bundle bone changes the alveolar ridge. During the healing phase, the horizontal dimensional change (29%–63%) is more prominent than

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**Conflict of Interest**
No potential conflict of interest relevant to this article was reported.

**Author Contributions**
Conceptualization: Jung-Tae Lee, Yoonsub Lee; Formal analysis: Jung-Tae Lee, Yoonsub Lee; Investigation: Dajung Lee; Methodology: Dajung Lee; Project administration: Sungtae Kim, Jin-soo Ahn; Writing - original draft: Dajung Lee; Writing - review & editing: Yoonsub Lee.

the vertical change (11%–22%) [1]. In particular, different portions of bundle bone contribute more to the resorption of the buccal bone area [2]. Alveolar bone loss is also considered to result from continuous mechanical overloading. Bone resorption interferes with the ideal position of implant placement. In particular, the 1- and 2-wall types of bone defects are disadvantageous for bone grafting and subsequent implant placement [3].

To compensate for these dimensional changes, guided bone regeneration (GBR), which uses bone graft material around a bony wall, has been introduced. Several studies have been conducted on GBR using various materials. Urban et al. suggested GBR (vertical and horizontal alveolar ridge augmentation) as a standard choice for providing bone support to dental implants [4]. Osteogenesis, osteoinduction, and osteoconduction are important properties of bone graft materials. Despite these properties, autogenous bone has the disadvantage of causing discomfort to the patient during the acquisition process [5]. As an alternative method, demineralized freeze-dried bone allograft (DFDBA) has the drawbacks of bone resorption and inflammation in clinical use [6]. Buser et al. [7] reported that in the initial healing phase, an autogenous bone graft was superior to other bone substitutes such as DFDBA, tricalcium phosphate, and hydroxyapatite. Bovine- and porcine-based xenografts and synthetic bone graft materials have also been introduced. Owing to the similarity of histologic shape and collagen composition, xenografts from mammal species have been recommended as bone substitutes [8]. Thermo-chemical procedures remove organic components from bones to create a mineral scaffold with residual collagen [9].

Deproteinized bovine bone mineral (DBBM) is widely used in clinical practice. It contains 100% inorganic bovine bone and is a safe and biocompatible material with osteoconductive ability [10]. DBBM has porous particles (particle size: 0.25–2 mm) and it is produced by removing the organic components at high temperatures to minimize the immune response. After removing the organic components, DBBM has a similar architecture to human cancellous bone in terms of porosity, chemical composition, and crystallite. Furthermore, DBBM provides a large surface area and promotes the growth of blood vessels and osteogenic cells, resulting in increased bone formation [11,12]. A previous study reported that DBBM particles exhibited favorable results during a 7-month healing period after grafting in the extraction sockets [13]. DBBM is used for the recovery of both the extraction socket and periodontal tissue. Nine months after treatment of a single periodontal infrabony defect with DBBM, a decrease in probing depth and an increase in clinical attachment level were observed [14,15]. Nevins et al. also reported that DBBM could maintain the alveolar ridge and result in favorable healing of soft tissue 4–6 months after implant placement [14]. A previous study reported a 39% proportion of newly formed bone during sinus augmentation with DBBM [16]. However, the number of comparative studies among DBBMs still is not sufficient. Manufacturers advertise that their bovine bone graft materials have a similar shape and composition to human bone. However, it is not easy for clinicians to know the exact shape and composition of DBBMs on the market. This is because, in most cases, only the manufacturer has the exact information on the DBBM composition.

Two types of DBBM were analyzed in this study; one is used in the clinical field, while the other is produced through low-temperature processing with an extremely low heating rate. The aim of this study was to evaluate the preclinical results of these 2 commercially available DBBMs on alveolar bone defects in the lower jaw of dogs.


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## MATERIALS AND METHODS

### Experimental preparation

This *in vivo* preclinical study was designed according to the modified Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for preclinical research [17].

Six adult beagle dogs (age: 15 months; weight: 10–15 kg) with fully erupted permanent dentition were included in the present study. The study design and protocols were approved by the Institutional Animal Care and Use Committee (CRONEX, Seoul, Korea; approval no. 202003001). The timeline of this study is shown in **Figure 1**.

*Sample size*

The sample size was calculated based on estimations from a previous similar study by Netto et al. [18]. The type I error was set at 0.05 and the sample size was calculated to achieve 90% statistical power. The calculation was done using G*Power software version 3.1 [19].

