ORIGINAL ARTICLE doi: 10.2176/nmc.oa.2013-0295

Neurol Med Chir (Tokyo) 54, 647–653, 2014 Online March 27, 2014

Evaluation of Antibiotic-loaded Calcium Phosphate Bone Cement in a Cranium-infected Experimental Model

Yoshiaki SAKAMOTO,[1] Hiroko OCHIAI,[2] Ikuko OHSUGI,[3] Yoshikazu INOUE,[3] Yoko YOSHIMURA,[3] and Kazuo KISHI[1]

[1]*Department of Plastic and Reconstructive Surgery, Keio University School of Medicine, Tokyo;*
[2]*Department of Plastic and Reconstructive Surgery, National Hospital Organization Tokyo Medical Center, Tokyo;*
[3]*Department of Plastic and Reconstructive Surgery, Fujita Health University School of Medicine, Toyoake, Aichi*

Abstract

Treatment of calvarial defects has remained a challenge in reconstruction surgery, especially because of infection at these sites. We produced a bactericidal biomaterial for treating infected bone defects by using calcium phosphate bone cement mixed with antibiotics. We evaluated the usefulness of this material mixed with the antibiotic vancomycin in a cranium-infected rat model. The concentration of vancomycin used was 5.0 wt%, as reported in our previous study. In order to establish the rat model, a cranium defect (diameter, 5 mm) was made that was infected with methicillin-resistant *Staphylococcus aureus* (MRSA). Thirty-six rats were divided into 6 groups depending on whether an autologous graft or bone cement with or without antibiotic was used for the defect. After 1 and 4 weeks, abscess formation was checked, tissue bacterial counts were determined, and pathological examination was performed. At both 1 and 4 weeks, no MRSA was detected on tissue bacterial culture or pathological examination in groups that received bone cement with antibiotics. In groups that received bone cement without antibiotic, MRSA was detected, and the bone cement had compromised and disintegrated into several slices. In conclusion, bone cement that contains antibiotics appears to be effective not only for reconstruction in cases of cranial defect, but also in terms of preventing infection.

Key words: calcium phosphate cement, hydroxyapatite, antibiotics, methicillin-resistant Staphylococcus aureus (MRSA)

Introduction

Treatment of calvarial defects remains a distinct challenge in reconstructive surgery. Clinical circumstances often require the reconstruction of an infected and chronically scarred wound. Standard treatment for these cases involves reconstruction using the autologous bone. However, standard, autogenous bone donor sites may yield small amounts of tissue and expose the patient to significant donor-site morbidities such as infection, pain, hemorrhage, and nerve injury, which is the case in up to 10% patients with such defects.[1)]

Additionally, reconstruction using artificial bones, such as those constructed from titanium, hydroxyapatite, or other ceramics, is contraindicated because these constructs must be removed when infection spreads to the artificial bones. However, we hypothesized that if the infection were controlled, it might be possible to use artificial bones that would effectively eradicate infection.

The bactericidal or bacteriostatic effects of the antibiotic generally depend on the local concentration of the antibiotics. Therefore, a local delivery system enabling a sustained high concentration of antibiotics might be effective for controlling infections in calvarial defects.

In infected hip or knee replacements, treatment with calcium phosphate cement (CPC) beads with a high dose of antibiotics has proven useful.[2,3)] This method can probably be applied for cranial reconstruction. However, before clinical application of the procedure, an animal experiment is required

Received September 3, 2013; Accepted October 25, 2013

to confirm the efficacy of this treatment. Therefore, in this study, we evaluated the effect of antibiotic-loaded CPC in the methicillin-resistant *Staphylococcus aureus* (MRSA)-infected experimental rat model.

Materials

I. Bone cement

In this study, we used Ceratouch bone cement (Ngk Spark Plug Co., Ltd, Aichi), which consists of powder [tetracalcium phosphate (TTCP) and anhydrous dibasic calcium phosphate] and a sclerosed solution (dextran sulfate sodium). Both the materials were mixed to produce hydroxyapatite (HA), according to the following reaction: $2Ca_4(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2$. This reaction is slow, which allows us to mold the cement before it hardens after approximately 90 minutes at 25°C (i.e., room temperature, *in vitro*), and approximately 9 minutes at 37°C (i.e., *in vivo*). While hardening, the consistency of the cement remains at 50–200% for 17 minutes at 25°C. After 8 hours, the Ceratouch reaches its maximum compressive strength of approximately 50 Mpa.

