

## ORIGINAL ARTICLE

### Histopathological Evaluation of the Materials Used for Mastoid Obliteration in Rats: Allogeneic Bone, Allogeneic Cartilage, Demineralized Bone Matrix and Hydroxyapatite Cement

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**Hypothesis:** This study was conducted to evaluate the histopathological changes in bone, cartilage, demineralized bone matrix (Regenafil) and hydroxyapatite cement (Mimix) used in mastoid obliteration (MO) after 1 year.

**Background:** Many techniques and obliterating materials have been described for use in MO. The ideal material for MO maintains good biological support during healing and is then replaced gradually by the newly formed autogenous tissue. Although there are many reports on the obliterating materials used for MO, few published comparisons of the long-term changes associated with natural and artificial materials are available.

**Materials and Methods:** Forty rats received mastoid bulla obliteration using the four materials. Each materials group was subdivided into two groups according to whether the mucosa was removed or conserved. New bone formation, state of the obliterating materials, inflammatory response and mucocoele formation were evaluated on each side.

**Results:** In the mucosa-removal groups, the newly formed bone areas were significantly greater in the bone, cartilage and Regenafil groups than in the Mimix group ( $p = 0.001$ ). In the mucosa-conservation groups, the newly formed bone areas were significantly greater in the bone and cartilage groups than in the other groups ( $p < 0.001$ ). The bone and Regenafil groups showed significant differences in new bone formation between the mucosa-removal and mucosa-conservation groups ( $p = 0.016$  and  $p < 0.001$ , respectively). All bone grafts exhibited definite collapse into the bulla at the edge of the surgically created bony defect, compared with the groups obliterated with other materials. The Mimix group showed a marked inflammatory response around the residual material. Mucocoele formation was significantly greater in the bone and Regenafil than in the other materials ( $p < 0.001$ ).

**Conclusion:** Our study shows that cartilage, bone and Regenafil are appropriate graft materials for MO in the rat and that cartilage is suitable for MO in the mucosa-conservation condition. Mimix HA cement was unsatisfactory for MO in the rat.

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The surgical management of aggressive cholesteatoma usually requires canal wall down (CWD) mastoidectomy to ensure complete removal of the lesion. If not obliterated, a CWD mastoid cavity can cause persistent otorrhea that can be difficult to control even with topical antibiotic therapy and frequent cleaning of the cavity<sup>[1]</sup>. Other problems associated with a CWD mastoid cavity may include slow healing, recurrent infection, impaction with debris and difficulty using a hearing aid.

Mastoid obliteration (MO) is indicated to reduce the size of the cavity and is, ideally, conducted as a primary procedure at the time of the CWD mastoidectomy. However, for patients with a problematic mastoid cavity and chronic otorrhea and nonhealing, MO can be performed as a secondary revision procedure<sup>[1]</sup>. Many techniques and obliterating materials have been described for MO. The ideal material for MO maintains good biological support during healing and is then replaced gradually by the newly formed autogenous

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tissue. Various autogenous or artificial materials have been used in MO. Autogenous materials, including bone putty and chips of bone and cartilage, are suitable for reconstruction because of their high biocompatibility, lack of harmful reactions and low risk of infection.<sup>[2]</sup> However, because of the limited availability of autogenous materials, artificial biomaterials, such as demineralized bone matrix (DBM) and hydroxyapatite (HA), are useful alternatives to autogenous materials<sup>[3-6]</sup>. Although many obliterating materials have been reported, there are few published comparisons of the long-term changes associated with natural and artificial materials<sup>[2,3,7]</sup>. The purpose of this study was to evaluate histopathological changes after 1 year in allogeneic bone, allogeneic cartilage, DBM and HA cement used in rats. We also examined morphology of four obliterating materials in relation to the presence of the mucosa because the mastoid mucosa is sometimes retained at the site of the MO.

