In vitrolin vivo evaluation of the efficacy of gatifloxacin-loaded PLGA and hydroxyapatite composite for treating osteomyelitis

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Molten 10 wt% gatifloxacin (GLFX-loaded poly(lactide-co-glycolide) (PLGA) was introduced into three-dimensionally interconnected pores and onto the surfaces of hydroxyapatite (HA) granules. The composite granules exhibited clinically sufficient bactericidal activities against *Streptococcus milleri* and *Bacteroides fragilis* from 3 h to 10 days. The composite granules were implanted in bone defects created by debridement of osteomyelitis lesions in rabbit mandibles. After 4-week implantation, inflammation in the composite granule-implanted group was significantly smaller than that in the debridement group ($p < 0.05$). Moreover, newly formed bone was observed in the pores and on the surface of HA granules of the composite. These findings show that GFLX/HA composite controls bacterial infection and supports bone regeneration for osteomyelitis treatment.

Keywords: Osteomyelitis, Bone regeneration, Drug delivery system, Poly(lactide-co-glycolide), Hydroxyapatite

INTRODUCTION

Osteomyelitis is induced by trauma, surgical stress, and bacterial invasion from other infected sites^{1–3}. Mandibular osteomyelitis is often caused by the progression of apical lesions and periodontitis, the spread of secondary infection from impacted teeth, and infection from extraction sockets. Acute inflammation in mandibular osteomyelitis likely progresses to chronic inflammation, which is an intractable symptom often recurring after the patient is cured⁴. The common treatments for osteomyelitis are systemic administration of antimicrobial agents *via* oral or intravenous routes and surgical therapy^{5,6}. However, when immune cells accumulate in the affected area during the immune response to bacteria, thereby causing tissue degeneration and ischemia, it is difficult to deliver antimicrobial agents to the affected area⁷. In such cases, a high dose of antimicrobial agents should be administered to obtain adequate medicinal effects, which also increases the incidence of various adverse events due to the side effects of the agents⁸. In addition, surgical removal of abnormal granulation and sequestra caused by osteomyelitis can often result in widespread bone defects⁹. Therefore, various treatment methods have been developed to overcome these problems.

One of the treatment methods developed is the direct introduction of antimicrobial agents *via* a drug delivery system (DDS) that can maintain the local concentration of antimicrobial agents. Although polymethyl methacrylate (PMMA) beads were used as a DDS in a previous study¹⁰, the beads could not provide

long-term sustained release of the agent¹¹ and also posed harmful effects due to the residual monomer¹². In addition, because PMMA does not have osteoconduction capacity, the development of composites of PMMA and a bone-filling material that acts as a scaffold for bone regeneration has been pursued^{13–17}.

To this end, our research group has developed osteoconductive materials for treating mandibular osteomyelitis and promoting bone regeneration at the site of bone defects using bioabsorbable polymers as a DDS for carrying low-dose antimicrobial agents that are released locally and sustainably. Miyai *et al.*¹⁵ developed a composite consisting of 1 wt% equivalent gatifloxacin (GFLX), a new quinolone antimicrobial agent, and poly(ϵ -caprolactone) (PCL) as a carrier of GFLX, which were loaded onto β -tricalcium phosphate (β -TCP) disks with interconnected micropores. The composite was then implanted into a rabbit mandibular osteomyelitis model and the effects examined. As a result, because PCL has a low absorption rate, the composite remained in the affected area for a long period and inhibited new bone formation. However, because of the shape of the β -TCP disks, the composite could not be tightly implanted into affected areas with complicated shapes. Tamazawa *et al.*¹⁶ used poly(lactide-co-glycolide) (PLGA) as a DDS for carrying 1 wt% GFLX and β -TCP granules (porosity ranging 73–82%) as a bone-filling material. Although the composite was tightly implanted into the affected area, the GFLX sustained release rate was high because of the high absorption rate of PLGA, resulting in decreased local antibacterial activity. To solve this problem, the antibacterial activity was increased by increasing the concentration of GFLX from 1 to 10%. Inflammation in the affected area was thus

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suppressed¹⁷). However, the results showed that the β -TCP granules disappeared in some cases before new bone formation occurred because of the high absorption rate of the granules¹⁸).

In this study, we prepared a composite consisting of 10 wt% GFLX carried by PLGA and high-porosity hydroxyapatite (HA) granules. HA granules, which have three-dimensionally interconnected micropores and are hardly absorbed in the body, were adopted as a bone-filling material to decrease the absorption rate.