The hardened Ceratouch has a porosity of 35%, and both open and closed pores have a ratio of 17.5%. Each pore is < 0.1 μm in diameter.

II. MRSA and Antibiotics

MRSA was clinically isolated from a surgical wound of a patient who had undergone neurosurgery, and the isolate was allowed to grow overnight on mannitol salt agar, after which individual colonies were incubated after 12 hours at 35°C. The cultured cells were then suspended in phosphate buffered saline (PBS) at a concentration of approximately 1.0×10^6 colony-forming units (CFU)/ml.

Vancomycin (Shionogi Pharmaceutical, Osaka) was used as the antibiotic in our study; the optimal concentration to be mixed with CPC was 5.0 wt%, as obtained in our previous study.[4]

III. Sutures

Rats, mice, and other rodents are convenient models for handling; however, inducing infections in these animals is difficult as infections tend to heal in most cases, even after inoculation with bacteria.[5,6] Therefore, it is necessary to prepare wounds surgically. For this, researchers have attempted to retain foreign bodies such as sutures,[7,8] sand,[9] or dextran beads.[10,11] Unlike sutures, sand and beads have not been used clinically. Therefore, in this study, we used sutures since their use has been reported in clinical medicine. We chose sutures from Surgion (Covidien, Mansfield, Massachusetts, USA), which are inert, non-absorbable sutures composed of the long-chain, aliphatic polymers Nylon 6 and Nylon 6-6.

Methods

Male Slc:SD rats (weight, 200 g) were used in this study. Rats were anesthetized intraperitoneally using sodium thiopental (30 mg/kg). After this, the hair on their heads was removed with clippers; through a midline skin incision, the parietal skull was exposed, and a cranial bone defect of 5 mm in diameter was created bilaterally using a surgical tome (Fig. 1). We then placed sutures at the defect sites. Either only PBS without MRSA (Groups 1, 3, 5) was injected or a 0.1 ml MRSA suspension (Groups 2, 4, 6) was injected into the bone defect. In each group, CPC with 5.0 wt% vancomycin (Groups 3 and 4) or CPC without vancomycin (Groups 5 and 6) was implanted into the defect. Finally, in Groups 1 and 2, the removed cranial bone was simply put back in place (Fig. 2). Thus, in all there were 6 groups with 3 rats each (Table 1). The procedure was performed on both sides of the rat cranium in each rat.

After surgery, all rats were housed in cages and provided food and water ad libitum. No dressing was applied. At the end of 1 and 4 weeks, the different groups of rats were sacrificed, and the calvarias were dissected to evaluate the degree of the defect repair. Six sides of three rats were assigned to each experimental group.

Of those, 2 × 2 mm of granulation tissues were taken from three sides of the rats in each group. These samples were transferred to a sterile bottle containing 10 ml of physiological saline and were homogenized for 2 minutes. A mannitol salt agar plate was inoculated with 10,000 times dilution of

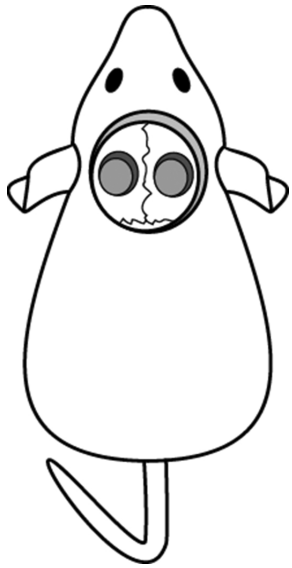

Fig. 1 Schema of the experimental model.