## **Materials and Methods**

### ***Experimental Animals***

Forty female Sprague Dawley rats (7-8 weeks old) with a normal Preyer reflex were purchased from Samtako Bio Korea Co., Ltd (Osan, Korea). The animals had free access to water and standard rat diet. All experimental protocols were approved by the Animal Research Committee, School of Medicine, Dong-A University (Busan, Korea). Animal care was under the supervision of the Institute of Laboratory Animals, School of Medicine, Dong-A University.

### ***Obliterating Materials***

Allogeneic corticocancellous bones were harvested from the femoral heads of rats (7-8 weeks old) under sterile conditions. At harvest, the bones were rinsed thoroughly and frozen at -80 °C to reduce the immunogenicity of the bone graft. After 2 weeks, the bones were thawed at room temperature and broken into 1-2 mm chips.

Allogeneic cartilage was harvested under sterile conditions from the xiphoid process of rats (7-8 weeks old). The cartilage chips were collected using the same method as described above for bone chips. The size of the cartilage chips ranged from 0.5 to 1 mm.

The DBM used in this study was Regenafil™ paste (Regeneration Technologies, Inc., Alachua, FL, USA), which uses a gelatin system to deliver human DBM for use in surgery. The gelatin is biologically inert and is resorbed entirely from the graft site. Regenafil paste was warmed to 45 °C and inserted into the bulla.

Mimix(r) HA cement (Walter Lorenz Surgical, Jacksonville, FL, USA), a calcium phosphate powder that forms HA bone cement when mixed with anhydrous citric acid in water, was used. It was supplied in a sterile 5 g vial formulation and used according to the manufacturer's instructions. The cement requires 30-45 seconds for mixing and develops into a paste-like consistency, which is malleable for 3-4 minutes before setting, and then requires only 4-6 minutes to harden completely.

### ***Group Design***

The rats were divided into four graft groups (bone, cartilage, Regenafil and Mimix groups), and each material group was subdivided into two groups according to whether the mucosa was removed or conserved. Each subgroup comprised five rats.

### ***Obliteration Technique***

Adequate anesthesia was obtained with intraperitoneal injection of ketamine at 10 mg/kg. Aseptic surgical procedures were used with sterile instruments in an animal operating room. The MO was performed on the right side in all rats. The hair was cut around the surgical site, and 1% lidocaine and a 1:100,000 dilution of epinephrine was injected in the soft tissues over the bulla for local anesthesia and hemostasis, respectively. A skin incision was made posterior and inferior to the auricle. Using blunt and sharp dissection, the bulla was exposed. The lateral wall was then removed at the level just posterior to the external auditory canal, creating a 4-5 mm defect in the lateral wall. In the mucosa-removal groups, a curette was used to remove the mucosa from the inside surfaces of the bulla. Each material was then used to obliterate the bulla space. Soft tissues and skin were closed over the bulla with absorbable sutures to hold the material in place.

### Histological Preparation and Evaluation

The animals were killed 1 year after the obliteration using hyperbaric carbon dioxide. The sacrificed animals were decapitated, and the heads were fixed immediately in 10% neutral pH formalin. After 24 hours of fixation, the specimens were decalcified in pH 7.0 10% ethylene-diamine tetraacetic acid for 2 weeks. Once decalcification was complete, the temporal bones were harvested en bloc and embedded in paraffin. A microtome was used to produce 5 ( $\mu$ -thick section through the center of the bulla parallel to its long axis. The histological sections were stained with hematoxylin and eosin.

The prepared slides were submitted to two independent blinded observers to evaluate the histology. New bone formation, state of the obliterating materials, inflammatory response and mucocoele formation were the parameters to be evaluated on each slide. The observers estimated the percentage of each graft demonstrating evidence of new bone formation by examining the histological section through the midportion of the graft. The average of the two estimations for each individual graft was used to assign a grade to the graft using a five-point scale (Table 1)<sup>[8]</sup>. The presence of cartilage or chondrocytes, active osteoblasts, osteoid tissue, newly formed bone or marrow and associated fat cells were the criteria for evidence of the process of endochondral bone formation.<sup>[5]</sup> In the mucosa-conservation groups, the observers assigned the percentage of mucocoele formation, using a five-point scale (Table 2).