*Operation 1: induction of alveolar defects*

All surgical procedures were performed under general anesthesia. General anesthesia was induced through intravenous injection with a 1:1 mixture of 2 mL/10 kg Zoletil 50 (tiletamine hydrochloride + zolazepam hydrochloride; Virbac S.A., Carros, France) and Rompun (xylazine hydrochloride; Bayer, Leverkusen, Germany). Additional respiratory anesthesia was provided by administering a 2:1 Terrell solution (isoflurane; Piramal Critical Care Inc., Seoul, Korea) and oxygen. Scaling was performed before surgery.

In this study, defects were made in the lower jaw of dogs. Local anesthesia was induced by injecting 1:100,000 epinephrine-containing lidocaine (Huons, Seongnam, Korea). An intracrevicular incision was performed on the surgical sites: the mandibular second, third, and fourth premolar areas (P2, P3, and P4).

Hemi-sections of 3 premolars (P2, P3, and P4) were made using a rotary dental instrument. The mesial roots of P2, P3, and P4 were extracted with extraction forceps. Root canal therapy of the P2, P3, and P4 distal roots, including pulp removal with a 25-mm #15 K-file (MANI,

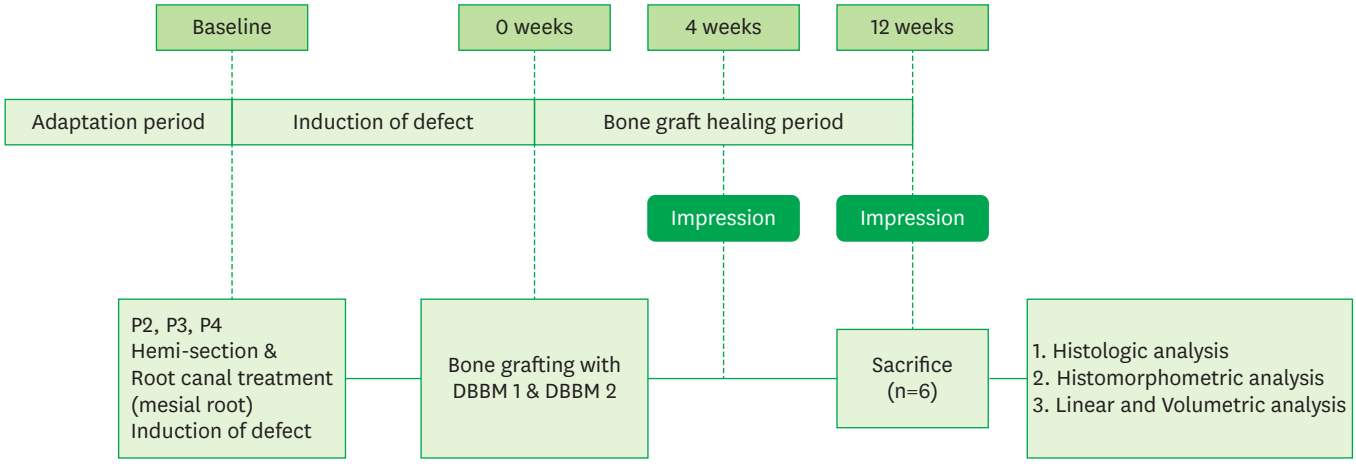


**Figure 1.** Flow diagram of the study.
DBBM: deproteinized bovine bone mineral.


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Inc., Utsunomiya, Tochigi, Japan), was conducted. A gutta-percha master cone was applied to the root canal through cold condensation. Additional accessory cones with root canal sealer (AH Plus; Dentsply, DeTrey GmbH, Konstanz, Germany) were placed around the master cone. The treated root canal was sealed with a dental sealing material (IRM; Dentsply Sirona, Milford, DE, USA). An alveolar defect (5×5×5 mm) was surgically created on the extracted sites of each premolar according to previous studies [20,21] (**Figure 2A and B**). After creation of the alveolar defect, the flap was sutured with 4-0 Vicryl (Ethicon Inc., Somerville, NJ, USA).

*Operation 2: guided bone regeneration 4 weeks after alveolar defect induction*

GBR was performed 4 weeks after the creation of the alveolar defect. Bone substitutes and resorbable membranes were arranged into the following 2 groups on the alveolar bony defects of both lower sides.