The effectiveness of this new material for treating mandibular osteomyelitis was examined by *in vitro* tests including pH, GFLX sustained release, and bactericidal activity analyses. For *in vivo* tests, the material was implanted into a rabbit mandibular osteomyelitis model, in which the effects of the material on inflammation in mandibular osteomyelitis and bone formation were histopathologically and radiographically examined, and GFLX concentrations in tissues and serum were measured.

MATERIALS AND METHODS

Preparation of GFLX-containing PLGA/HA granule composite

The following materials were used: new quinolone antimicrobial agent GFLX (gatifloxacin G0278, LKT Laboratories, St. Paul, MN, USA) as the antimicrobial agent, bioabsorbable synthetic polymer PLGA (PLGA5015, Wako Pure Chemical Industries, Osaka, Japan) as the sustained release carrier, and HA granules (Neobone®, DK12-001, Covalent Materials, Nara, Japan) as the scaffold for bone regeneration. The HA granules used in this study had porosities of 72–78%, an average pore size of 150 μ m, and granule sizes of 1–2 mm. PLGA used in this study had a lactic acid/glycolic acid ratio of 50/50 and a molecular weight of 15,000. This ratio was selected according to the examination of various lactic acid/glycolic acid ratios carried out by Tamazawa *et al.*¹⁶).

One gram of PLGA powder was placed in a glass tube and melted at 140°C using an aluminum block heater (ND-M11, Nissin Laboratory Instruments, Tokyo, Japan). This melting temperature was 47°C lower than the decomposition temperature of GFLX (187°C). After confirming the melting of PLGA, 10 wt% GFLX was mixed with PLGA and stirred uniformly at 2,000 rotations/min for 40 $\pm$ 5 s using a stirrer (GRANDE GRC2000, Argofile Japan, Tokyo, Japan) to fabricate GFLX-containing PLGA. The GFLX-containing PLGA/HA granule composite (GFLX/HA composite) was fabricated by immersing the HA granules in GFLX-containing PLGA one by one for 1 min, after which they were removed and allowed to stand at room temperature. The composite was then sterilized using ethylene oxide gas. The weight of the HA granules before and after immersion in GFLX-containing PLGA was measured to determine the amount of GFLX contained in the GFLX/HA composite. Loading of the GFLX-containing PLGA into the HA granule was

confirmed by surface and cross-sectional images around the center of the HA granules before and after loading using scanning electron microscopy (SEM; Miniscope® TM-1000, Hitachi, Tokyo, Japan).

In vitro pH changes

To determine the pHs of GFLX (2.0 mg), PLGA (20.0 mg), HA granules (23.0 mg), and the GFLX/HA composite (weight, 45 $\pm$ 5.0 mg; GFLX content, 2.2 $\pm$ 0.2 mg), all materials were separately immersed in 5.0 mL of Hank's balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) and then incubated at 37°C. The supernatant (2.0 mL) was collected at 1, 3, 5, 7, 10, 14, 21, and 27 days, and the pH of each sample was measured using a pH meter (Navi pH meter F55, Horiba, Kyoto, Japan) ($n=5$). The pHs are shown as the mean of three measurements for each sample. The weights of the HA granules, GFLX, and PLGA used in the pH and GFLX sustained release analyses were determined according to the mean weight of GFLX, PLGA, and HA granules contained in a sample from the GFLX/HA composite used in pH analyses.

In vitro GFLX release of the GFLX/HA composite

The amount of GFLX eluted from GFLX/HA composite containing 10 wt% GFLX in Hanks' solution was measured at an absorbance at 286.2 nm with no background subtraction using a UV-Vis spectrophotometer (Model V-550, Jasco, Tokyo, Japan) after 1, 3, 5, 7, 14, 21 and 28 days ($n=5$).

In vitro bactericidal properties of the GFLX/HA composite

The bactericidal properties of the GFLX/HA composite against *Streptococcus milleri* NCTC7331 and *Bacteroides fragilis* NCTC9343 (both obtained from the Public Health Laboratory Service, London, UK), which were used to generate the rabbit mandibular osteomyelitis model, were examined using the Kirby-Bauer (KB) method (KB disk method)¹⁹. Bacterial suspensions were prepared by culturing *S. milleri* aerobically at 37°C for 24 h in heart infusion broth (Eiken Chemical, Tokyo, Japan) and *B. fragilis* anaerobically at 37°C for 48 h in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical, Tokyo, Japan) using the gas pack method (AnaeroPack® Kenki, Mitsubishi Gas Chemical Company, Tokyo, Japan). The GFLX/HA composite after immersion for 1–10 days in the Hank's solution under the same condition as that of the pH analyses was placed in a circular area with a diameter less than 6.35 mm on the Pearlcore® heart infusion broth (Eiken Chemical) inoculated with *S. milleri* (1 $\times$ 10⁸ CFU/mL). The culture was then incubated aerobically at 37°C for 24 h. Similarly, the GFLX/HA composite was placed on GAM agar (Nissui Pharmaceutical) inoculated with *B. fragilis* (1 $\times$ 10⁸ CFU/mL), and the culture was then incubated anaerobically at 37°C for 48 h using the gas pack method. The bactericidal properties were then determined by measuring the diameters of inhibition zones. KB disks (Eiken Chemical) containing 5 μ g of