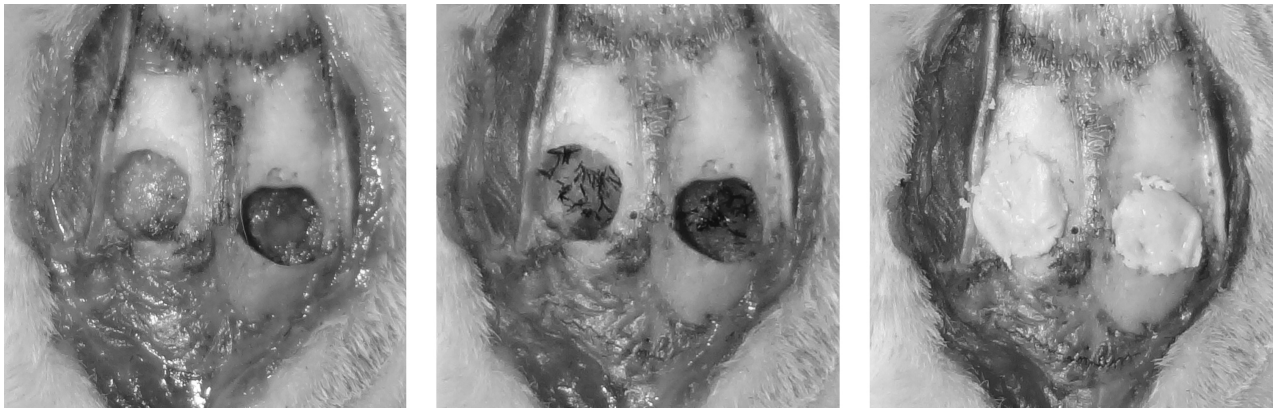

Fig. 2 Intraoperative view of the rat cranium *Left*: defect, *Center*: immediately after sutures are placed at the defect site, *Right*: immediately after the implantation of the calcium phosphate cement at the defect site.

0.1 ml of the solution and incubated for 24 hours at 37°C, after which time total viable counts were taken.

In the other three sides of the three rats from each group, the calvariae were harvested, fixed in 10% neutral buffered formalin, and processed for paraffin embedding. The specimens were sectioned in the coronal plane at a thickness of 5 μm and stained with hematoxylin-eosin for observation under a conventional qualitative, bright-field light microscope.

Table 1 The difference in each group

	Injection	Reconstruction
Group 1	PBS without MRSA	Autologous cranium
Group 2	MRSA suspension	Autologous cranium
Group 3	PBS without MRSA	CPC with vancomysin
Group 4	MRSA suspension	CPC with vancomysin
Group 5	PBS without MRSA	CPC without vancomysin
Group 6	MRSA suspension	CPC without vancomysin

CPC: calcium phosphate cement, MRSA: methicillin-resistant *Staphylococcus aureus*, PBS: phosphate buffered saline.

Results

I. Macroscopic appearances

All groups that were not infected with MRSA and those that received the antibiotic had intact skin and no pyogenic mass in the subcutaneous layer at both 1 and 4 weeks.

Group 2 that received the autologous cranium and infection but no antibiotic had damaged skin and pyogenic masses in the subcutaneous layer after 1 week, but the wound was repaired and intact without pyogenic masses after 4 weeks. However, the grafted autologous cranium had changed in color and seemed to have undergone necrosis.

Similarly, Group 6 that received CPC and infection but no antibiotic, had damaged desiccated skin with pyogenic masses in the subcutaneous layer after 1 week. However, after 4 weeks, the CPC had disintegrated, and granulation was observed in the calvarial defect (Figs. 3, 4).

II. Histology

The histologic analysis of Group 1 that received PBS and the autologous graft showed fibrous connective tissue around the grafted bone and neutrophils concentrated around the suture at 1 week. Similarly, both Groups 3 and 5 that did not receive infection but had undergone reconstruction with CPC with or without the antibiotics showed fibrous connective tissue and neutrophils around the artificial bone in their 1-week histologic analysis.

The microscopic analysis at 1 week in Groups 2, 4, and 6 that had received infection indicated the presence of many neutrophils around the grafts and sutures. However, the number of neutrophils in Group 4 that received CPC with vancomycin was less than that in the two other groups (Fig. 5).

In contrast, the microscopic analysis at 4 weeks, of Groups 1, 3, and 5 that were not infected, showed few neutrophils; however, that of Groups 2 and 6 has still showed neutrophil permeation, indicating the presence of MRSA infection. Additionally, the grafted autologous bone in Group 2 was thinner than that in Group 1. The artificial bone in Group 6 lost its original shape. However, in Group 4 that had received the antibiotic and CPC, the microscopic analysis indicated that the neutrophils had disappeared and the artificial bone had retained its shape. Fibrous connective tissue was observed between the conventional bone and the artificial bone, as observed in Groups 3 and 5 (Fig. 6).

