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Influences of mechanical barrier permeability on guided bone augmentation in the rat calvarium

Takanobu Yamamoto¹⁾, Akira Hasuike^{2,3)}, Ryosuke Koshi^{2,3)}, Yasumasa Ozawa¹⁾,
Manami Ozaki¹⁾, Tatsuya Kubota¹⁾, and Shuichi Sato^{2,3)}

¹⁾Division of Applied Oral Sciences, Nihon University Graduate School of Dentistry, Tokyo, Japan

²⁾Department of Periodontology, Nihon University School of Dentistry, Tokyo, Japan

³⁾Division of Advanced Dental Treatment, Dental Research Center, Nihon University School of Dentistry, Tokyo, Japan

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Abstract: We used radiological and histological analyses to evaluate the effects of mechanical barrier permeability in a rat model of calvarial guided bone augmentation (GBA). The calvaria of 20 rats were exposed, and one of four types of plastic caps (an occlusive cylindrical plastic cap; a plastic cap with no top; a plastic cap with three holes; and a plastic cap with four holes) was randomly placed on both sides. Newly generated bone in the plastic caps was evaluated with micro-computed tomography (micro-CT) and histological analysis. Micro-CT volumetric analysis and decalcified hematoxylin and eosin-stained sections showed that GBA barrier permeability was inversely associated with the quantity of augmented bone obtained. Masson's trichrome staining showed that collagen in newly generated bony tissue was more mature in plastic caps with three holes than in those with more-permeable or more-occlusive barriers. Bone augmentation was inhibited in specimens exhibiting invasion of soft tissue through penetrating holes, and barrier permeability was associated with the quantity of augmented bone developed. In conclusion, moderate barrier permeability is optimal for development of mature augmented bone.

Keywords: alveolar ridge augmentation; bone regeneration; dental implants; skull; X-ray microtomography.

Introduction

The demand for dental implants is increasing, and dental clinicians are therefore seeing a growing number of patients with insufficient bone for implant installation. Guided bone augmentation (GBA), the most common clinical approach for increasing bone volume, guides bone cells to the area beyond the original skeletal envelope. Although dental clinicians overwhelmingly prefer this technique because of its simplicity and safety, GBA has some limitations in achieving sufficient bone volume for installation of dental implants. In particular, vertical GBA remains a challenging procedure.

The use of standardized animal models is essential in addressing the limitations of GBA. The rat calvarium can be used as a preclinical model of GBA to evaluate bone regeneration and test new scaffolds or bioactive molecules before progressing to larger animals and potential application to humans (1,2). Our group developed a preclinical rat GBA model that uses plastic caps placed on calvarial defects (3). This model is well-standardized and normalized and permits sequential radiographic examination. We previously used this model to examine the bone regenerative effects of a growth factor (4), bone substitutes (5), and hormones (6). Bone dynamics were assessed longitudinally by using *in vivo* micro-computed tomography (micro-CT), and the osteogenic potency of these factors was shown in a GBA setting. These added

Correspondence to Dr. Akira Hasuike, Department of Periodontology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Fax: +81-3-3219-8349 E-mail: hasuike.akira@nihon-u.ac.jp

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factors raised the volume of newly generated bone in plastic caps from a range of 20-30% to 60-70% (4-7). Although these treatments were highly successful, challenges remain in achieving efficient vertical bone augmentation.

The traditional process of GBA involves placement of mechanical barriers over an osseous defect, to impede epithelial and connective tissue proliferation into the defect (7). In this context, the optimal barrier for guiding desirable cells must be sufficiently occlusive. An occlusive barrier prevents undesirable fibrous tissue invasion but may inhibit inflow of stem cells, growth factors, or blood, which may explain why GBA remains challenging.

