Original

Evaluation of Guided Bone Regeneration Using the Bone Substitute Bio-Oss® and a Collagen Membrane in a Rat Cranial Bone Defect Model

Shin Kasuya¹⁾, Shihoko Inui¹⁾, Nahoko Kato-Kogoe¹⁾, Michi Omori¹⁾, Kayoko Yamamoto¹⁾, Kazuya Inoue¹⁾, Yuichi Ito¹⁾,
Yoichiro Nakajima¹⁾, Azumi Hirata²⁾ and Takaaki Ueno¹⁾

¹⁾ Division of Medicine for Function and Morphology of Sensor Organs, Dentistry and Oral Surgery, Osaka Medical College, Osaka, Japan

²⁾ Department of Anatomy and Cell Biology, Osaka Medical College, Osaka, Japan

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Abstract: The value of using the xenogeneic bone substitute Bio-Oss® and a collagen membrane for guided bone regeneration (GBR) was evaluated. Five-millimeter bone defects were created in the cranial bones of 15, 15-week-old, male Sprague-Dawley rats, five of which were left unfilled (Group A), five were filled with Bio-Oss® (Group B), and five were filled with Bio-Oss® and a collagen membrane (Group C). They were evaluated by micro-computed tomography (micro-CT) Weeks 8 after bone defect creation. The animals were euthanized in Week 8, and tissue samples were taken. The sampled tissue was evaluated by the Kawamoto technique for preparing nondecalcified frozen sections. On micro-CT, radiopacities in the bone defects were evident in Groups B and C, but not in Group A. The amount of radiopacity tended to be greater in Group C than in Group B. Histologically, no new bone formation was observed in Group A. In Groups B and C, new bone formation around the Bio-Oss® was apparent and some osteoblastic cells were observed along the new bone. The Bio-Oss® was not absorbed up to Week 8, suggesting that it may be a potential bone regeneration scaffold. The combined use of a collagen membrane anchored the Bio-Oss® in close contact with the cranial bone, suggesting that it may help create a favorable environment for bone formation. The combination of Bio-Oss® and a collagen membrane in GBR may be useful for bone defect regeneration.

Key words: Bio-Oss®, Guided bone regeneration, Collagen membrane, Kawamoto technique, Micro-computed tomography

Introduction

Prosthetic treatment with dental implants has recently been increasing in popularity for the replacement of missing teeth. Compared with removable dentures or bridges, it provides more satisfactory mastication and occlusion, and the number of patients requesting implant treatment is rapidly increasing. However, this treatment cannot be performed if the dental implant insertion site lacks sufficient bone. The best way to resolving this problem is to carry out guided bone regeneration (GBR) prior to implant treatment¹⁻³⁾. The gold standard for osteoplasty is autologous bone grafting, using bone harvested from inside or outside the mouth as a graft⁴⁾.

However, this has the disadvantages of requiring two-stage surgery, postoperative impairment of the donor site, limitations on the amount of bone that can be harvested, and rapid resorption of autologous bone. Bone substitutes for use instead of autologous bone have been developed with the aim of overcoming these problems, but no ideal bone substitute for use in clinical osteoplasty currently exists.

Bio-Oss® is a xenogeneic bone substitute with a biological composition and microstructure resembling those of human bone. It has been found to possess high osteoinductive potential^{5,6)}, and there are great expectations for its clinical application in GBR. Alveolar bone, where GBR is performed, does not have a flat structure but is irregularly shaped, meaning that bone substitutes may fall out or fail to adhere closely to alveolar bone. Collagen membrane is therefore

sometimes used as a barrier membrane at osteoplasty sites. However, few studies have addressed the histological efficacy of the combined use of a collagen membrane and Bio-Oss® filling *in vivo*.

In this study, the bone regeneration process when the xenogeneic bone substitute Bio-Oss® was used in combination with a collagen membrane was evaluated in a rat cranial bone defect model. The evaluation methods used were micro-computed tomography (micro-CT)^{7,8)}, a non-invasive method of analyzing bone morphology and structure within a short time, and the Kawamoto technique of rapid observation of hard tissue as nondecalcified frozen sections, with no possibility of the absorption or loss of hard tissue⁹⁻¹¹⁾.

