Original

**Pilot Study of Bone Augmentation in Rat Calvaria Using Silicone
Molds with Recombinant Human Bone Morphogenetic
Protein-2-containing Atelocollagen Sponge**

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Abstract : Bone augmentation is required for cases of highly absorbed alveolar ridge in the placement of dental implants. Various biomaterials and/or growth factors have been used to induce new bone formation; however, it remains difficult to obtain the required bone shape. In this study, we used cylindrical silicone molds coupled with recombinant human bone morphogenetic protein (rhBMP) to induce a specifically shaped bone. The cylindrical mold was made of silicone rubber impression material with a 10 mm outer diameter, 5 mm inner diameter, and 5 mm height. RhBMP-2 was applied using an atelocollagen sponge placed into the silicone mold; it was then implanted into the parietal subperiosteal region of 8-week-old male Wistar rats. Histological and micro-computed tomography (micro-CT) analyses were performed to detect new bone formation at 2, 4, and 8 weeks after the surgery. Micro-CT analysis indicated a disc-shaped bone formation adjacent to the periosteum at 2 weeks after surgery. Newly formed bone was also detected near the parietal bone at 4 weeks. At 8 weeks, an almost cylindrical bone was formed in the silicone mold, and histological observation confirmed that the newly formed bone completely adhered to the host parietal bone. Hematopoietic bone marrow was also detected in the newly formed bone. A specifically made silicone mold might provide the scaffold required to induce a specific shape of newly formed bone under the combined induction of rhBMP.

Key words : recombinant human bone morphogenetic protein-2 (rhBMP-2), silicone mold, atelocollagen sponge, onlay grafting

Introduction

In oral and maxillofacial surgery, bone grafting is used to recover bone defects due to malformation, cysts, or tumors. Recently, sinus lifting has been selected to augment bone volume for dental implant installation in cases of highly atrophic alveolar ridge, particularly

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in the maxillary molar region¹⁻⁵⁾. Autogenous bone grafting has been performed in many of these cases as the long-standing “gold standard”⁶⁾, however, this method of grafting requires another operation field for bone harvesting, such as the iliac region, potentially increasing the risk of morbidity⁷⁻¹⁰⁾. Achieving adequate osseointegration and stable function after the implant placement requires that the newly formed bone is well incorporated into the original host bone, it is of suitable quantity, and also of the desired shape. To this end, various biomaterials and / or growth factors have been investigated for many years as alternative clinical methods to bone grafting¹¹⁻¹⁵⁾.

Urist¹⁶⁾ first reported the concept of bone morphogenetic protein (BMP) as an osteoinductive factor; Wozney *et al*¹⁷⁾ and Wang *et al*¹⁸⁾ were the first to purify and clone this protein. Subsequently, many studies have applied BMP effectively clinically, and atelocollagen is now an accepted and potent carrier of BMP for the induction of newly formed bone¹⁹⁻²²⁾.

Alam *et al*²³⁾ and Hosoya *et al*²⁴⁾ recently induced bone formation to a desired shape using BMP/collagen in silicone molds. Herein, we aimed to similarly obtain newly formed bone to a specified shape and quality for implant placement using BMP and silicone molds in rat calvaria.

Materials and methods

1. Preparation of implant materials

A silicone rubber material (vinyl polysiloxane impression material, GC Dental Products Co., Tokyo, Japan) was used to make the molds for inducing bone tissue formation. A cylindrical silicone mold (10 mm outer diameter, 5 mm height) was made using a made-to-order template. The inner diameter of the mold was then standardized to 5 mm (Fig. 1). The mold was sterilized with ethylene oxide gas, and an atelocollagen sponge (TERUPLUG[®], Olympus Terumo Biomaterial Co., Tokyo, Japan) was cut so as to just fill the inner space of the mold. Under aseptic conditions, 10 µg of rhBMP-2 (PeproTech, Inc., Rocky Hill, NJ, USA) dissolved in 10 µl of physiological saline was added to the atelocollagen sponge. In the control group, only physiological saline was applied.

2. Animals and surgical procedures

The experimental protocol used was reviewed and approved by the Animal Care Committees of Showa University and Hirosaki University. Eight-week-old male Wistar rats (weight 250–300 g) were divided into two groups: BMP-treated group (N = 15) and control group (N = 6). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (2.5 mg per 100 g body weight). All surgeries were performed under sterile conditions.