Before their common use with dental implants, osseous grafting procedures using allografts, xenografts, and alloplastic bone grafts for reconstruction of deficient edentulous ridges were a focus of maxillofacial surgery. Numerous ridge augmentation techniques have been proposed for correction of bone deficiencies. Many clinicians prefer particulate grafts to block grafts, as the latter take longer to revascularize and remodel and require a longer healing period. Titanium mesh has been used since the 1970s for particulate bone graft containment when reconstructing large edentulous ridges. Titanium mesh has been used to maintain space and confine graft material to the recipient site. As the demand for implant treatment increases, bone augmentation with titanium mesh has become a common therapeutic option, not only for full, but also for localized, edentulous ridges and has been broadly classified as a type of GBA.

Some commercially available titanium meshes are macroporous and far from occlusive, as pore diameters reach the millimeter range. Thus, traditional GBA procedures may substantially differ from actual clinical practice. Rocuzzo et al. (8) compared autogenous onlay osseous grafting alone with alveolar ridge augmentation that used autogenous bone plus titanium mesh. The mean increase in vertical bone was 5 mm in the titanium mesh group and only 3.4 mm control group. Interestingly, the sites with titanium mesh coverage exhibited 13.5% bone resorption, while the control group had 34.5% bone resorption. These findings suggest that undifferentiated cells, bioactive molecules, or blood supply applied from the periosteal covering of the barrier may help accelerate bone regeneration within the space secluded by the nonocclusive barrier. Therefore, the properties required for barriers used in GBA need to be identified.

We used radiological and histological analyses to evaluate the effects of mechanical barrier permeability in a rat calvarial model of GBA. Plastic caps with holes to increase permeability were placed on calvarial defects,

and the dynamics of bone augmentation were examined by micro-CT and histological assessment.

Materials and Methods

Animals

This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry (AP15D026). Twenty nine-week-old male Fischer rats (weight, 250-300 g) with no injuries or congenital defects were used. All animals were housed in individual cages (temperature 22°C, 55% humidity, 12-h/12-h light/dark cycle) and had ad libitum access to food and water.

Experimental GBA surgical procedure

Rats were pre-medicated by inhalation of isoflurane, which was followed by general anesthesia with intraperitoneal injection of a mixture of 0.15 mg/kg dexmedetomidine hydrochloride, 2.0 mg/kg midazolam, and 2.5 mg/kg butorphanol tartrate. An intraperitoneal injection of 0.5 mL of a 1:80,000 dilution of lidocaine (Xylocaine; Astra Zeneca, Osaka, Japan) was administered to control bleeding and provide additional anesthesia. The surgical areas were shaved, and the skin was wiped with 70% ethanol before surgical incision. To raise the skin off the calvarium, a 6.0-cm skin incision was made over the linea media. A cutaneous flap was raised laterally with a small sharp periosteal elevator. The parietal area was exposed under aseptic conditions, and the periosteum was elevated to expose the bone. In each rat, a trephine bur with an inner diameter of 5 mm was used to make a circular groove on each side of the median sagittal suture of the parietal bone, under profuse irrigation with sterile saline. To induce bleeding, five small holes were drilled with a No. 2 round bur (Fig. 1A). After thoroughly rinsing the area with sterile saline to remove any bone fragments, a cylindrical plastic cap (standardized columnar shape, 1.5 mm in height and 5.0 mm in outer diameter) was placed on the circular grooves (Fig. 1B). The GBA sites were divided into four experimental groups, in accordance with the type of cylindrical plastic cap used (Fig. 2), as follows: 1. An occlusive cylindrical plastic cap (occlusive; control); 2. An open (nonocclusive) plastic cap (open); 3. A plastic cap with three holes drilled with a No. 4 round bur (three holes); 4. A plastic cap with four holes drilled with a No. 4 round bur (four holes).

The characteristics of these plastic caps are shown in Table 1. The size of the holes in the three-hole and four-hole caps was identical to that used in commercially available titanium mesh (diameter, 1.4 mm). The barrier permeability of the four-hole cap was the same as that of commercially available titanium mesh, while the

Fig. 1 A circular groove was made with a trephine bur, and five small holes were drilled with a round bur, to allow for marrow penetration (A); one of the four types of plastic caps was seated on each of the respective defects (B).