Materials and Methods

Animal examination

Fifteen, 15-week-old, male Sprague-Dawley (SD) rats of body weight 283.7-305.8 g were divided into three groups: in Group A (n = 5), bone defects were left unfilled; in Group B (n = 5), they were filled with Bio-Oss® (Geistlich, Wolhusen, Switzerland); and in Group C (n = 5) they were filled with Bio-Oss® and a collagen membrane (Zimmer, Carlsbad, CA, USA). Each group underwent micro-CT in Week 8 after bone defect creation, and tissue samples were collected in Week 8.

The Institutional Animal Care and Use Committee of Osaka Medical College approved all research protocols (approval ID: 27110), including surgical procedures and animal care.

Surgical method

Inhalation anesthesia was induced with isoflurane (Forane® inhalation anesthetic, ABBVIE, Tokyo, Japan), the skin of the

Correspondence to: Dr Shin Kasuya, Division of Medicine for Function and Morphology of Sensor Organs, Dentistry and Oral Surgery, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki City, Osaka, 569-8686 Japan; Tel: +81726846438; Fax: +81726846833; E-mail: ora086@osaka-med.ac.jp

Figure 1. Methods of surgery in the present study. A: A tissue punch is used to make a 5mm-diameter bone defect. B: Calvarial bone defect group C: Bio-Oss® (arrow head) filling group. D: Bio-Oss® with a collagen membrane (arrow head) filling group. Bars=5mm

calvarium was thoroughly disinfected, and local anesthesia was performed with 2% xylocaine (containing epinephrine 1:80,000). A sagittal incision was made on the left side of the calvarium with a no. 15 scalpel, and the periosteum was detached to expose the cranial bone surface. A biopsy punch (Kai Medical, Tokyo, Japan) was then used to mark a circle 5 mm in diameter, and this mark was used to create a cylindrical bone defect reaching the cerebral dura mater using a dental steel bur (Maillefer steel bur round, Densply, Tokyo, Japan) (Fig. 1A). The defect was thoroughly washed, and the animal was allocated to Group A (Fig. 1B), Group B (Fig. 1C), or Group C (Fig. 1D), after which the periosteum was sutured with 4-0 Vicryl (Ethicon, Cincinnati, OH, USA), and the skin was sutured with 3-0 silk suture (Mani®, Tochigi, Japan). In Week 8, the animals were euthanized, a skin incision was made, and the cranial bone was excised en bloc together with the periosteum.

Micro-CT analysis

A LaTheta LCT-200 micro-CT scanner for experimental animals (Hitachi-Aloka Medical Systems, Tokyo, Japan) was used for micro-CT scanning. This device consists of a desk-top micro-CT scanner and a computer workstation, and the tissue or animal to be examined is placed in the holder provided with the scanner.

SD rats placed under isoflurane inhalation anesthesia were immobilized in the holder, and scanning was performed under scanning conditions of tube voltage 50 kV, tube current 0.5 mA, resolution 80 $\mu\text{m} \times 80 \mu\text{m}$, and 80 $\mu\text{m}/\text{voxel}$. Scanning was performed in the horizontal, coronal, and sagittal planes, and observations were made from the surface of the 5-mm cranial bone defect to the surface of the cerebral dura mater. Scans were performed 8 weeks after bone defect creation. From these data, threshold values were standardized, and the volumes of the radiopacities in the observed regions were measured.

The image reconstruction software VG Studio MAX 2.2 (Volume

Figure 2. Micro-computed tomography (CT) images in the midline area of each defect. A: Calvarial bone defect group. B: Bio-Oss® filling group. C: Bio-Oss® with a collagen membrane filling group. There is no opacity in the defect area of the control group (A). There is opacity in the defect area of the Bio-Oss® filling group and the Bio-Oss® with a collagen membrane filling group (B, C). The arrows show the defect areas. Bars=1000 μm .

Graphics, Heidelberg, Germany) was used for volume rendering and to construct three-dimensional (3D) images from there slice data.

Histological analysis

The tissue samples were fixed by immersion in 10% neutral buffered formalin and washed in water, after which frozen blocks were prepared using cryoembedding medium (Super Cryoembedding Medium, Section-Lab Co. Ltd., Hiroshima, Japan) and hexane (-80°C). Nondecalfied frozen thin sections, 3- μm -thick, were prepared from the frozen blocks using a CM3050S cryostat (Leica, Tokyo, Japan) with a tungsten carbide blade (Leica Microsystems, Section-Lab Co. Ltd.) and a special adhesive film (Cryofilm type 2C (9)®; Section-Lab Co. Ltd.). The sections were washed in water and hematoxylin and eosin (H-E) staining was carried out.