The skin of the head was shaved and then disinfected with ethanol and iodine. A curved horizontal incision (1.5 cm) was made in the parietal region between the ears and the periosteum was elevated to form a pocket and expose the bone surface. On the occasion of elevating the periosteum, a 1.0 cm periosteal incision was made at the posterior border of the parietal bone, and then the periosteum was ablated forward to make a subperiosteal pocket large enough to fit the implanted silicone mold. A silicone mold containing BMP/collagen was

Fig. 1. Illustration of the silicone mold. It has a cylindrical shape with a 5 mm inner diameter, 10 mm outer diameter, and 5 mm height.

Fig. 2. Illustration of the implantation. The silicone mold was implanted into the subperiosteal region of parietal bone.

implanted into the subperiosteal pocket, and then the periosteum and skin were sutured with nylon thread in each layer (Fig. 2). The periosteal flap was sutured using single interrupted sutures and the skin flap was sutured tightly using a simple continuous suture. At 2, 4, and 8 weeks after surgery, rats were sacrificed and specimens (heads) were fixed in 10% neutral buffered formalin.

3. Micro-CT Examination

Before histological preparation, computerized X-ray tomography was performed with a micro-CT scanner (inspeXio SMX-90CT Micro Focus X-Ray CT System, Shimadzu, Kyoto, Japan). Whole head specimens were placed in the sample holder and scanned (90 kV, 110 μ A). From the micro-CT scan datasets, three-dimensional models were built for visualization and morphological observation. Evaluation was performed in the sagittal plane at the area with the maximal diameter of the silicone mold.

The volume of newly formed bone in each silicone mold was measured with a three-dimensional analyzing software (TRI/3D; RATOC, Tokyo, Japan). Bone density area inside the silicone mold was measured as the region of interest and evaluated as the bone volume. Values of bone volume in each group (2, 4, 8 weeks, $n = 5$ in all groups) were tested by one-way analysis of variance (Sheffe Test). Statistical analyses were carried out using IBM SPSS Statistics20 software (Japan IBM, Co., Tokyo, Japan).

4. Histological examination

After the fixation in formalin, the silicone molds were removed. The specimens were decalcified by using Plank-Rychlo solution and embedded in paraffin. Sections at 5 μm thickness were prepared and stained with hematoxylin and eosin, and then examined under the light microscope (ZEISS Axioskop 2 plus).

Results

1. Macroscopic observation

In the BMP-group at 8 weeks after surgery, cylindrical-shaped, newly induced bone was detected after removing the silicone mold. The bone was firmly adhered to the host parietal bone (Fig. 3). In the control group, no bone formation was detected. Soft connective tissue resembling a scar was observed in the silicone mold.

2. Micro-CT scan analysis

In the BMP-treated group, disc-shaped bone formation was observed adjacent to the periosteum at 2 weeks after surgery (Fig. 4A). At 4 weeks, new bone formation was observed adjacent to the original parietal bone, growing from the periosteal side and connecting to the parietal bone side (Fig. 4B). At 8 weeks, the inside of the silicone mold was almost filled with cylindrically shaped bone. The bone was composed of compact trabecular bone (Fig. 4C).

The three-dimensional measurement of volume revealed mean values for the newly formed bone at 2, 4, and 8 weeks of 4.15 mm³ (SD 4.12), 28.03 mm³ (SD 13.98), and 56.88 mm³ (SD 10.81), respectively (Fig. 5). Significant differences were recognized between 2 weeks and 4 weeks, and between 4 weeks and 8 weeks ($P < 0.05$). In the control group, bony tissue was not detected even at 8 weeks after surgery.

3. Histological observation

At 2 weeks after surgery, newly formed bone was observed in the subperiosteal region. The bone was characterized as woven bone (Fig. 6A, 6B). No apparent hematopoietic bone marrow

Fig. 3. Macroscopic findings at 8 weeks in the BMP-group. Bone formation was cylindrical shaped.

Fig. 4. Micro-CT finding in the BMP-group

- A : 2 weeks. Disc-shaped bone formation was observed in the upper region of the silicone mold.
 B : 4 weeks. Bone formation was observed in the region of the host parietal bone.
 C : 8 weeks. Bone formation was observed in all regions of the silicone mold. Bar = 3 mm.

Fig. 5. Relationship between the newly formed bone volume and the weeks after implantation. Significant differences were recognized between 2W and 4W, and between 4W and 8W. (Columns : mean, bars : SD, *: $P < 0.05$)

was detected at this stage (Fig. 6B).

At 4 weeks, newly formed bone was observed adjacent to the periosteum and near the host parietal bone (Fig. 7A). This bone showed a trabecular structure, and osteocytes, located in the lacuna, were more obvious than those detected at 2 weeks (Fig. 7B). At this stage, the newly formed bone was not completely attached to the host parietal bone, however, many osteoblasts were observed along the surface between the host parietal bone and the newly formed bone

Fig. 6. Two weeks in the BMP-group

A : New bone formation was observed in the subperiosteal region. P : periosteum, BN : bone. Bar = 1 mm.