**Table 1.** Scoring of new bone formation

Score	New bone formation
0	No new bone
1	1-25% of graft involved in new bone formation
2	26-50% of graft involved in new bone formation
3	51-75% of graft involved in new bone formation
4	>76% of graft involved in new bone formation

**Table 2.** Scoring of mucocoele formation

Score	New bone formation
0	No new bone
1	1-5% of graft involved in mucocoele formation
2	6-15% of graft involved in mucocoele formation
3	16-25% of graft involved in mucocoele formation
4	>26% of graft involved in mucocoele formation

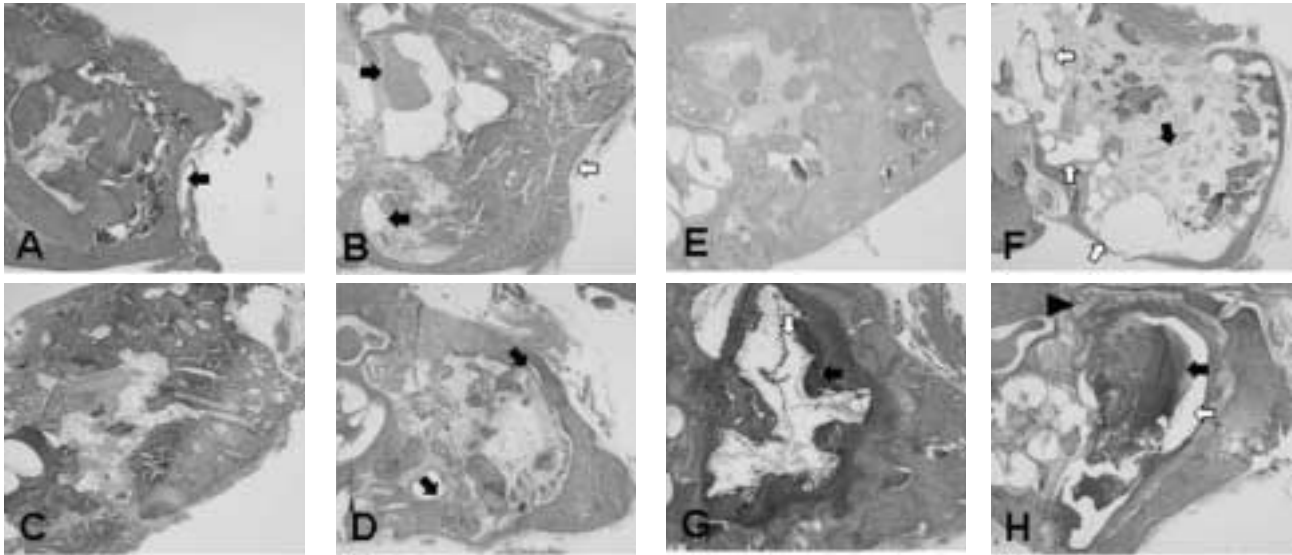
### Statistical Analysis

Data were analyzed using the t test (for comparison between two groups) and one-way analysis of variance (for comparison among four groups) with the Statistical Package for Social Sciences for Windows 12.0. Differences identified in the one-way analysis of variance were analyzed using the Tukey and Duncan tests. A value of  $p < 0.05$  was considered statistically significant.