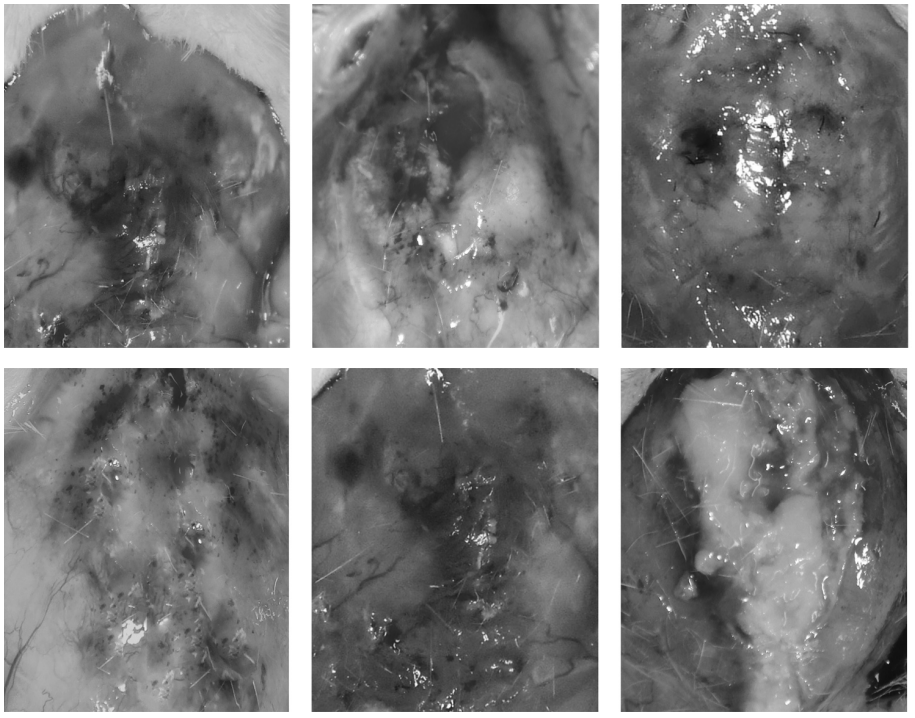

Fig. 3 Macroscopic appearance of the cranium at 1 week, (from *left*) Groups 1–6. Note that Groups 2 and 6 had detected pyogenic masses in the subcutaneous layer. The other four groups, pyogenic masses could not be detected.

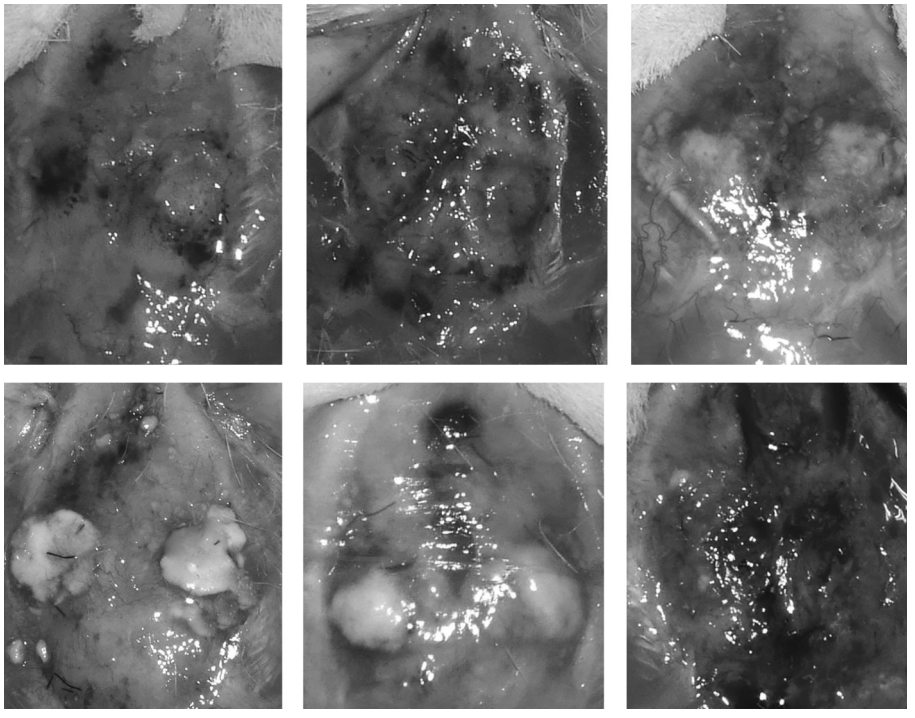

Fig. 4 Macroscopic appearance of the cranium at 4 weeks, (from *left*) Groups 1–6. Note that the grafted autologous cranium had changed in color and seemed to have undergone necrosis in Group 2. In Group 6, the CPC had disintegrated, and granulation was observed in the calvarial defect. The other four groups, both grafted autologous cranium and CPC were seen to be intact. CPC: calcium phosphate cement.