Fig. 2 Photographs of the plastic caps used in this study.

three-hole cap was less permeable. Forty sites in 20 rats were randomly assigned to one of four groups; thus, each group included 10 sites.

After surgery, the skin was closed with 5-0 resorbable sutures (Alfresa Pharma CO., Osaka, Japan). The day of surgery was designated as day 0, and the rats were euthanized at 6 or 12 weeks after surgery.

Micro-CT analysis

A micro-CT system (R_mCT2 system; Rigaku, Tokyo, Japan) was used for repeat micro-CT imaging at 0, 4, 8, and 12 weeks after surgery. Under light anesthesia with 1.5% isoflurane (Mylan, Canonsburg, PA, USA), rats were placed on the stage, and micro-CT images were obtained by using exposure parameters of 90 kV and 100 μ A. Three-dimensional images were reconstructed with i-View software (i-View Image Center, Tokyo, Japan). Bone volume within regions of interest (diameter: 5.0 mm, height: 3 mm) was measured with specialized software (Kitasenjyu Radist Dental Clinic I-View Image Center, Tokyo, Japan). Then, enhanced bone volume was

Table 1 Characteristics of the plastic caps used in the experimental groups

Group	Area of top (mm ²)	Permeability rate (%)
Occlusive	0	0
Three-hole	9.04	30.39
Four-hole	10.58	40.53
Open	15.20	100

Fig. 3 Schematic diagram showing the method for detecting the red reaction in Masson's trichrome (MT) staining. The percentage of red reaction in MT staining (%) was calculated in relation to the inner volume of the plastic caps.

calculated by subtracting the volume on day 0 from the respective values at later time-points.

Histological and histomorphometric analyses

Ten of the 20 rats were euthanized with excess CO₂ gas inhalation at 6 or 12 weeks, i.e., after the last micro-CT scan. Each group (five sites each) was assigned to the 6-week or 12-week group. The skin was dissected, and calvarial bone defects with the fixed plastic cap were resected, fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin wax, processed into 5- μ m sections, and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT). MT staining was used to detect collagen and lamellar bone formation (9). A light microscope was used for histological and morphometric assessments of sections, and the data were analyzed with ImageJ software (National Institutes of Health, Rockville, MD, USA). For each histological section stained with HE, the percentage of areas of newly generated bone (percentage of newly generated bone) was calculated relative to the area bounded by the plastic cap and parent bone, which was defined as 100%. Within the area confirmed as newly generated bone in HE staining, formation of mature collagen and lamellar bone was detected as reddish dyed tissue in MT staining. We calculated the percentages of areas of reddish tissue in MT staining (percentage of red reaction in MT staining) relative to the inner area of the plastic cap (Fig. 3).

Table 2 Volumetric measurements from micro-CT analysis (mm³)

Bone volume (mm ³)	4 weeks		8 weeks		12 weeks	
Occlusive	6.40	(1.01)	10.91	(1.11)	11.62	(1.42)
Three-hole	5.81	(0.75)	7.72	(0.94)	11.13	(1.21)
Four-hole	5.85	(1.55)	7.85	(1.64)	9.08	(1.19)
Open	4.15	(0.45)	5.39*	(0.40)	5.76*#	(0.71)

Values are means (SD). *n* = 10 at 4 weeks, *n* = 5 at 8 and 12 weeks. **P* < 0.05 vs occlusive, #*P* < 0.05 vs three-hole.

Fig. 4 Micro-CT radiographic images at 4, 8, and 12 weeks after surgery, by type of plastic cap used.

Statistical analysis

Means and standard deviations were calculated for volumes obtained from micro-CT images, percentages of newly generated bone in HE staining, and percentages of red reaction in MT staining. Data were analyzed for statistical significance by using GraphPad Prism 7 software (GraphPad Inc, La Jolla, CA, USA) and one-way ANOVA and the Bonferroni *post-hoc* test. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Healing progressed well in all animals, and no complications or signs of postoperative infection were observed in any experimental animal.