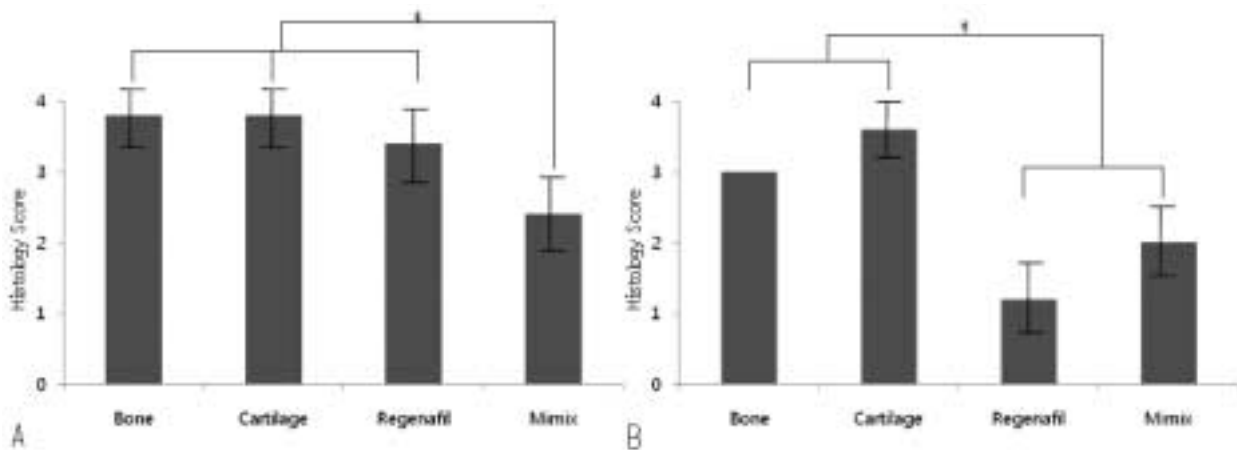
### Results

In the bone, cartilage and Regenafil groups with the mucosa removed, the mastoid bullae were filled with bone tissue, including the implanted materials, 1 year after obliteration (Figure 1A, 1C and 1E, respectively). In the mucosa-removal groups, the histology scores of the newly formed bone areas were significantly higher in the bone, cartilage and Regenafil groups than in the Mimix group ( $p = 0.001$ ; Figure 2A). In the mucosa-conservation groups, the scores for newly formed bone areas were significantly higher in the bone and cartilage groups than in the other groups ( $p < 0.001$ ; Figure 2B). The bone and Regenafil groups showed significant differences in scores for new bone formation between the mucosa-removal and mucosa-conservation groups ( $p = 0.016$  and  $p < 0.001$ , respectively; Figure 3). The other materials caused no significant difference in new bone formation between the mucosa-removal and mucosa-conservation groups. In the cartilage group, the mastoid bullae were filled with new bone regardless of mucosa removal, and the difference in new bone formation between the two groups was the smallest for the four materials (Figure 1C and 1D).

After 1 year, all bone grafts exhibited definite collapse into the bulla at the edge of the surgically created bony defect compared with the groups obliterated with other materials (Figure 1). In the cartilage group, the process of endochondral bone formation was observed sporadically (Figure 1C and 1D). In the Regenafil group with removed mucosa, no residual Regenafil was observed (Figure 1E), but the implanted Regenafil remained in the mucosa-conservation group even after 1 year (Figure 1F). In the Mimix group, the implanted Mimix remained, regardless of mucosal removal (Figure 1G and 1H).