DBBM 1 (n=12): InterOss®, SigmaGraft Inc., Fullerton, CA, USA
DBBM 2 (n=12): A-Oss®, Osstem Implant Co., Seoul, Korea
Collagen membrane: Bio-Gide®, Geistlich, Wolhusen, Switzerland

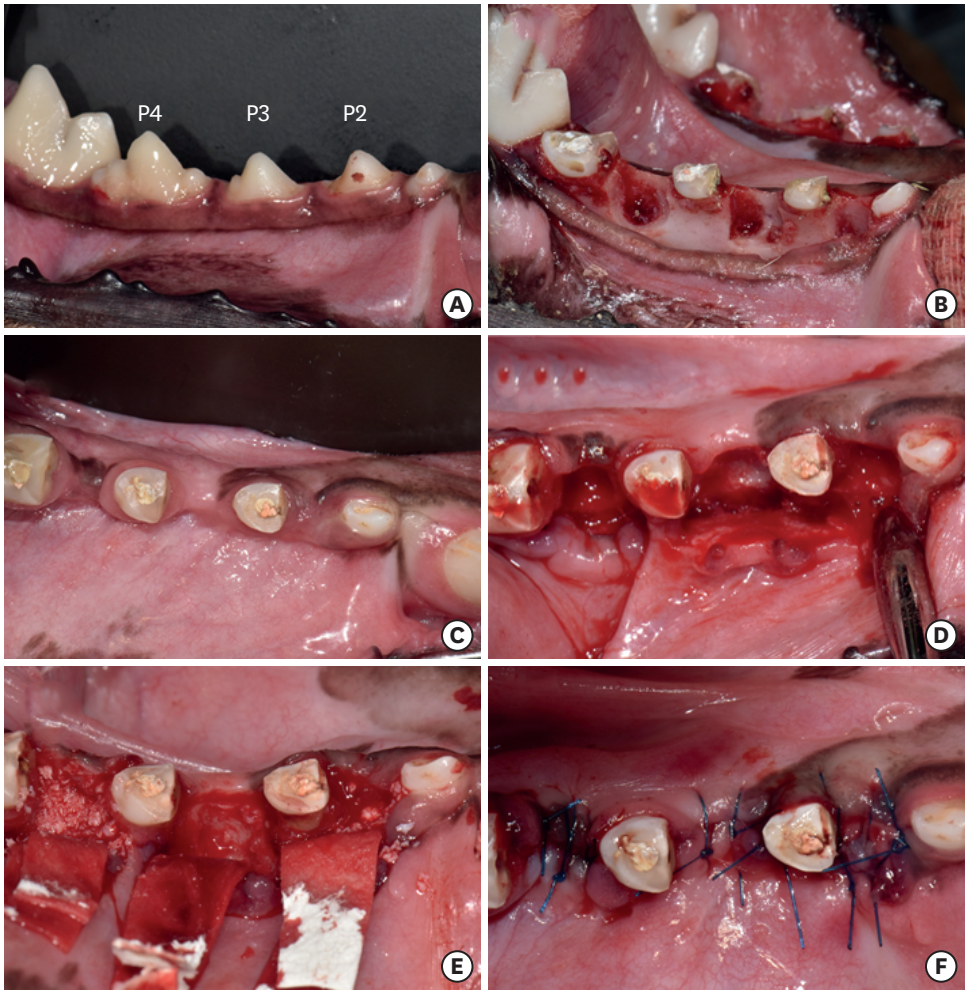


**Figure 2.** Clinical photographs of the experimental sites. (A) Mandibular surgical sites. P2: second premolar, P3: third premolar, P4: fourth premolar area. (B) Hemi-section, defect creation (mesial extraction site), and endodontic treatment (distal root). (C) Four weeks after defect creation. (D) Opened flap. (E) Bone graft material and resorbable membrane application. (F) Suture.


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The opened flap was sutured using the same suture material as in operation 1. After the operation, 0.2 mL/kg antibiotics (enrofloxacin, KOMI Biotril® 100 Injection; Komipharm Co. Ltd., Siheung, Korea) and 0.4 mg/kg analgesics (meloxicam, Metacam®; Labiana Life Science, S.A., Barcelona, Spain) were administered for 3 days. The sutures were removed after 10 days. During the healing period, chemical plaque control was performed using 0.2% chlorhexidine (Hexamedine; Bukwang Pharmaceutical, Seoul, Korea). At 12 weeks after surgery, the dogs were sacrificed using 1 mL of Succipharm (suxamethonium chloride hydrate [50 mg]; Komipharm Co. Ltd.), and the experimental sites were obtained and fixed in 10% buffered formalin (**Figure 2C-F**).