GFLX were used as the control.

Preparation of osteomyelitis in a rabbit mandible

Osteomyelitis was induced in the left mandible of 16 male New Zealand white rabbits (body weight, 2.5–3.0 kg) in accordance with the Satoh-Heimdahl method²⁰. General anesthesia was induced by an intramuscular injection of a mixture of 0.2 mL of butorphanol tartrate (Vetorphale®, Meiji Seika Pharma, Tokyo, Japan), 0.4 mL of medetomidine hydrochloride (Domitor®, Nippon Zenyaku Kogyo, Tokyo, Japan), and 2.0 mL of midazolam (Dormicam®, Astellas Pharma, Tokyo, Japan) into the thighs. After confirming the induction of anesthesia, the left submental and malar regions were shaved, disinfected with 70% ethanol, and locally anesthetized with 1/80,000 epinephrine-containing 2% lidocaine hydrochloride (Xylocaine Dental® Cartridges, Dentsply-Sankin, Tokyo, Japan). An approximately 1-cm transverse incision was made along the zygomatic and auricularis muscle fibers, and the bony part of the anterior fossa masseterica was exposed by pressing the masseter in the posterior direction. The periosteum was then incised and stripped to expose the bone surface. A small cavity with a diameter of 2.3 mm reaching the bone marrow was drilled on the exposed bone surface using a dental round bur (#8). A small swab impregnated with *S. milleri* or *B. fragilis* (1.0×10^8 CFU/mL) was then placed into the small cavity. After the periosteum and skin were sutured with 5-0 VICRYL® (Johnson & Johnson, New Brunswick, NJ, USA) and 5-0 nylon (Nihon Chosen, Chiba, Japan) by a single suture, respectively, the rabbits were allowed to recover in a room for infected animals for 4 weeks with feeding to generate the mandibular osteomyelitis model.

Implantation of the GFLX/HA composite in a rabbit mandible

Four weeks after infection, the area with osteomyelitis was exposed similarly to the preparation of the model (Fig. 1A). The sites of abnormal granulation and bone tissues affected by infection were debrided as much as possible, and the surface of the bone cavity was

cleaned using a dental round bur (#8). After irrigation with saline, the bone cavity was tightly filled with the GFLX/HA composite (71.3 ± 21.1 mg) (Fig. 1B), which was covered with an absorbable collagen membrane (BioMend®, Zimmer Dental, Carlsbad, CA, USA) to prevent connective tissue invasion. The periosteum and skin were sutured with 5-0 VICRYL® and 5-0 nylon, respectively. These rabbits were designated as the GFLX/HA composite group ($n=4$). In the GFLX-PLGA group ($n=4$), the bone cavity was tightly filled with GFLX-containing PLGA granules (80.3 ± 30.8 mg) after bone debridement, covered with an absorbable collagen membrane, and closed. In the HA granule group ($n=4$), the bone cavity was filled with HA granules. In the debridement group ($n=4$), the bone cavity was treated by bone debridement alone, covered with an absorbable collagen membrane, and closed.

All rabbits were allowed to recover for 4 more weeks with feeding and then sacrificed with an intramuscular injection (1.5 mL/kg) of sodium pentobarbital solution (Somnopenityl®, Kyoritsu Seiyaku, Tokyo, Japan) to collect mandible samples. Lateral and cross-sectional (coronal views at the dotted line in the lateral images) images of the buccal face of the left mandible were obtained using microcomputed tomography (micro-CT). Paraffin-embedded samples prepared by conventional methods were cut into thin sections (4 μ m thick) and stained with hematoxylin-eosin for histopathological examination. Oral tissues, subcutaneous tissues, salivary glands, lymphoid tissues and serum were collected from rabbits in the GFLX/HA composite group to determine the amount of GFLX transferred to the tissues and serum.