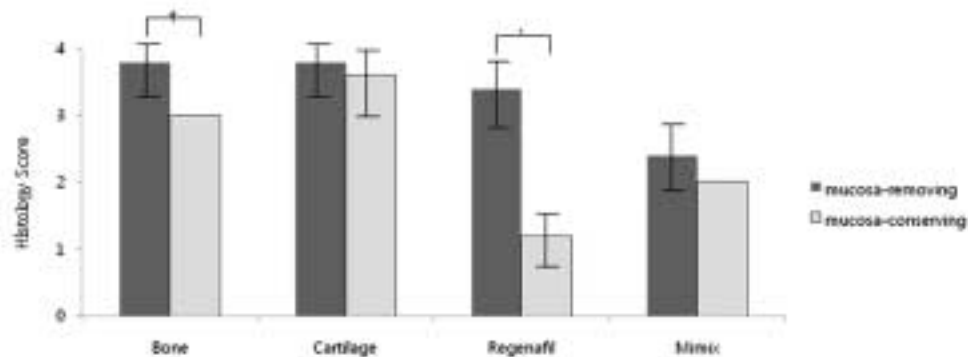
**Figure 1.** Light microscopy of four obliterating materials. (A) The bone group with removed bulla mucosa. The mastoid bulla is filled with implanted bone chips and new bone. There is definite collapse (filled arrow) into the bulla at the edge of the surgically created bony defect. (B) The bone group with conserved bulla mucosa showing a number of mucocoeles (filled arrows). There is definite collapse (open arrow) into the bulla at the edge of the surgically created bony defect like the mucosa-removal group. (C) The cartilage group with removed bulla mucosa. The bulla has been obliterated completely by newly formed bone. (D) The cartilage group with conserved bulla mucosa. There are a small number of mucocoeles (filled arrows) even up to 1 year after obliteration. (E) The Regenafile group with removed bulla mucosa. Vigorous new bone formation is evident without the inflammatory response. (F) The Regenafile group with conserved bulla mucosa showing a low level of new bone formation. Implanted Regenafile (filled arrow) remained up to 1 year after obliteration. Regenafile induced the marked formation of the mucocoeles (open arrows). (G) The Mimix group with bulla mucosa removed showing a marked inflammatory response (filled arrow) around the residual material (open arrow). The image shows a low level of new bone formation and a high level of residual Mimix. (H) Mimix group with conserved bulla mucosa showing a marked inflammatory response (filled arrow) around the residual material (open arrow). The image shows a low level of new bone formation and a high level of residual Mimix. There are a small number of mucocoeles (filled arrowheads) (hematoxylin and eosin; original magnification, x20).



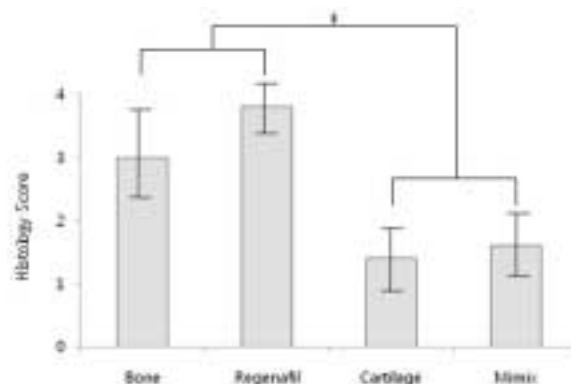
**Figure 2.** Comparison of new bone formation between the four obliterating materials. (A) The mucosa-removal groups. \* The histology scores for newly formed bone areas were higher in the bone, cartilage, and Regenafile groups than in the Mimix group (one-way ANOVA;  $p = 0.001$ ). (B) The mucosa-conservation groups. \* The histology scores for newly formed bone were significantly higher in the bone and cartilage groups than in the other groups (one-way ANOVA;  $p < 0.001$ ).

In the Mimix group, histological findings performed at 1 year showed a marked inflammatory response around the residual material in all specimens regardless of mucosal removal (Figure 1G and 1H).

The bone, cartilage and Regenafile groups showed no evidence of inflammation, such as lymphocyte infiltration, in the bullae.



**Figure 3.** Comparison of new bone formation between the mucosa-removal and mucosa-conservation groups. The bone (\*) and Regenafl (†) groups differed significantly between the mucosa-removal and mucosa-conservation groups (t test;  $p = 0.016$  and  $p < 0.001$ , respectively).



**Figure 4.** Comparison of mucocoele formation between the four obliterating materials in the mucosa-conservation group. \* The histology scores for mucocoele areas were higher in the bone and Regenafl groups than in the other groups (one-way ANOVA;  $p < 0.001$ ).