## Histological and histomorphometric analyses

The cross-sectioned specimens were embedded in acrylic resin (Technovit 7200 VLC; Heraeus Kulzer, Wehrheim, Germany). Each specimen was cut with a thickness of 45 μm. Goldner trichrome staining was performed for each specimen. A digital scanner (Pannoramic 250 Flash III; 3D HISTECH, Budapest, Hungary) was used for digitization.

Two image analysis programs—Case Viewer (3DHISTECH Kft., Budapest, Hungary) and ImageJ (Bethesda, Maryland, USA)—were used for histological and histomorphometric analyses. A region of interest (ROI: 5×5 mm defect area) was set on the histological images. The quality of the grafted area from each histologic specimen of both experiments was evaluated by quantifying the relative composition of the total augmented area (%) with respect to vital bone area (VBA), residual bone substitutes area (RBA), fibrovascular tissue area (FVA), and bone marrow (**Figure 3A**).

## Micro-computed tomography (CT) scanning

Micro-CT (SkyScan 1173; Bruker microCT, Kontich, Belgium) with a resolution of 24.9 μm (achieved using the scanner at 130 kV and 60 μA) was performed to analyze the fixed block specimens. The cross-section data were reconstructed with NRecon and Dataviewer (Bruker microCT) software to create 3-dimensional (3D) shapes. The CTAn software (Bruker microCT) was used to calculate the total volume of the augmented area (ROI: 5×5×5 mm) (**Figure 3B**).

## Linear and volumetric analyses

*Dental impression*

Dental impressions were obtained twice (at 1 month and 3 months post-surgery) using impression materials (Aquasil Ultra LV and Aquasil Ultra XLV; Dentsply DeTrey, Konstanz, Germany) and individual trays made by a 3D printer. Dental casts were poured out of dental stone (GC Fujirock type 4; GC Corporation, Tokyo, Japan) and digitized using a dental scanner (ZEISS COMET 5M, Oberkochen, Germany).

The scanned STL file was superimposed on the basis of static points (non-moving reference point: canine and first molar) using a 3D metrology problem (Geomagic Design X and Control X; 3D Systems, Rock Hill, SC, USA). The volumetric and linear measurements were performed in the overlapping state (**Figure 3C and D**).

*Linear measurements: buccal area*

The linear measurements in this study were performed according to the methods described in previous studies [22,23]. When the STL files were matched, a longitudinal slice that divided the ridge into 2 equal areas was made. Thereafter, the long axis of the residual distal root (P2, P3, and P4) was selected as a vertical line on the sectional image. The perpendicular