III. Bacterial counts

The number of viable bacteria present in all biopsy specimens is shown in Fig. 7. In Groups 1, 3, and 5, at both 1 and 4 weeks, no MRSA colony was observed. In Groups 2 and 4, the colony counts decreased observed at 4 weeks had reduced compared to that at 1 week, and the difference was significant ($p < 0.05$). In contrast, there was no significant difference in colony counts in Group 4 between 1 and 4 weeks ($p = 0.28$). The colony count in Group 4 was smaller than that in the other groups, and the difference was significant ($p < 0.05$).

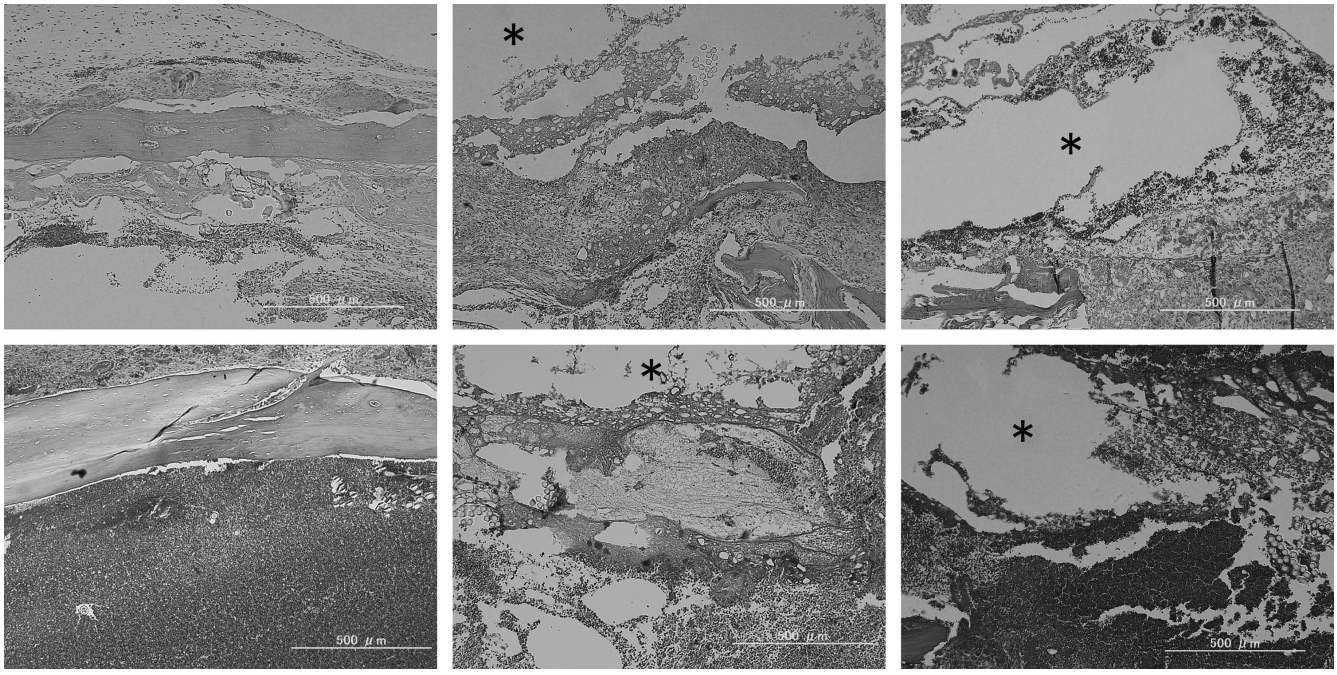

Fig. 5 Histologic analysis using hematoxylin and eosin stain at 1 week. *The mark* "*" identifies bone cement. *Above*: Groups 1, 3, and 5. *Below*: Groups 2, 4, and 6. Note that Groups 1, 3, and 5 showed fibrous connective tissue and neutrophils around the grafted bone. Groups 2, 4, and 6 indicated presence of many neutrophils around the grafts and sutures.