Analysis of micro-CT images

Micro-CT images showed a gradual, time-dependent increase in radiopacity contrast in all four groups (Fig. 4). Table 2 shows volumetric values obtained from micro-CT images at 4, 8, and 12 weeks. The values decreased as barrier permeability increased, in all three observation periods. Volumes in the open group were consistently lower than those in the other three groups. Specifically, volumes significantly differed between the open and occlusive groups at 8 and 12 weeks and between the open

and three-hole groups at 12 weeks. Volume markedly increased between 4 and 8 weeks in the occlusive group and between 8 and 12 weeks in the three-hole group.

Histological analysis

HE and MT staining showed soft-tissue invasion in all groups but the occlusive group; soft-tissue growth was most invasive in the open group (Figs. 5, 6). Consequently, new bone formation was minimal in the open group and suppressed in the four-hole group, because soft-tissue invasion was greater than in the three-hole group. New bone formation along the inner edges of plastic caps was seen in the occlusive, three-hole, and four-hole groups. In the three-hole and four-hole groups, no bone formation near holes was confirmed.

Quantitative results from HE and MT staining are shown in Table 3. New bone formation, as indicated by HE staining, was consistent with volumetric values obtained from micro-CT images. In all groups, the area of newly generated bone was larger at 12 weeks than at 6 weeks. High barrier permeability was associated with a smaller area of newly generated bone at 6 and 12 weeks. At 6 weeks, the percentage of new bone formation was significantly higher in the occlusive and three-hole groups than in the other groups. At 12 weeks, the percentage of new bone formation was significantly higher in the

Fig. 5 Representative histological results of hematoxylin and eosin staining of sections at 6 and 12 weeks, by experimental group.

Fig. 6 Representative histological results of Masson's trichrome staining at 6 and 12 weeks, by experimental group.

Table 3 Histomorphometric area measurement: percentage of newly generated bone, as indicated by HE staining and percentage of red reaction in MT staining (%)

%	6 weeks				12 weeks			
	Newly generated bone in HE		Red reaction in MT		Newly generated bone in HE		Red reaction in MT	
Occlusive	23.22	(3.71)	0.32	(0.04)	25.81	(3.02)	0.28	(0.08)
Three-hole	13.25*	(1.34)	0.37*	(0.13)	25.09	(4.07)	0.62*	(0.15)
Four-hole	6.59*#	(1.43)	0.14	(0.02)	17.42	(2.72)	0.27#	(0.04)
Open	4.23*#	(1.11)	0.05*#	(0.02)	15.26*#	(1.20)	0.34	(0.08)

HE staining: hematoxylin and eosin staining; MT staining: Masson's trichrome staining.

Values are means (SD). $n = 5$, * $P < 0.05$ vs occlusive; # $P < 0.05$ vs three-hole.

occlusive and three-hole groups than in the open group.

The area of red reaction in MT staining was larger in the three-hole group than in the other groups at 6 and 12 weeks. The area of red reaction was significantly larger in the three-hole group than in the open and four-hole groups at 6 weeks, and as compared with the occlusive and four-hole groups at 12 weeks. The area of red reaction at 6 weeks significantly differed between the occlusive and open group. In the three-hole group, the overlap of areas of red reaction and blue reaction was greater than that for the scattered red-reaction segments seen in the other groups.

Discussion

This study examined the effect of barrier permeability on the quality and quantity of augmented bone. Micro-CT analysis and histological observation showed that greater GBA barrier permeability resulted in less augmented

bone. No or minimal bone regeneration was observed in the open group. In addition to the quantitative results for augmented bone, the area of red reaction in MT staining was larger for the three-hole specimens.