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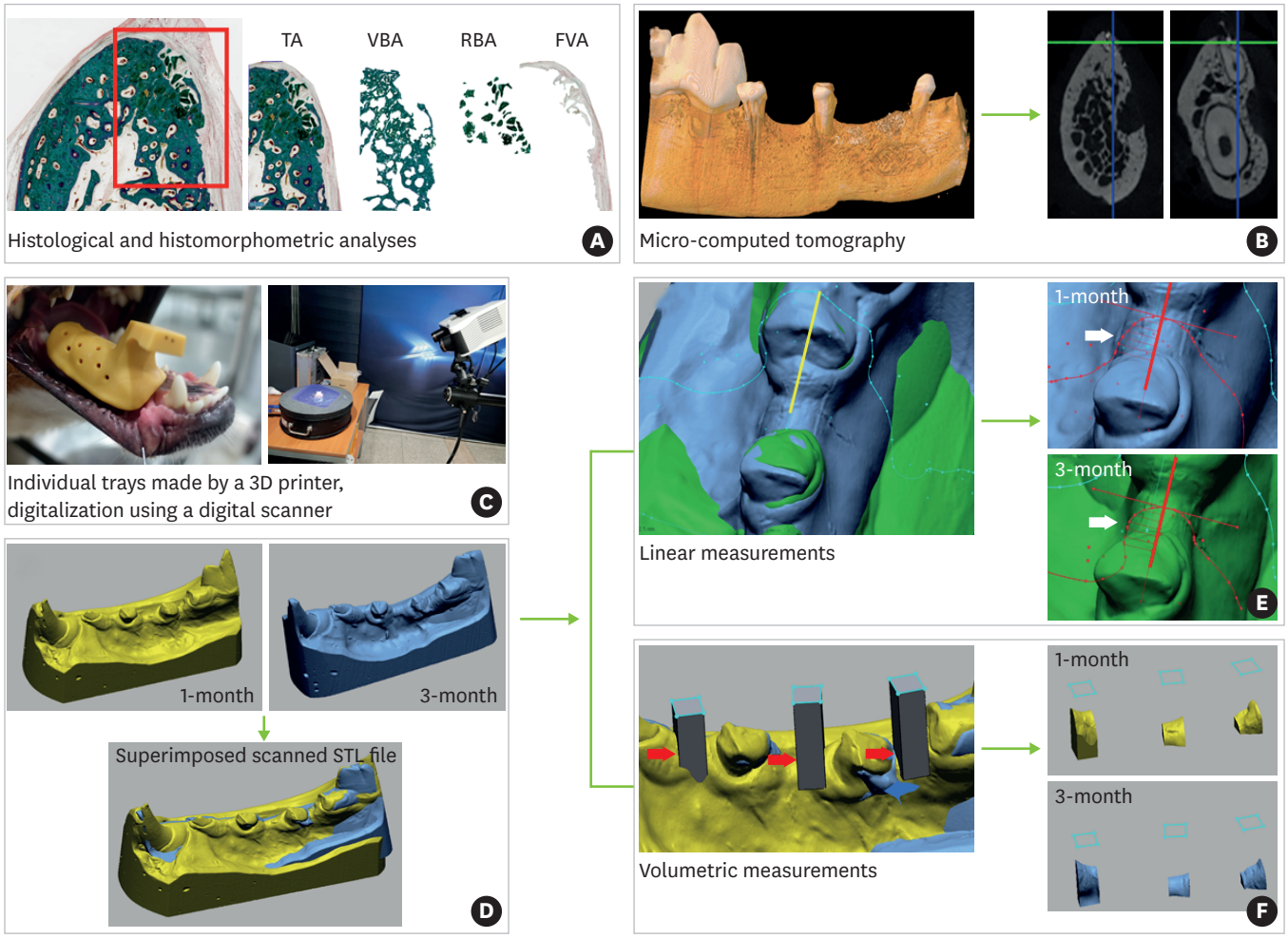


**Figure 3.** Schematic illustration of analyses (A) Histological and histomorphometric analyses. (B) Micro-CT. (C) Pre-fabricated individualized trays with a 3-dimensional printer; digitalized work of the master cast with a dental stone using dental scanner. (D) Scanned STL files (1 and 3 months), and superimposed files. (E) Linear measurements: long axis of the residual distal root (yellow line) → applying an imaginary line on the surgical site according to the long axis of residual distal root (red line) → setting perpendicular lines at 0.5, 1.0, 1.5, and 2.0 mm from the alveolar crest → measuring the distance to the buccal surface (white arrows). (F) A virtual block with a ROI (2.5×3.0 mm) was created including the surgical area (red arrows). Each volume of the 2 periods overlapping with this virtual block was measured.
CT: computed tomography, ROI: region of interest, VBA: vital bone area, RBA: residual bone substitute area, FVA: fibrovascular tissue area, TA: total area.

lines were drawn 0.5, 1.0, 1.5, and 2.0 mm from the most coronal area of the alveolar ridge. From the vertical line to the buccal contours, linear measurements were performed on each perpendicular line (0.5, 1.0, 1.5, and 2.0 mm) (**Figure 3E**).

*Volumetric measurements: buccal area*

An ROI (2.5×3.0 mm) was selected on the buccal area of each surgical site (mid-crestal line to the buccal aspect) from the top view of the merged images. A virtual block with the ROI dimensions was created including the surgical areas. Each volume from 2 periods (1 month and 3 months later) overlapping with this virtual block was measured. The amount of change between these 2 periods was calculated using the method of a previous study [24] (**Figure 3F**).

## Statistical analysis

Statistical analysis was performed using the SPSS version 22.0 (IBM Corp., Armonk, NY, USA). The normality of the distribution was confirmed by the Shapiro–Wilk test. The


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independent *t*-test or Mann–Whitney test was used for comparisons between 2 groups (DBBM 1 and DBBM 2) for linear, volumetric, and histomorphometric analyses, if the parameters followed a normal distribution. To evaluate differences between the periods (1 and 3 months later), the paired t-test or Wilcoxon signed-rank test was conducted. The threshold for statistical significance was 5%.