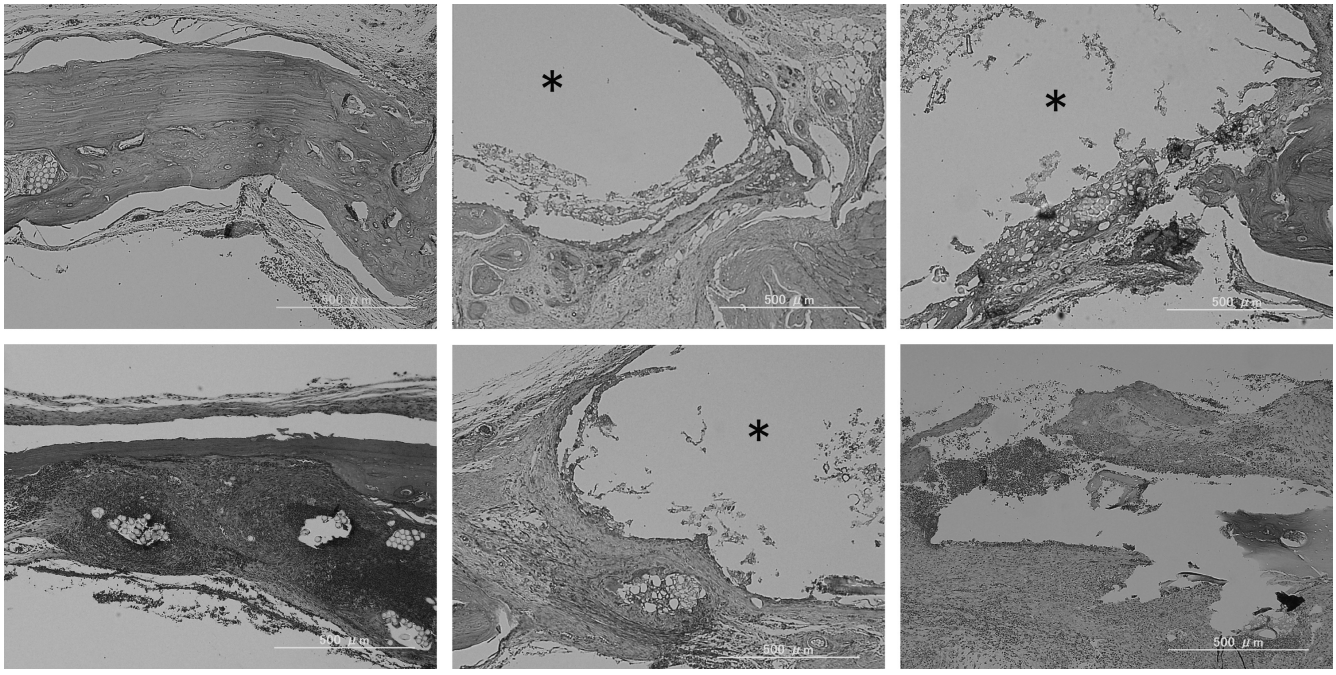

Fig. 6 Histologic analysis using hematoxylin and eosin stain at 4 weeks. *The mark* "*" identifies the bone cement. *Above*: Groups 1, 3, and 5. *Below*: Groups 2, 4, and 6. Note that Groups 1, 3, 4 and 5 showed few neutrophils. Especially, Group 4 indicated that the neutrophils had decreased sharply compared to at 1 week and the artificial bone had retained its shape. Instead, Groups 2 and 6 had still shown neutrophil permeation. The grafted autologous bone in Group 2 was thinner than that in Group 1. The artificial bone in Group 6 lost its original shape.