The present results show that barrier permeability affects the quantity of newly formed augmented bone. This finding is consistent with the previous observation that nonosteogenic cells move into a defect through pores in the GBA barrier faster than do osteogenic precursor cells, thereby resulting in minimal bone formation under the barrier. Rakhmatin et al. (10) evaluated the optimal porosity of titanium meshes for enhancement of bone regeneration. They examined six types of titanium meshes of different thicknesses and pore sizes and three commercially available membranes in rat calvarial defects. The greatest bone volume was achieved by using 100- μ m-thick membranes with larger pores, which is inconsistent with the present findings. They concluded

that membrane porosity is essential for bone regeneration, especially during the initial phase of healing. In the present study, a No. 4 round bur was used to drill the holes in tops of caps. The hole size was identical to that used in commercially available titanium meshes. Future studies should investigate how variations in hole size affect bone augmentation, particularly holes on the micron and nanometer scales.

On MT staining, the area of red reaction was larger in the three-hole specimens. MT staining visualizes collagen extent and has better photographic contrast as compared with HE staining. The normal lamellar pattern is absent in woven bone, in which collagen fibers are laid down in a disorganized manner. In MT staining, the blue reaction is mainly localized to woven osteoid tissue, in which collagen fibers are haphazardly distributed, while the red reaction is associated with calcified lamellar bone formation. Thus, a large area of red reaction reflects an advanced stage of ossification. Interestingly, moderate barrier permeability was associated with increased maturity of augmented bone. In the three-hole group, the area of red reaction overlapped the area of blue reaction.

Pore location may affect bone maturity level. In the four-hole group, the pores were closer to the cap edge than in the three-hole group. This enabled soft tissue to migrate into the plastic caps along the sidewall and could impede bone maturation.

In our animal GBA model, we raised a full-thickness flap, which included the periosteum and covered the plastic cap. The periosteum placed on the plastic cap might be a good source of osteogenic precursor cells and growth factors. Many studies have examined the ability of mesenchymal stem cells (MSCs) in bone regeneration (11,12). Although MSC implantation has beneficial effects on bone regeneration, the survival of implanted MSCs is limited. One study suggested that paracrine mechanisms triggered by growth factors and cytokines secreted by the implanted MSCs were responsible for the beneficial effects of MSC implantation (13). Thus, growth factors and cytokines rather than the cells themselves may play important roles. Intramembranous ossification is characterized by invasion of capillaries into the mesenchymal zone and by the emergence and differentiation of mesenchymal cells into mature osteoblasts. We previously investigated angiogenesis in a rabbit GBA model and a rat calvarial bone defect model and confirmed that angiogenesis occurred in newly generated tissue beyond blood vessels arising from existing calvarial bone (14,15). In animals with penetrated caps, angiogenesis occurred on both the dura side and periosteal side. Ossification and maturation may be induced by angiogenesis from both

sides and by the influx of growth factors and cytokines from the periosteum. The greater the penetrated area of the barrier, the more nutrients that can be transported, although the possibility of intrusion by non-osteogenic soft tissue is increased. In the present study, the three-hole cap might have achieved the optimal balance between accelerated nutritive influx and incursion of soft tissue and thus yielded the most-mature bony tissue.

Porous meshes have been used clinically for fixation of bone substitutes, which raises the question of compatibility between barriers and bone substitutes. Using autologous iliac bone grafts in a rabbit calvarial model, Ikeno et al. (16) examined the effects of shield permeability on bone augmentation by comparing titanium cylinders with open conditions, titanium mesh, an expanded polytetrafluoroethylene membrane, and titanium plate. The volume of augmented bone did not significantly differ between these groups. Future studies should investigate the effect of GBA barrier permeability on bone regeneration with several categories of bone substitutes.

In conclusion, the present findings indicate that GBA barrier permeability is inversely associated with the quantity of newly formed augmented bone. Furthermore, the maturity level of augmented bone was highest in specimens with moderate barrier permeability. In future, smart barriers capable of selectively filtering out beneficial factors, e.g., growth factors and microvasculature, should be used to investigate further the differential effects on GBA.

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Conflict of interest

The authors have no conflict of interest to declare.

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