Statistical analysis

The statistical software used was GraphPad Prism7 (USACO, Tokyo, Japan). Significance was tested by one-factor analysis of variance (ANOVA) for intergroup comparisons, followed by Dunnett's test for multiple comparisons. Statistical values are presented as means $\pm$ standard deviations.

Results

The state of bone defect regeneration was observed by analyzing

Figure 3 . Three-dimensional (3D) images. A: Calvarial bone defect group. B: Bio-Oss® filling group. C: Bio-Oss® with a collagen membrane filling group. There is no opacity in the defect area of the control group (A). There is opacity in the defect area of the Bio-Oss® filling group and the Bio-Oss® with a collagen membrane filling group (B, C). The arrow heads show the defect areas. Bars=1000µm.

Figure 4. The hard tissue volume in the bone defect area. The volumes of hard tissue in the observed regions are measured in each group. * P=0.074, ** P<0.05 compared with the volume of hard tissue of control.

radiopacities on micro-CT images.

As shown in Fig. 2, although no radiopacities were evident in the bone defects of the control group, radiopacity formation did occur from the cut edges of the cranial bone. Granular radiopacities were observed in the bone defects that were filled with Bio-Oss®. Radiopacity formation from the cut edges of the cranial bone was also present. Granular radiopacities were also observed in the bone defects that were filled with Bio-Oss® and a collagen membrane, and radiopacity formation from the cut edges of the cranial bone was also present. A comparison of the defects filled with Bio-Oss® and those filled with Bio-Oss® and a collagen membrane found that there was a tendency for more granular radiopacities to be present in those filled with Bio-Oss® and a collagen membrane.

Three-dimensional reconstruction was also used to observe and analyze the state of bone defect regeneration in three dimensions. As shown in Fig. 3, bone regeneration from around the defect was evident in the control group. The defects filled with Bio-Oss® and those filled with Bio-Oss® and collagen membranes were full of granular radiopacities. Three-dimensional observations showed that these granular radiopacities tended to occupy a wider extent of the defects filled with Bio-Oss® and a collagen membrane compared with the defects filled with Bio-Oss®.

The volumes of the radiopacities in the defect areas were $0.0246 \pm 0.0031 \text{ cm}^3$ in the Bio-Oss® filling group and $0.0301 \pm 0.0040 \text{ cm}^3$ in the Bio-Oss® with a collagen membrane filling group. (Fig. 4).

H-E staining of samples from the control group did not show any hard

Figure 5 . Histological pictures of the bone defects in each group. A: Calvarial bone defect group. B: Bio-Oss® filling group. C: Bio-Oss® with a collagen membrane filling group (CB: Calvarial bone, Black arrow heads: newly formed bone, White arrow heads: defect area). Cross-sectional images through the center points of the bone defect areas. Bars=1000µm.

tissue formation in the defects. H-E staining of samples from the defects filled with Bio-Oss® showed osteoblastic cells in some areas of tissue

with a trabecular architecture surrounding the Bio-Oss®. H-E staining of samples from the defects filled with Bio-Oss® and a collagen membrane also showed osteoblastic cells in some areas of tissue with a trabecular architecture surrounding the Bio-Oss®. New bone formation was only observed on the periostium side of the central areas of the defects filled with Bio-Oss® and a collagen membrane. Some osteoblastic cells were observed around new bone which contacts Bio-Oss®. The amount of Bio-Oss® remaining was clearly greater in the defects filled with Bio-Oss® and a collagen membrane. Histological observations showed more new bone formation and osteoblastic cells in defects filled with Bio-Oss® and a collagen membrane than in those filled with Bio-Oss® (Fig. 5)

Discussion

Rapid advances in implant treatment mean that it is now increasingly popular as a safe, reliable method of treatment. The number of patients with periodontal disease is continuing to increase, and the shift to an aging society is exacerbating this trend, meaning that, even with optimal prosthetic treatment, an increasing number of patients can be expected to suffer from alveolar bone resorption as a result of tooth loss. This will lead to more patients requiring GBR prior to implant treatment. GBR techniques that are simple to perform, are not stressful for patients, and provide sufficient alveolar bone with less patient burden and a simple procedure.

