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Bone formation of middle ear cavity using biphasic calcium phosphate lyophilized with *Escherichia coli*-derived recombinant human bone morphogenetic protein 2 using animal model

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ABSTRACT

Objective: The aim of the study was to analyze the value of *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (ErhBMP-2) coated biphasic calcium phosphate (BCP) for the obliteration of middle ear bone defect after mastoid surgery.
Methods: Twenty-four specific pathogen-free Sprague-Dawley rats were randomly assigned to the BCP group ($n = 12$) and BCP-ErhBMP-2 group ($n = 12$; in which BCP scaffold of the granular type was coated with ErhBMP-2). In both groups, BCP scaffold was used to surgically fill the middle ear bulla. New bone formation was evaluated by measuring bone density (%) after 4 and 8 weeks in all rats in both groups.
Results: At 4 weeks, new bone was visible at the periphery and center of the middle ear cavity in both groups. In the BCP group, a moderate amount of fibrous tissue had infiltrated into the interspace of the scaffolds. New bone almost totally filled the interspace in the BCP-ErhBMP-2 group. At 8 weeks, copious new bone formation had occurred. Histometric measurements showed that bone density in the BCP group was smaller than in the BCP-ErhBMP-2 group at 4 weeks (25.10% and 38.43%, respectively; $p < 0.05$) and 8 weeks (25.54% and 34.18%, respectively; $p < 0.05$).
Conclusions: New bone formation was greater in the presence of BCP-ErhBMP-2 scaffolds. ErhBMP-2 coated BCP scaffolds is a potentially useful material for middle ear obliteration after mastoidectomy.
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1. Introduction

Mastoid surgery is common in the treatment of chronic otitis media. The surgery creates a bone defect and the resulting large cavity can cause various complications. Many techniques aim to decrease the cavity size and bone defect [1]. While autologous tissues like inferior based flap or bone pate are often used for the obliteration, the tissue is often insufficient to fully obliterate postoperative bone defect and can dissolve over time.

There has been much interest in developing scaffolds for the bone regeneration. Scaffolds comprised of biphasic calcium phosphate (BCP), a composite of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), have shown excellent biocompatibility [2,3].

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a protein derived from a subgroup of the transforming growth factor β family [4]. It accelerates ossification by controlling proliferation and differentiation of osteoblasts from progenitor cells and the biosynthesis of bone matrices [5]. More recently, *Escherichia coli*-derived rhBMP-2 (ErhBMP-2) has been developed and demonstrated effective in osteoinduction [6].

The BCP composite has shown good results concerning osteoconduction [7]. However, the effects of ErhBMP-2 coated BCP on bone regeneration in a mastoid obliteration model have not been determined.

This study analyzed whether BCP coated with ErhBMP-2 might be useful for the obliteration of middle ear bone defect after mastoid surgery.

2. Materials and methods

2.1. Animals

Twenty-four specific pathogen-free, Sprague-Dawley rats weighing 200–250 g (Orient Bio, Gyenggi, Korea) were assigned

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to the study. The Institutional Animal Care and Use Committee (IACUC) approved the protocols for this study, and the animals were cared for in accordance with the Guidelines of Animal Experiments of the Dongguk University Ilsan Hospital. Animals were housed in a room maintained at a temperature of 23 ± 3 °C and a relative humidity of 50 ± 10%. Artificial lighting was applied from 08:00 to 20:00 with 13–18 air changes per hour. Animals were examined by otomicroscopy (Omi Pico; Carl Zeiss, Jena, Germany) to document the normal middle ear status and were kept isolated in an infection free zone for 2 weeks before experiment.

2.2. BCP scaffold

ErhBMP-2 was obtained from Cowellmedi (Busan, Korea). Nonglycosylated ErhBMP-2 was obtained in the form of inclusion bodies and was refolded in vitro into the active dimer form, as previously described [8]. Microporous BCP granules (0.5–1 mm diameter; Bio-C; Cowellmedi) with a 30:70 ratio of HA to β-TCP were used. The total porosity was 70% as measured using the Brunauer–Emmett–Teller method with helium adsorption. BCP granules packed into glass ampules and sterilized by γ-irradiation.

ErhBMP-2 was coated to microporous BCP granules as previously established methods [7]. ErhBMP-2 solution (0.67 mL in 1.5 mg/mL buffer) was pipetted into an ampule containing 1 g of the BCP granules and lyophilized in a freezer drier (Shinil, Seoul, Korea). The solution was frozen by placing the ampule on precooled shelves and cooling it to −43 °C. The formulations were maintained at this temperature for 3 h, after which they were dried in a condenser at −40 °C (primary drying) and kept in a pressure chamber at 5 mTorr for 2 h. Secondary drying was performed on a shelf using the following sequence: −20 °C for 4 h, −10 °C for 4 h, 0 °C for 2 h, and 20 °C for 20 h. The chamber pressure was constant throughout the procedure. The morphology of granules of microporous BCP was observed with field emission scanning electron microscopy using a S-4700 apparatus (Hitachi, Tokyo, Japan) (Fig. 1).