In all mucosa-removal groups, no significant mucocoele formation was seen around the implants on histological examination. The bone and Regenafl groups with conserved mucosa showed marked formation of mucocoeles and reduced new bone formation in proportion to the mucocoele formation. Mucocoele formation was significantly greater in the bone and Regenafl than in the groups treated with other materials ( $p < 0.001$ ; Figure 4).

## Discussion

When used for MO, the bone lends itself for use as a paté or chip. The main problem with bone grafts is that they lose volume with time, leading to the development of a cavity<sup>[3,9]</sup>. The process of remodeling usually reduces the size of the bone graft by 15%- 25%, thus impairing the outcome of the reconstruction<sup>[10]</sup>. Insufficient amounts of material, donor site morbidity, poor quality and limited

shapes and sizes of available bone are common problems when autogenous bone is used<sup>[11]</sup>. However, many reports have described the successful use of autogenous bone grafts for MO after long-term follow-up<sup>[2,7,12-14]</sup>. The bone grafts have good capacity for proper healing, resistance to infection, capability to restore hearing and resolution of the symptoms of a problematic cavity<sup>[2]</sup>. Histopathological analysis of cadaveric MO showed that the bone chips and paté used as the MO tissue retained their bulk and identity 5 years after surgery<sup>[7]</sup>. Roberson et al.<sup>[12]</sup> reported that MO with autogenous cranial bone had a bone graft success rate of 95% of 62 cases during a mean follow-up of 18.5 months. Ramsey et al.<sup>[13]</sup> reviewed 60 cases where an inferiorly pedicled, periosteal-pericranial flap in conjunction with autogenous bone paté, produced a dry, trouble-free cavity in 90% of the cases with a minimum follow-up of 1 year.

In our current study, the mastoid bullae were filled completely with bone tissue at 1 year in the bone group. However, the bone graft at 1 year exhibited definite collapse into the bulla at the edge of the surgically created bony defect. To solve this problem, the surgeon should consider covering the bone graft in its entirety with a pedicled flap or a cartilage plate. Many investigators have emphasized the need for complete coverage of the bone material with fascia, a pedicled flap or a cartilage plate<sup>[1,12,13]</sup>. Using MO and canal wall reconstruction with a composite multi-fractured osteoperiosteal flap, Ucar<sup>[14]</sup> found that new bone formation filled the mastoid cavity within 2 years. This technique offers a good blood supply to the bone chips used in MO and may be considered as a MO method for use with a bone graft.

Cartilage has a long history of use as a graft material in ear surgery<sup>[2,4,15]</sup>. Cartilage is nourished largely by diffusion and seems to offer high resistance to both the lack of vascularization and infection. Clinical studies show that cartilage is well tolerated by the middle ear and long-term survival is the norm<sup>[15]</sup>. Cartilage is also cost effective and easily harvested from the tragus, conchal bowl and cyma area. Numerous reports have described the use of cartilage as an obliterating material<sup>[2,4]</sup>. Tympanomastoid obliteration using tragal or conchal cartilage has good integration properties<sup>[4]</sup>. A technique to partially obliterate the mastoid cavity with autogenous cartilage chips reliably produces dry, trouble-free cavities<sup>[4]</sup>. In our study, obliterating the mastoid bulla completely with the cartilage chips produced excellent results, including vigorous new bone formation, little resorption and no inflammatory response. Unlike the method using bone grafts, there was no definite collapse into the bulla at the edge of the surgically created bony defect.

Despite its many advantages, a major disadvantage of autogenous cartilage is the limited amount that can be obtained from the concha and tragus. Adequate amounts of cartilage are often not available to sufficiently obliterate the mastoid cavity. One method to supplement cartilage is to use costal or nasal septal cartilage, but morbidity of the donor site cannot be

avoided. In the past, this problem was dealt with by using homograft cartilage, but the fear of transmitting infectious diseases has curtailed its use. Another method is to use artificial cartilage, but many studies are ongoing to test the clinical use of synthetic cartilage. Because they cannot provide the full amount of obliterating material needed, cartilage chips are placed first in the critical areas, requiring the use of other materials to complete the obliteration<sup>[4,16]</sup>.