B : Higher magnification of newly formed bone in Fig. 6A. Bar = 300 μ m.

Fig. 7. Four weeks in the BMP-group

A : Periosteum, newly formed bone (NB), and host parietal bone (HB) were seen. Bar = 800 μ m.

B : Interface of the HB and NB. Many osteoblasts were observed lining the bone surface. Undifferentiated mesenchymal cells were also seen. Bar = 100 μ m.

Fig. 8. Eight weeks in the BMP-group

A : The newly formed bone firmly adhered to the host parietal bone. Interface of the host parietal bone and newly formed bone (arrowheads), showing complete incorporation. Bar = 800 μ m.

B : Higher magnification of the connecting region between the newly formed bone and parietal bone (arrowheads). Bar = 200 μ m.

C : Bone marrow tissue (asterisk) was observed in this stage. Bar = 200 μ m.

(Fig. 7B).

At 8 weeks, the newly formed bone showed a lamellar structure and it was firmly adhered to the host parietal bone (Fig. 8A, B). The connecting line between the newly formed bone and the host parietal bone was evident (Fig. 8B). Bone marrow was also distinctly present at this stage (Fig. 8C).

Discussion

Many kinds of materials have been used as the carrier for BMP-induced osteogenesis, such as hydroxyapatite (HA)²⁵⁾, β -tricalcium phosphate (β -TCP)²⁶⁾, and atelocollagen¹⁹⁻²²⁾. In this

study, we selected atelocollagen sponge as a carrier because it is absorbable and it is approved for clinical adaptation²⁰⁾.

One of the most important clinical objectives is to induce new bone with a definite and desirable shape. Alam *et al*²³⁾ reported that replacement and incubation of muscle tissue with a vascularized flap inside a BMP-containing silicone mold in the thigh of rat induced ectopic bone formation in the mold. Similarly, Hosoya *et al*²⁴⁾ induced bone formation in the silicone mold with a vascularized flap from fat tissue instead of muscle tissue, and the newly formed bone replicated the shape of the silicone mold. We modelled our present study on the same principle to form the desired shape of newly induced bone.

We further attempted to induce the bone by means of “onlay grafting” to the intact bone surface to simulate alveolar ridge bone augmentation without introducing a bone defect. Murata *et al*²¹⁾ reported that onlay augmentation using rhBMP-2 is much more difficult than inlay grafting, and attributed that to mechanical stress caused by the covering soft tissues preventing the volume and the shape of the newly formed bone to be achieved at the site of onlay grafting. We instead proposed that silicone molds have the required properties to resist such excessive mechanical stress.

In this study, the newly formed bone was first detected adjacent to the periosteum, and then was further formed at the region of the host parietal bone. Adeyemo *et al*²²⁾ indicated that osteoclasts were predominantly located at the graft–host interface in the early stage of onlay grafting in sheep mandible, and thus suggested that osteoclastic bone resorption on the host bone surface might be necessary when new bone formation is induced by BMP. In this study, we could not detect active bone resorption in the detected osteoclasts during the experimental period.

Osteogenic cells have been identified in the periosteum^{27–29)}, whereas few of these cells would be located on parietal bone due to the removal of the periosteum during the surgical operation. This outcome might explain the difference in initiation of the new bone formation between the two regions.

It was suggested that the atelocollagen sponge acted as a carrier of BMP and “biological scaffold” for osteoinduction, and that the silicone mold played the role of a “physical scaffold” to induce the desired bone shape. It was also reported that ectopic newly formed bone induced by rhBMP-2 in rat thigh was intact without resorption in the silicone molds up to 48 weeks²⁴⁾. In this study, active hematopoietic bone marrow was induced inside the newly formed bone in the silicone mold, indicating the maturation of bone.

Silicone molds are useful for maintaining induced bone; however, the mold should be removed after bone augmentation and before the installation of the dental implants, prompting the question as to whether the shape and volume of the augmented bone would be maintained after removal of the silicone mold. Further investigation is needed to establish effective clinical methods for applying BMP-induced bone formation.

In conclusion, newly formed bone could be induced in the parietal subperiosteal region of rats using rhBMP-2 with an atelocollagen sponge within a silicone mold. The new bone replicated

the shape of the silicone mold and was firmly adhered to the host bone.

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Conflict of interest disclosure

The authors declare that there are no conflicts of interest.

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