2.3. Study design and surgical procedure

The 24 rats were randomly assigned to the BCP group (n = 12) and BCP-ErhBMP-2 group (n = 12). Each rat was anesthetized with zoletil (50 μg/100 g; Virbac, Carros cedex, France) and a post-auricular incision was made. The dissection was extended more deeply to expose the ventral surface of the left side middle ear bulla, and a 4 mm-sized bony hole was made with an electric drill. The middle ear mucosa was removed, granules were filled in the middle ear bulla, and the bony hole was repaired with Durelon™ carboxyalte cement (3 M, Seefeld, Germany). The incision wound was closed layer-by-layer and healing occurred over 4–8 weeks.

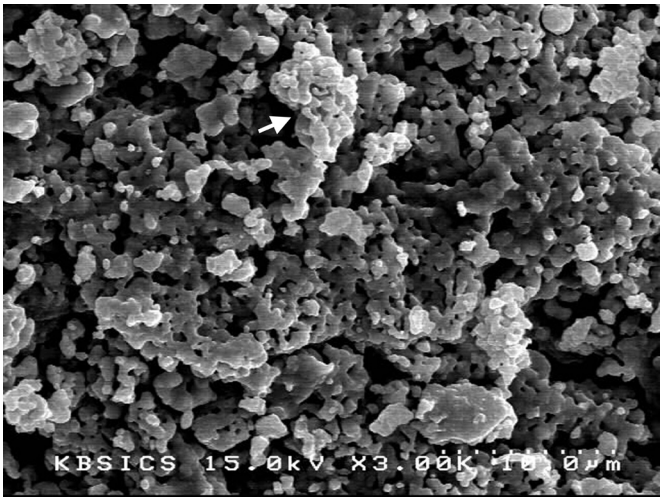

Fig. 1. Scanning electron microscopic photomicrograph of ErhBMP-2 coated BCP. Surface morphology showed rough granular shaped unit interconnected with pore structure (arrow).

2.4. Histologic & histometric analysis

For histopathologic examination, tympanic bullas were obtained at 4 and 8 weeks post-operatively from six rats in the BCP group and BCP-ErhBMP-2 group. After fixing with 10% formalin for 24 h, each bulla was decalcified and embedded in paraffin, sectioned to obtain 5 μm-thick slices, and the sections were stained with hematoxylin and eosin. The specimens were examined for inflammatory responses as well as new bone formation by light microscopy using a model DM LB microscope (Leica Microsystems, Wetzlar, Germany) equipped with a model DC300F camera (Leica Microsystems, Heerburgg, Switzerland). Images were saved as digital files. The photos from a single slide were merged and the area of the middle ear bulla was marked. The area with newly formed mineralized bone was measured as new bone area and residual scaffold, marrow, and fibrovascular tissue were excluded. Bone density was determined as the percentage of new bone area in the area of the middle ear bulla. At least three different slides from a single sample were averaged to determine the bone density of a sample.

2.5. Statistical analysis

All data are expressed as mean ± standard deviation. Student's t test was used to determine statistically significant differences between the BCP group and BCP-ErhBMP-2 group at each time point. $P < 0.05$ for the null hypothesis was accepted as indicating a statistically significant difference. Statistical analysis used SPSS version 11 (SPSS, Chicago, IL, USA).

3. Results

3.1. Histologic analysis

At 4 weeks, middle ear cavity was filled with scaffolds and graft particles were well maintained. The new bone formation was identified both at the periphery and the center of the middle ear cavity filled with scaffold. The new bone was interconnected and encircled the scaffolds in the BCP group and BCP-ErhBMP-2 group. Osteoblastic cell lining was observed at the margin of new bone area contacting the scaffolds. In the BCP group, a moderate amount of fibrous tissue infiltrated into the interspace of the scaffolds (Fig. 2A). However, in the BCP-ErhBMP-2 group, most of the interspace of the scaffolds was filled with mineralized new bone, and fibrous tissue was minimal (Fig. 2B). At 8 weeks, scaffolds were visible in the middle ear cavity. In the BCP group, a moderate amount of fibrous tissue was still visible and resorption of the scaffolds in the fibrous tissue area was minimal (Fig. 2C). However, in the BCP-ErhBMP-2 group, most of the interspace of the scaffolds was filled with mineralized new bone, and fibrous tissue was minimal. Copious new bone formation had occurred. The newly formed bone was lamellar and deposited directly onto the scaffold surface to form an anastomosing network of trabeculae. Also, considerable amount of scaffolds was resorbed (Fig. 2D).