In histopathological sections showing the middle portion of lesions in each group, the areas of inflammatory lesions and sequestra within and outside the debrided area as well as the area of new bone in the debrided area were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The cure rate was obtained according to the number of rabbits not showing inflammatory lesions and sequestra in histopathological sections (cured inflammation) and

Fig. 1 Osteomyelitis in the rabbit mandible and implantation of the GFLX/HA composite. A: Four weeks after the induction of osteomyelitis in the rabbit mandible; B: GFLX/HA composite implanted into the bone defect after debridement of osteomyelitis.

that of rabbits showing bone formation in the debrided area (bone formation).

All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals under the approval of the Animal Care Committee of the School of Life Dentistry, Nippon Dental University (approval numbers 11–18 and 12–32).

GFLX concentrations in tissues and serum

Resected and collected oral tissues (tongue, lower gingiva, palate mucosa, and buccal mucosa), subcutaneous tissues (neck subcutaneous tissue and dorsum subcutaneous tissue), salivary glands (submandibular gland and parotid gland), and lymphoid tissues (submandibular lymph node) were minced using scissors into fragments of approximately $1 \times 1 \text{ mm}^2$ size and $0.4 \pm 0.2 \text{ g}$ weight. These tissue fragments were placed in a zirconia container with zirconia grinding balls, mixed with $500 \mu\text{L}$ of phosphate-buffered saline (PBS)(–), and homogenized at 5,000 rpm for 5 min using a mixer mill (Type MM301, Retsch, Haan, Germany). The mixture was then placed into a centrifuge tube with a $0.2\text{-}\mu\text{m}$ filter (Ultrafree®-MC, Merck Millipore, Billerica, MA, USA) and centrifuged (LC200, Tomy Seiko, Tokyo,

Japan) at 3,000 rpm and 4°C for 20 min to obtain the supernatant. The amounts of GFLX in the supernatant and the serum were determined by high-performance liquid chromatography (HPLC) (LC-10, Shimadzu, Kyoto, Japan) as described elsewhere^{15–17} and shown as the mean of the experimental group ($n=4$). The lower gingiva, salivary glands, and lymphoid tissues were collected from both the left and right sides, and the amount of GFLX transferred to these tissues was measured separately.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD). Significant differences in inflammation and bone formation in the debrided area observed among groups were examined using the Kruskal-Wallis test. The results were compared using the Mann-Whitney test. p Values < 0.05 were considered statistically significant.

RESULTS

Preparation of the GFLX/HA composite

Figure 2 shows light microscopic and SEM images of an HA granule and the GFLX/HA composite. The HA granule (Fig. 2A) had $100\text{--}200\text{-}\mu\text{m}$ -diameter macropores that were interconnected by micropores of $40 \mu\text{m}$

Fig. 2 HA granules before and after immersion in GFLX-containing molten PLGA. A: An HA granule before immersion in GFLX-containing molten PLGA; B: cross-sectional image of an HA granule before immersion in GFLX-containing molten PLGA; C: HA granule after immersion in GFLX-containing molten PLGA; D: cross-sectional image of an HA granule after immersion in GFLX-containing molten PLGA.

diameter or less (Fig. 2B). For the GFLX/HA composite, the surface of the HA granule was covered with GFLX-containing PLGA (Fig. 2C), and GFLX-containing PLGA was loaded into the pores of the granule (Fig. 2D).

In vitro pH changes

Figure 3 shows the results of pH analyses. On day 1, the pH of Hank's solution containing PLGA decreased to 7 or lower, which was slightly lower than that of the other substances. The pHs of Hank's solution containing PLGA and the GFLX/HA composite rapidly decreased and became acidic from day 3 to day 7. The decrease in the pHs of Hank's solution containing PLGA and the GFLX/HA composite slowed from day 7 onwards, and the pH of Hank's solution containing the GFLX/HA composite remained fairly constant from day 14 onwards. However, the pH of Hank's solution containing PLGA continued to decrease and reached 3 or lower on day 21. Conversely, the pHs of Hank's solution containing the

Fig. 3 *In vitro* pH changes. pH changes Hank's solution after immersing the GFLX/HA composite, HA granules, GFLX, and PLGA in Hank's balanced salt solution.

HA granules and GFLX slightly decreased from day 3 to day 7 and then remained fairly constant. Their pHs were approximately 7, indicating neutrality, even on day 27.

In vitro GFLX sustained release profile

Figure 4 shows the release profile of GFLX from GFLX/HA composite containing 10 wt% GFLX. The release of GFLX was slight until day 3. However, the release speed of GFLX gradually increased from day 3 to day 7, and the GFLX concentration significantly increased from day 7 to day 21.