## RESULTS

### Histological and histomorphometric analyses

In the histological analysis, no adverse inflammation associated with the bone graft material was observed in any samples. The resorbable membranes were well positioned over the entire ROI (5×5 mm; defect area on the histological image). In both groups, bone remodeling occurred and new bone formed from the basal bone. Newly formed mature bone was observed between the particles. It is difficult to distinguish new bone from basal bone based on histologic images. Therefore, this category was referred to as "vital bone." The dome-shaped augmented area was well maintained, and residual bone graft particles were also maintained in both groups (**Figure 4**).

A histomorphometric analysis was performed to quantify the amount of residual bone graft material and the retained volume. As a result of evaluating the ratio of VBA, RBA, and FVA to the total area of the ROI, the VBA and RBA ratios in DBBM 2 (38.18% and 3.47%, respectively)

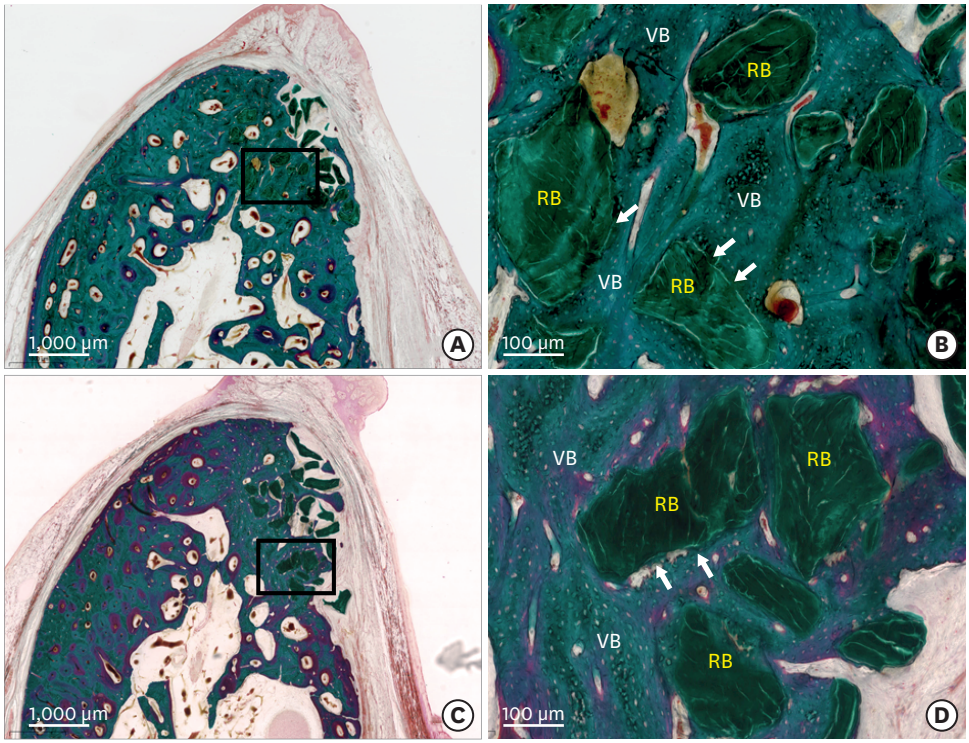


**Figure 4.** Histological staining in the DBBM 1 and 2 groups. (A and B) DBBM 1. (C and D) DBBM 2. The dome-shaped augmented area and residual bone graft particles were maintained in both groups. Newly formed mature bone was observed between the particles in both groups (white arrows). The scale bar represents 1,000 μm (A, C) and 100 μm (B, D).
DBBM: deproteinized bovine bone mineral, VB: vital bone, RB: residual bone substitute.


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**Table 1.** Areal measurements of the histomorphometric analysis (%)

| Group | VBA/TA | RBA/TA | FVA/TA | Bone marrow/TA |
|---|---|---|---|---|
| DBBM 1 | 33.74±10.85 | 3.41±3.97 | 23.31±2.49 | 39.54±7.90 |
| DBBM 2 | 38.18±9.99 | 3.47±4.19 | 19.68±2.46 | 38.66±9.55 |

Values are expressed as mean ± standard deviation.
DBBM: deproteinized bovine bone mineral, VBA: vital bone area, TA: total area, RBA: residual bone substitute area, FVA: fibrovascular tissue area.

were higher than those of DBBM 1 (33.74% and 3.41%, respectively). DBBM 1 showed higher values of FVA and bone marrow (23.31% and 39.54%, respectively) than DBBM 2 (19.68% and 38.66%, respectively), as presented in **Table 1**. There was no significant difference between the 2 groups.