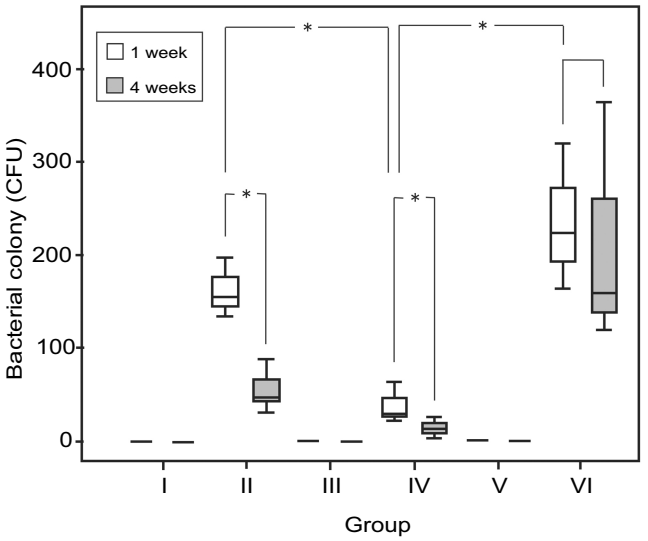

Fig. 7 Bacterial colony counts in each group. *The mark* "*" indicates $p < 0.05$, which is a significant difference.

Discussion

Currently, the commercially available antibiotics delivery system is antibiotics-loaded polymethyl methacrylate bone cement.[12,13] The most frequently used treatment has been CPC, which consists of TTCP and educted HA by a hydration reaction.[14–16]

According to previous *in vitro* study, the compressive strengths of CPC is lowered with antibiotics than without antibiotics and is < 40 Mpa.[4] This suggests that mixed cements would be difficult to use in joint arthroplasty, as vertebra spacers, for example, or for supplementation of femur defects. This is because these sites require compressive strengths of approximately 133 Mpa.[17] In contrast, the compressive strength of the skull is at least 31.1 Mpa.[18] Therefore, CPC with antibiotics might be useful in skull reconstruction.

Therefore, in the present study, infection sites were prepared at the cranium in the rat, and the efficiency of antibiotic-loaded CPC was verified. To our knowledge, this is the first report to examine antibiotics-loaded CPC *in vivo*.

Our study showed that antibiotic-loaded CPC was useful for controlling infection. VCM activity is considered to be time-dependent; that is, antimicrobial activity depends on the duration that the serum drug concentration exceeds the minimum inhibitory concentration (MIC) of the target organism. The half-life of VCM was 4–5 hours, and VCM activity from single local administration with maximum dose was observed only for 12 hours.[19] Instead, our previous preliminary study noted that sustained release of VCM with exceeding the MIC from antibiotic-loaded CPC was observed for about 20 days.[4] It has been suggested that CPC has a microstructure and nano-network pass, which are useful for bone conduction, and would allow antibiotics diffusion.[2–4]

From the same reason, the CPC might be useful for other time-dependent antibiotics such as cephem-based and penicillin-based antibiotics might be useful as a drug carrier. However, our previous preliminary study noted that each antibiotic showed different influences on the characteristics of the bone cement.[4] We are going to study about the release rate of various antibiotics and mechanical properties of bone cements.

Considering clinical use, the most effective method is reconstruction for cranial defect after debridement; thus, in combination with antibiotics-loaded CPC and debridement, a high rate of efficiency is expected. Furthermore, the other advantage is that simultaneous cranial reconstruction can be performed without donor site morbidity. In principle, the alloplastic have to be removed from the infection area, and cannot be used until controlling the infection. Otherwise, the use of the autologous graft tissue is recommended for simultaneous reconstruction. The use of antibiotics-loaded CPC can prevent the repeat operation and donor site morbidity.

Our study had certain limitations. This is an *in vivo* study using rats, and there are considerable biological differences between rats and humans. In particular, rats, mice, and other rodents mount strong immune defenses againt local infections, even after inoculation with bacteria.[5] In fact, our results also show that an autologous bone graft in Group 2 that received infection with MRSA alleviated the infection, although the thickness of bone grafts would be reduced by absorption. However, as all the specimens were prepared and tested in a uniform and reproducible manner and significant differences were observed between autologous bone graft, bone cement with antibiotics, and bone cement without antibiotics, we believe that these results provide useful information.

Additionally, long-term examinations, particularly on the factors that limit antibiotic exposure to the infection site in bone conduction, will be necessary.

In summary, this *in vivo* study demonstrated theoretical advantages with bone cement mixed with antibiotics. Unlike other cements that have been studied, bone cement with a microstructure and nano-network may be useful not only as a filler for cranial reconstruction, but also as a drug carrier. Future studies should examine longer release times of the antibiotics or the use of other drugs, such as bone morphogenetic protein.