DBM is a potential alternative or supplement to autogenous bone grafts. DBM has been used with success in orthopedic and craniofacial reconstruction, and has high success rates for union of fractures, filling cavity defects and production of both laminar and woven bone within the matrix. DBM does not contain osteoprogenitor cells, and the efficacy of DBM as a bone graft substitute or extender may be influenced by a number of factors, including the sterilization process, the carrier, the total amount of bone morphogenetic protein present and the ratios of the different bone morphogenetic proteins present<sup>[17]</sup>. Regenafile paste uses a gelatin delivery system to deliver human DBM in surgery. The gelatin is biologically inert, permits revascularization, and is resorbed entirely from the graft site. The handling characteristics of Regenafile permit positioning of the graft with lessened risk of graft migration, and it is simple to use in the operative field.

The DBM has been used successfully in MO in many surgical areas. Leatherman et al.<sup>[5]</sup> evaluated the use of human DBM as a graft material for MO in rats 9 weeks after obliteration and observed a high level of new bone formation, good wound healing, minimal inflammatory response and lack of short-term resorption. In another study, the same research group applied human DBM in 11 patients receiving MO and evaluated its use for a mean follow-up of 14.5 months<sup>[16]</sup>. The DBM as a graft extender in MO allowed them to create a dry, smooth-contoured canal in all patients. We observed satisfactory new bone formation after 1 year. Unlike Mimix, Regenafile showed no inflammatory response for 1 year and, like the two natural materials, a high level of new bone formation.

Our results suggest that DBM is an acceptable graft alternative for revision MO when the autogenous material is insufficient. Differences in the osteoinductive potentials of commercially available DBMs have been reported<sup>[17]</sup>. Clinical reports on the use of DBM indicate that preparations of this material can have varying effects on bone formation<sup>[17]</sup>. We examined the efficacy of MO using only Regenafile, and further studies are needed to compare the efficacy of various kinds of commercially available DBMs in MO.

The most promising alloplastic material for bone substitution is HA, a bioactive ceramic with a molecular structure similar to that of human bone<sup>[10]</sup>. Its peculiarity lies in its osteogenicity and it does not induce any phylogistic reaction of the foreign body type<sup>[10]</sup>. A number of investigators have reported that porous HA granules offer the best results in MO<sup>[3,9,18-20]</sup>. Takahashi and Nakano<sup>[18]</sup> reported details of experimental studies in guinea pigs where implanted HA granules did not undergo morphological changes in the bullae for 1 year. The bullae were filled completely with bone tissue, including the HA granules, the bone and HA granules interdigitated tightly. Yung<sup>[19]</sup> reported an excellent outcome of MO using HA granules and an inferiorly based periosteal flap in humans. Estrem and Highfill<sup>[20]</sup> had an 87% success rate after MO with only HA granules in patients followed up for a mean of 42.7 months.

HA cement is easily applied as a paste that hardens in 6-20 minutes and is easily contoured, a property that is highly desirable in MO surgery<sup>[21]</sup>. HA cement is a calcium phosphate preparation that hardens as microporous HA<sup>[22]</sup>. Dornhoffer and Simmons<sup>[23]</sup> used Mimix for MO in a gerbil model. After 6 weeks, most specimens showed new woven bone ingrowth at the bone-implant interface, with active osteoblasts and viable lacunae cells and no inflammatory response. Hussain et al.<sup>[24]</sup> performed MO and canal wall reconstruction with HA cement and the postauricular flap. Only 3 of their 29 patients developed complications, 1 of whom required revision during the mean follow-up period of 21.6 months. Mahendran

and Yung<sup>[21]</sup> reported that, in their clinical experience with MO and HA cement, the material is disappointing because of a high infection rate, especially when compared with the excellent outcome of HA granules. In their series, there was a significant incidence of postoperative infection, and 50% of the patients required revision surgery and removal of the foreign material during a minimum follow-up of 1 year. They suggested that HA cement has an unacceptable rate of postoperative infection, possibly because it is nonporous and does not allow fluid or blood accumulated beneath it to escape<sup>[9]</sup>.