## Micro-CT scanning

DBBM 1 occupied 40.44% and DBBM 2 accounted for 39.01% of the bone volume of the total ROI. Although DBBM 1 showed a slightly higher percentage of bone volume, the difference was not statistically significant (**Table 2**).

## Linear and volumetric analyses

Linear measurements conducted at 1 month and 3 months after surgery showed that the values significantly decreased after 3 months at all measurement points (i.e., 0.5, 1.0, 1.5, and 2.0 mm). The linear change between 1 and 3 months was smaller in DBBM 1 than in DBBM 2. However, the intergroup difference was only significant at 1.5 mm (**Table 3**).

The volume change of DBBM 1 between 1 month and 3 months (29.64%±17.83%) was slightly higher than that of DBBM 2 (29.42%±15.27%); however, this change was statistically insignificant (**Table 4**).

**Table 2.** Volumetric analysis of micro-CT (%)

| Group | Bone volume ratio |
|---|---|
| DBBM 1 | 40.44±11.71 |
| DBBM 2 | 39.01±6.63 |

Values are expressed as mean ± standard deviation.
CT: computed tomography, DBBM: deproteinized bovine bone mineral.

**Table 3.** Linear changes according to observation period after surgery

| Group | 1 month later | 3 months later | Change between 1 month and 3 months later |
|---|---|---|---|
| DBBM 1 | | | |
| A (0.5 mm) | 1.22±0.29 | 1.06±0.23[b] | 0.16±0.18 |
| B (1.0 mm) | 1.79±0.33 | 1.55±0.18[b] | 0.23±0.21 |
| C (1.5 mm) | 2.29±0.51 | 1.92±0.32[b] | 0.37±0.31[a] |
| D (2.0 mm) | 2.71±0.75 | 2.18±0.54[b] | 0.52±0.39 |
| DBBM 2 | | | |
| A (0.5 mm) | 1.24±0.30 | 0.95±0.18[b] | 0.29±0.24 |
| B (1.0 mm) | 1.73±0.26 | 1.48±0.27[b] | 0.25±0.34 |
| C (1.5 mm) | 2.27±0.53 | 1.70±0.41[b] | 0.57±0.37 |
| D (2.0 mm) | 2.74±0.74 | 2.09±0.67[b] | 0.65±0.41 |

Values are expressed as mean ± standard deviation.
DBBM: deproteinized bovine bone mineral.
[a]Significantly different between the 2 groups (the statistical significance level was 5%, $P<0.05$).
[b]Significantly different from 1 month later (the statistical significance level was 5%, $P<0.05$).


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Table 4. Volumetric analysis of scanned images (%)

| Group | Change between 1 month and 3 months later on scanned images of dental casts |
|---|---|
| DBBM 1 | 29.64±17.83 |
| DBBM 2 | 29.42±15.27 |

Values are expressed as mean ± standard deviation.
DBBM: deproteinized bovine bone mineral.

## DISCUSSION

An *in vivo* test was performed on beagle dog defects to determine the predictable bone tissue reaction, stable biocompatibility, and safety of 2 DBBM materials. During the follow-up period of 3 months, no inflammatory reaction was observed. The findings for sequential healing were similar between the 2 DBBMs for RBA, FVA, and the bone marrow area in the histological analysis. The VBA ratio of DBBM 2 was higher than that of DBBM 1. The linear change between 1 and 3 months was smaller in DBBM 1 than in DBBM 2. However, there were no significant differences between the 2 groups in the histologic and histomorphometric analyses except for the measurement point value at 1.5mm in DBBM 1. The volume change was also almost the same in both DBBMs (DBBM 1: 29.64%, DBBM 2: 29.42%). DBBM has osteoconductive properties and provides a scaffold for vital bone formation. Khalid et al. reported that new bone formation using bovine bone was better than that with other synthetic bone materials (33% and 13%–28%, respectively). In this study, the 2 DBBMs showed similar vital bone formation to a previous study (DBBM 1: 34.25% and DBBM 2: 38.59%, respectively) [25].