3.2. Histometric analysis

Bone density was determined as the percentage of new bone area in the area of the middle ear bulla. Histometric measurements

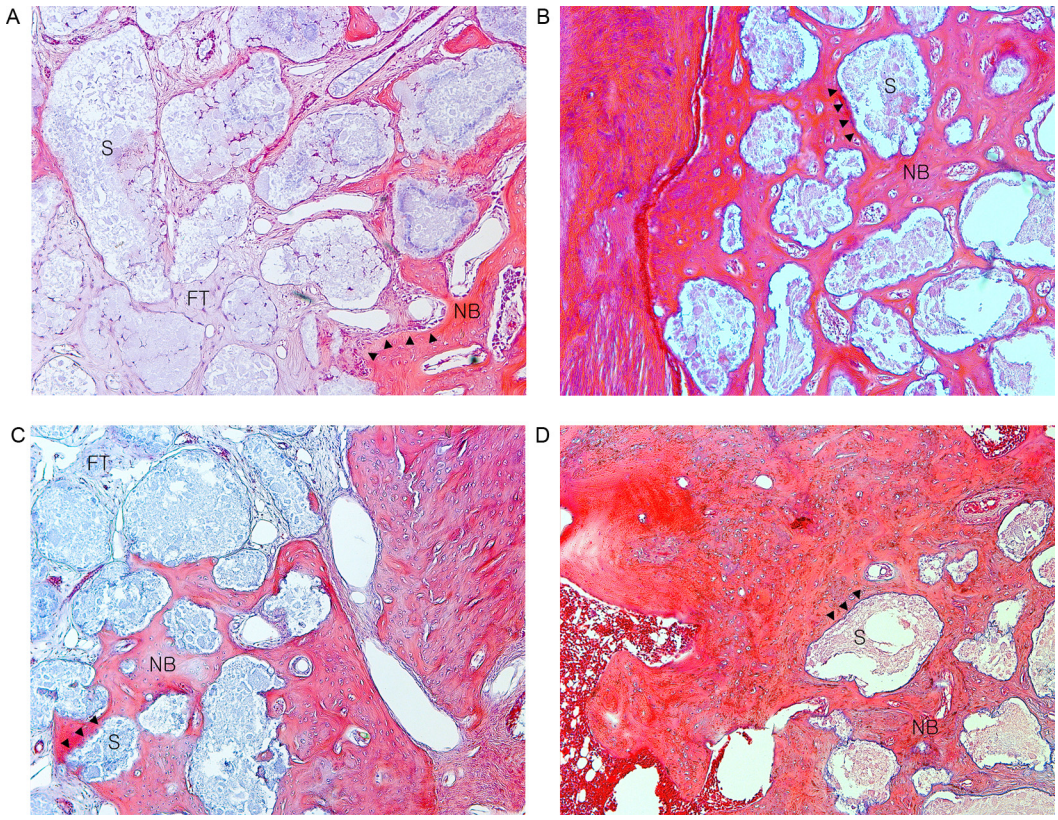

Fig. 2. Representative photomicrographs of new bone formation in the middle ear cavity. (A) BCP group at 4weeks. (B) BCP-ErhBMP-2 group at 4weeks. (C) BCP group at 8weeks. (D) BCP-ErhBMP-2 group at 8 weeks; NB, new bone; FT, fibrous tissue; S, scaffold; arrowheads indicate osteoblastic cell lining. (H&E stain, 400×).

are summarized in Fig. 3. The bone density in BCP group was significantly less than in the BCP-ErhBMP-2 group at 4 weeks 25.10% and 38.43%, respectively; $p < 0.05$) and 8 weeks (25.54% and 34.18%, respectively; $p < 0.05$). The area of newly formed bone was significantly greater in the BCP-ErhBMP-2 group compared to the BCP group.

4. Discussion

The present study evaluated the effect ErhBMP-2-coated BCP on bone formation of middle ear cavity using a rat model. The findings indicate that ErhBMP-2-coated BCP promotes new bone formation in the middle ear cavity.