Our histological findings showed a marked inflammatory response and a low level of new bone formation with Mimix. The most likely reason for the severe inflammatory response in use of Mimix may relate to the insufficient space inside and between the HA particles. It appears that the poor formation of the pores inside and between the HA particles results in insufficient revascularization, which causes an inflammatory response. The pores inside and between the HA granules allow ingrowth of bone and fibrous tissue<sup>[9]</sup>. A prerequisite of rapid osteoconduction is rapid revascularization between the host bone and graft bone<sup>[25]</sup>. Regenafile paste uses a gelatin delivery system that permits revascularization, which could be suitable for MO. Successful outcomes during long-term follow-up have been reported for HA cement used in a relatively small area with good blood supply, such as for the repair of temporal bone defects, scutumplasty and ossiculoplasty<sup>[22,26,27]</sup>. Because the environment for MO comprises a relatively large area with poor blood supply, HA cements used for MO produce variable results. Saunders<sup>[27]</sup> suggested that the use of HA bone cements should be limited to relatively large defects that do not lend themselves to autogenous repair. Mimix HA cement may be considered a material to partially obliterate the mastoid cavity.

Many researchers have studied the materials used for MO in the mucosa-free condition. The surgeon should remove the mastoid mucosa completely before MO, although it may occasionally remain in the site for MO if the mastoid cavity in this area is obliterated. Because

of this clinical reason, we hypothesized that the outcome can vary depending on the obliterating material and whether the mastoid mucosa remains. To our knowledge, the obliterating material for MO has not been evaluated in the mucosa-conservation condition during long-term follow-up. The excellent results obtained with cartilage chips suggest that bone chips and Regenafile should not be used in MO in the mucosa-conservation condition. The cartilage chips in the mucosa-conservation condition induced a high level of new bone formation and a low level of mucocele formation. In the bone and Regenafile groups, a high level of mucocele formation was a problem.

The amount of autogenous cartilage that can be obtained from the concha and tragus is limited. During surgical intervention, attempts were first made to obtain adequate cartilage for obliteration from the local operative area. Our results suggest that the cartilage placement plays an important role in the results of MO. Dornhoffer<sup>[4]</sup> suggested that the most important areas of cartilage placement for partial MO are the perilyabyrinthine cells and adjacent retrofacial cells because they are the most frequent sites of debris and granulation collection leading to revision surgery. A combination of cartilage and other materials, such as bone and DBM, for MO can overcome the limited amount of the obliterating materials. After solving the problem relating to the amount of obliterating materials, cartilage placement would first be performed at the doubtful area where the mastoid mucosa may remain before MO. Because it is a reliable graft material, the cartilage chips could also be used in the epitympanic area because this area is probably the most critical area for preventing recurrent retraction and cholesteatoma<sup>[28]</sup>. At our hospital, the epitympanoplasty with MO has been performed since 1994, and the cartilage chips for MO have been used for both the epitympanic area and where the mastoid mucosa is likely to remain<sup>[29]</sup>. Our current results support the idea that this is a reasonable method.

## Conclusion

Our study shows that cartilage, bone, and Regenafile are appropriate graft materials for MO in the rat and that cartilage is suitable for MO in the mucosa-conservation condition. Mimix HA cement was unsatisfactory for MO in the rat because it causes a marked inflammatory response. Our study is expected to be helpful in selecting the obliterating materials for use in MO.

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