In a previous study, DBBMs showed a slow degradation rate and relatively low bone regeneration capacity [26]. Non-mineralized bone substitutes promote osteoblast differentiation by releasing bone morphogenic proteins. However, these non-mineralized bone materials have inferior mechanical properties. In contrast, DBBM, which is mineralized, has higher mechanical stability [27]. Bio-Oss, a bovine bone substitute, had significantly slower resorption than other bone graft materials [28]. Another study suggested that no significant resorption of Bio-Oss was observed up to 6 years [29]. In addition, previous studies explained that this delayed resorption of residual bone could apply in clinical use as a scaffold that compensates for autogenous bone resorption [30,31]. The bone volume with DBBM 1 and 2 (40.4% and 39.0%, respectively) was determined by observing the 3D structure of vital bone using a micro-CT scanner aimed at the ROI. There were no significant differences in most linear and volumetric parameters between the 2 groups. This finding suggested that the overall bone volume of both groups was similar, and the vital bone ratio in DBBM 2 was higher than that in DBBM 1. It can be considered that the structure and porosity (pore and interconnection size) of DBBMs may have affected the results of this study. After bone grafting, bone healing might have occurred by creeping substitution in both groups. Bone graft substitutes might begin to rebuild by osteoclastic resorption and the creation of new vascular channels, followed by osteoblastic activity for new bone formation in this study [27,32]. However, further studies involving immunohistochemical analysis to observe the levels of osteoprotegerin and receptor activator of nuclear factor-κB (RANK) and its ligand RANKL are required to determine the exact mechanism [33].

The clinical results of DBBM could be different depending on the composition of components, the architectural shape, and the manufacturing process. Therefore, several studies have compared the properties of different DBBMs. A previous study comparing sintered and non-sintered bovine bone blocks demonstrated that the sintered bovine bone


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block exhibited significantly less bone resorption than the non-sintered one [34]. Kim et al. [35] conducted a study on 2 particulate DBBMs (Bio-Oss vs. InterOss) in a dog model and reported that the materials had similar bone regeneration properties. Park [36] also described the basic characteristics of 3 commercially available bone substitutes: Bio-Oss, BBP (Oscotec, Seoul, Korea), and Osteograft (DIZG, Berlin, Germany). BBP showed a relatively large amount of residual protein, indicating that this material can cause inflammation [36]. Previous studies demonstrated that the maintenance of the graft material mainly depends on several factors, such as the porosity of the particles, the surface area, and the purity of the material [25,37]. In previous studies, bovine bone substitutes were mainly produced at low (<450°C) or high (>450°C) temperatures. Low-temperature bovine bone has large granules and high porosity. It allows better adherence of osteoblasts and the persistence of protein structures. On the contrary, high-temperature bovine bone provides relatively low porosity and small granules with a low possibility of residual protein [38,39]. Kübler et al. [39] found that high-temperature bovine bone showed higher proliferation and differentiation rates of osteoblasts than low-temperature bovine bone. For this reason, the relatively smooth surface of the granules did not enable adherence of the osteoblasts. In this study, both DBBMs were produced by a low-temperature process. The similarity of the manufacturing process of DBBM 1 and 2 may explain the lack of significant differences in the results.

Scanned images were involved in the histomorphometric and volumetric analyses. This technique is effective for measuring alterations of soft and hard tissues in the oral cavity. Scanned images are easy to obtain from subjects with alginate impressions. This method was used in previous studies. Sanz Martin et al. [40] evaluated the volumetric changes after implant placement by analyzing the scanned cast model with software. There are limitations of scanning casts, such as 1) errors during the impression procedure, 2) the limited accuracy of reproducing soft tissue, 3) discomfort, and 4) storage requirements. In this study, the linear analysis was conducted from the alveolar crest to 2.0 mm. The reason for this was that the soft tissue of the dogs' vestibule may affect the impression procedure at a distance of more than 2.0 mm. It could be necessary to use an intra-oral scanner in further studies.

This study has a few limitations. Since there are individual differences between animals and humans, it is necessary to evaluate the efficacy of DBBMs through long-term clinical trials. There were insufficient data on the histological changes over time. Further research comparing these DBBMs with high-temperature bovine bone is needed. In addition, this study lacked a comparison with Bio-Oss, which is the gold standard of DBBM. These limitations should be addressed in following studies.

Within the limitations of this study, DBBM 1 and 2 showed similar results through *in vivo* testing. Based on the results of this study, it can be considered that bovine bone materials derived through low-temperature processing may have resistance to resorption. These findings might be helpful for the clinical application of GBR and manufacturing of more advanced bone graft materials in the future.

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