Conflicts of Interest Disclosure

The authors did not receive any equipment, materials, or financial support from outside, to make this study. None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this manuscript.

References

1) Banwart JC, Asher MA, Hassanein RS: Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. *Spine* 20: 1055–1060, 1995
2) Kisanuki O, Yajima H, Umeda T, Takakura Y: Experimental study of calcium phosphate cement impregnated with dideoxy-kanamycin B. *J Orthop Sci* 12: 281–288, 2007
3) Niikura T, Tsujimoto K, Yoshiya S, Tadokoro K, Kurosaka M, Shiba R: Vancomycin-impregnated calcium phosphate cement for methicillin-resistant *Staphylococcus aureus* femoral osteomyelitis. *Orthopedics* 30: 320–321, 2007
4) Sakamoto Y, Ochiai H, Ohsugi I, Inoue Y, Yoshimura Y, Kishi K: Mechanical strength and in vitro antibiotic release profile of antibiotic-loaded calcium phosphate bone cement. *J Craniofac Surg* 24: 1447–1450, 2013
5) Bunce C, Wheeler L, Reed G, Musser J, Barg N: Murine model of cutaneous infection with gram-positive cocci. *Infect Immun* 60: 2636–2640, 1992
6) Ogaard AR, Lausund P, Berdal BP: Observations on Pseudomonas aeruginosa proteolytic and toxic activity in experimentally infected rats. *NIPH Ann* 15: 99–109, 1992
7) Boon RJ, Beale AS: Response of *Streptococcus pyogenes* to therapy with amoxicillin or amoxicillin-clavulanic acid in a mouse model of mixed infection caused by *Staphylococcus aureus* and *Streptococcus pyogenes*. *Antimicrob Agents Chemother* 31: 1204–1209, 1987
8) Gaar E, Naziri W, Cheadle WG, Pietsch JD, Johnson M, Polk HC: Improved survival in simulated surgical infection with combined cytokine, antibiotic and immunostimulant therapy. *Br J Surg* 81: 1309–1311, 1994
9) Fallon MT, Shafer W, Jacob E: Use of cefazolin microspheres to treat localized methicillin-resistant *Staphylococcus aureus* infections in rats. *J Surg Res* 86: 97–102, 1999
10) Barg N: Comparison of four antibiotics in a murine model of necrotizing cutaneous infections caused by toxigenic *Streptococcus pyogenes* and *Staphylococcus aureus*. *J Antimicrob Chemother* 42: 257–260, 1998
11) Kernodle DS, Kaiser AB: Comparative prophylactic efficacy of cefazolin and vancomycin in a guinea pig model of *Staphylococcus aureus* wound infection. *J Infect Dis* 168: 152–157, 1993
12) van de Belt H, Neut D, Schenk W, van Horn JR, van der Mei HC, Busscher HJ: Infection of orthopedic implants and the use of antibiotic-loaded bone cements. A review. *Acta Orthop Scand* 72: 557–571, 2001
13) Lewis G: Properties of antibiotic-loaded acrylic bone cements for use in cemented arthroplasties: a state-of-the-art review. *J Biomed Mater Res Part B Appl Biomater* 89: 558–574, 2009
14) Gilardino MS, Cabiling DS, Bartlett SP: Long-term follow-up experience with carbonated calcium phosphate cement (Norian) for cranioplasty in children and adults. *Plast Reconstr Surg* 123: 983–994, 2009
15) Larsson S: Calcium phosphates: what is the evidence? *J Orthop Trauma* 24(Suppl 1): S41–S45, 2010
16) Schbettler R, Sahl JP, Alt V, Pavlidis T, Dingeldein E, Wenisch S: Calcium phosphate-based bone substitutes. *Eur J Trauma* 30: 219–229, 2004
17) Carter DR, Spengler DM: Mechanical properties and composition of cortical bone. *Clin Orthop Relat Res* 192–217, 1978
18) Evans FG, Lissner HR: Tensile and compressive strength of human parietal bone. *J Appl Physiol* 10: 493–497, 1957
19) Levine JF: Vancomycin: a review. *Med Clin North Am* 71: 1134–1145, 1987

Address reprint requests to: Yoshiaki Sakamoto, MD, Department of Plastic and Reconstructive Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan.
e-mail: ysakamoto@z8.keio.jp