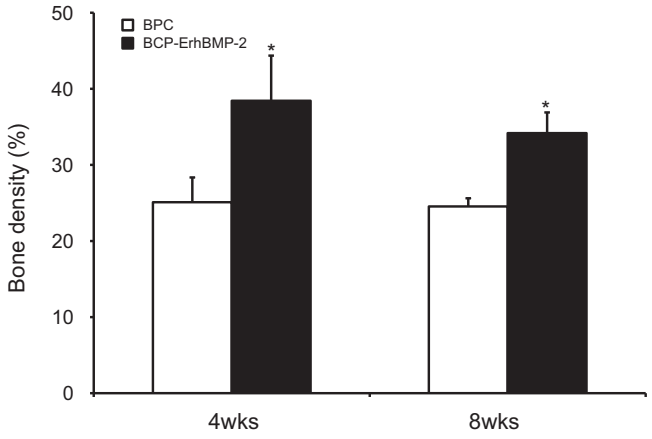

Fig. 3. Bone density (%) at each time point. The BCP-ErhBMP-2 group showed significantly increased bone density compared with BCP group at 4 weeks and 8 weeks. *Statistically significant difference ($p < 0.05$).

For the surgical treatment of chronic otitis media, mastoidectomy is often necessary to remove the irreversible mucosal lesion in the middle ear and mastoid. After mastoidectomy, a large bone defect remains postoperatively and, especially, open cavity mastoidectomy can cause various complications including slow healing, recurrent infection, poor hearing aid fit, and a chronically draining ear. These complications occur at a rate of 10% of cases in the most expert of hands [9].

There are various surgical techniques to minimize the bone defect using various tissues [10–14]. Soft tissue free grafts and vascularized flaps are frequently used. However, they are easily resorbed, resulting in the formation of a large mastoid cavity. Autologous cartilage and bone chips are often insufficient for full obliteration and there is a risk of morbidity at the site from which they were acquired.

Recent reports have addressed bone regeneration using alloplastic materials, such as HA [15,16]. Since the porosity and structural pattern of the HA scaffold can be modulated and has a good mechanical property, it is often used as the scaffold for bone regeneration [13]. Also, it is very slowly resorbed and replaced by newly regenerated bone tissue. β-TCP is a bone component and so is often incorporated into the scaffolds used for bone regeneration. Since it is resorbed easily in vivo, β-TCP is often mixed with other polymers such as HA to improve osteoconductive activity and biocompatibility. The HA/β-TCP composite shows better bioactivity than either single component [2,3]. Bio-C is a commercially available granular HA/β-TCP composite that has demonstrated good bone formation in rat calvarial defects [7] and in tooth extraction socket of patients [6].

BCP is regarded as safe material for the inner ear function. There are many experimental and clinical studies about mastoid obliteration using HA or TCP [15,17–20]. And most significant adverse effects were inflammatory response, granulation, and

osteitis of mastoid. However, there was no report of sensory neural hearing loss.

For large bone defects, a bloc type scaffold is often used. However, because the shape of bone defect in the mastoid cavity differs from patient-to-patient and the pore structure between blocs is irregular in the bloc type scaffold, granular scaffolds seem to be appropriate in mastoid obliteration.

Most bone defects of patients should be fully repaired by the structural and mechanical integrity of the scaffold. Also, the scaffold should support the mechanical strength needed during the regeneration process. In this study, the BCP scaffold maintained good mechanical property at 4 and 8 weeks after surgery.

There have been several reports about the external auditory canal or mastoid cavity reconstruction with calcium phosphate cement [21–23]. The pore structure of the scaffold is very important for the bone regeneration because ingrowth of osteoblasts and blood vessels to the center of scaffold mediates new bone formation. Appropriate porosity is advantageous to transport essential nutrients and oxygen to the center of the scaffold [24]. Because there are very few pore structures in the cement type of scaffold, if the bone defect is big, bone regeneration in the central area of scaffold can be hampered. Recent report showed that HA cement caused delayed failure of integration, infection, and severe bone inflammation [15].

In this study, BCP scaffolds displayed fully interconnected pore structures between granules, and the ingrowth of osteocytes and blood vessels to the central part of scaffolds was evident. BMP-2 is a protein derived from a subgroup of the transforming growth factor β family [4]. It accelerates ossification by inducing the proliferation and differentiation of osteoblasts and the biosynthesis of bone matrices [5]. Recently, ErhBMP-2 has been developed and was found to be more effective in preserving alveolar bone in a randomized clinical trial [6].

In this experiment, all the conditions and specimens were all pathogen-free. However, in the clinical situation, middle ear surgery is often done while infection is resent. When accompanied by infection and inflammation, osteoconduction and osteoinduction would be hampered and clinical outcome would be worse.

In conclusion, new bone formation was more copious in the BCP-ErhBMP-2 group than in the BCP group. And ErhBMP-2 seems to be a promising substance for osteoinduction for the mastoid obliteration. However, there are still some issues that should be clarified including ototoxicity, behavior in infectious conditions and optimization to prevent bony overgrowth. We think further study is needed to solve these problems and BCP-ErhBMP-2 scaffolds can be a good candidate for mastoid obliteration.

Disclosures

We report that there are no financial relationships or interests to disclose.

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