Conventionally, autologous bone has been used for GBR, but its disadvantages include postoperative damage to the harvesting site and rapid resorption of autologous bone. Hydroxyapatite, beta-tricalcium phosphate (β -TCP), and other biocompatible materials developed as bone substitutes are now in clinical use, but their success rates are unsatisfactory¹²⁾.

In this study, the effect of GBR using Bio-Oss® and a collagen membrane on bone regeneration, evaluated by radiological analysis using micro-CT and histological examination using the nondecalfied frozen section technique, was investigated. It was found that the volume of hard tissue was greater when using Bio-Oss® in combination with than without a collagen membrane in a rat cranial bone defect model after 8 weeks. The combination of Bio-Oss® and a collagen membrane may be useful for bone defect regeneration.

The performance of micro-CT has improved in recent years, and, since it offers a non-invasive, rapid method of observing bone structure, it is used for studies of hard tissues^{7,8)}. Unlike the methods used in conventional bone morphological examinations, such as thin-section preparation by undecalcified polishing technique, micro-CT takes only about 30 seconds and can provide observations of bone morphology over time. Image processing also enables the creation of three-dimensional reconstructions in which bone morphology can be observed and measured in any plane.

In this study, it was difficult to set the inhalation anesthetic depth, and there was artifact due to rat motion. Since the range setting depends on the position of the rat, it is necessary to set the body position in a standard manner that is easy to quantify.

The volume of radiopacity was greater in Group C, in which Bio-Oss® had been used in combination with a collagen membrane, than in Group B. This suggested that the collagen membrane may have helped retain the Bio-Oss® within the bone defect, and that it may be valuable for hard tissue regeneration.

On histological analysis, osteoblastic cells were present around the Bio-Oss® in Groups B and C. Although the defects had been filled with the same amount of Bio-Oss® in each group, in the tissue sections, the amount of Bio-Oss® was obviously greater in Group C, in which a collagen membrane had been used, than in Group B. The use of a collagen membrane may have helped to retain the Bio-Oss® and enable

its close adhesion. Although an increasing number of studies are now using histological observations to evaluate hard tissue¹³⁾, most have used either sections prepared using a decalcification solution such as ethylenediaminetetraacetic acid (EDTA) or polished specimens. Such methods may result in weakening of the bone and the degradation and loss of artificial bone, hindering the detailed evaluation of hard tissue. In the present study, therefore, the undecalcified frozen section preparation technique devised by Kawamoto et al. was used to evaluate the efficacy of the xenogeneic bone substitute Bio-Oss®⁹⁻¹¹⁾. This technique enables the rapid preparation of thin sections from frozen blocks without either decalcification or polishing, thus minimizing the absorption and degradation of bone and bone substitutes and enabling hard tissues to be observed in greater detail. Use of this method meant that it was possible to observe the state of bone regeneration in the defects and the state of bone formation around the Bio-Oss® more clearly than has been possible in previous studies.

In clinical practice, the blocking membrane used in osteogenesis makes space for bone regeneration.

It is used to block invasion of epithelial cells and connective tissue. The properties required of a barrier membrane include biocompatibility, cell blocking, easy intraoperative handling, and space-maintaining capacity, and titanium screws may be used to fix the barrier membrane in place. However, the use of titanium mesh or screws frequently requires surgery for their placement and removal, which is a complicated procedure, and this may result in the imposition of unnecessary external force on the GBR site and impose extra stress on the patient by prolonging operating time.

The absorbable collagen membrane used in the present study is easy to apply and does not require surgical removal, thus causing patients little stress, and it is becoming more widely used for this reason. According to Zitzmann *et al.*¹⁴⁾, sufficient osteogenesis at the protected site is achieved with the use of both absorbable and non-absorbable membranes, with no difference in the rate of bone formation. The results of the present study suggest that the combined use of a collagen membrane may help retain and stabilize Bio-Oss® within the defect, blocking invasion by epithelial cells and connective tissue and creating a favorable environment for bone formation.

This investigation of the bone regeneration process when the xenogeneic bone substitute Bio-Oss® was used in combination with a collagen membrane suggests that it may help create a favorable environment for bone formation. Some osteoblastic cells were observed around new bone which contacts Bio-Oss®. More detailed 3D studies using micro-CT and early postoperative histological diagnosis will be required to evaluate the bone regeneration process in greater detail in the future.

Conflict of Interest

The authors have declared that no COI exists.

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