In vitro bactericidal properties

Figure 5 shows the bactericidal properties of the GFLX/HA composite. Bactericidal activity of the GFLX/HA composite against *S. milleri* and *B. fragilis* was observed starting from 12 and 3 h after the beginning of the test, respectively.

Fig. 4 *In vitro* GFLX sustained release profile.

Fig. 5 *In vitro* bactericidal properties of the GFLX/HA composite against *S. milleri* (A) and *B. fragilis* (B).

Fig. 6 Micro-CT images of the rabbit mandible following various treatments.

A: Three-dimensional (3D) image of the GFLX/HA composite site; B: cross-sectional image of the GFLX/HA composite site; C: 3D image of the GFLX-PLGA site; D: cross-sectional image of the GFLX-PLGA site; E: 3D image of the HA granule site; F: cross-sectional image of the HA granule site; G: 3D image of the debridement site; H: cross-sectional image of the debridement site. Arrows indicate HA granules.

In vivo efficacy of the GFLX/HA composite in treating osteomyelitis

In the GFLX/HA composite and HA granule groups, HA granules were observed inside the bone cavity (indicated by arrows in Figs. 6A, B, E and F), and radiopaque regions that appeared to be immature new bone were observed around the HA granules (Figs. 6A

Fig. 7 Histopathological sections of the mandible after 4-week implantation.

A–D; GFLX/HA composite (B. magnified view of the boxed area in A; D. magnified view of the boxed area in C). E, F; GFLX-PLGA (B. magnified view of the boxed area in A). G, H; HA granules (B. magnified view of the boxed area in A). I, J; Debridement alone (B. magnified view of the boxed area in A).

and E). Moreover, part of the bone cavity was covered with thin, new bone in the GFLX/HA composite group (Fig. 6B). Although bone defects were observed in all groups, they were widespread in the debridement group (Figs. 6G and H). Cross-sectional images were obtained at the dotted line in the 3D image of each group. HA granules (indicated by arrows in Figs. 6A, B, E and F) were observed inside the bone cavity, and radiopaque regions that appeared to be immature new bone were observed around the HA granules (Figs. 6A and E). Part of the bone cavity was covered with thin new bone (Fig. 6B). Bone defects were observed in all groups.

Figures 7A–D show representative histopathological images from the GFLX/HA composite group. New bone formed in the debrided area (Fig. 7A), and the cortical-bone-equivalent area on the buccal side was covered with new bone (Fig. 7B). Moreover, new bone formed around the HA granules and in their pores in contact with the HA surface (Fig. 7C), and bone marrow-like tissues with new blood vessels were observed in the pores (Fig. 7D). Figures 7E and F show representative histopathological images from the GFLX-PLGA group. Granulation tissues formed in the debrided area, the amount of newly formed bone was small (Fig. 7E), and sequestra and lymphocytic invasion were observed (Fig. 7F). Figures 7G and H show representative histopathological images from the HA granule group. Inflammatory lesions associated with lymphocytic invasion were observed in

the debrided area (Fig. 7G), while new bone and new blood vessels formed in the pores of the HA granules (Fig. 7H). Figures 7I and J show representative histopathological images from the debridement group. Large granulation tissues and an abscess were observed mainly in the debrided area, and little new bone formed.

Table 1 shows the cure rate determined according to the number of rabbits showing cured inflammatory lesions and that of rabbits showing bone formation. The inflammatory lesions in and outside the debrided area did not completely disappear in all groups; namely, none of the rabbits showed cured inflammation. New bone formation in the debrided area was observed in all rabbits in the GFLX/HA composite and HA granule groups, while new bone formation was observed in three of four rabbits in the GFLX-PLGA group, and two of four rabbits in the debridement group.

Figure 8 shows a scatter plot of the area of inflammatory lesions in the debrided area. The inflammation area in the GFLX/HA composite group was significantly smaller than that in the debridement group ($p<0.05$). Figure 9 shows a scatter plot of the area of new bone in the debrided area. The newly formed bone area in the HA granule group was significantly larger than that in the other groups ($p<0.05$). Moreover, the newly formed bone area in the GFLX/HA composite group was significantly larger than that in the debridement group ($p<0.05$).

Table 1 Cure rate determined on the basis of inflammation and bone formation ($n=4$)

Group	Cured inflammation		Bone formation
	in debrided area	outside debrided area	in debrided area
GFLX/HA composite	0/4	0/4	4/4
GFLX-PLGA	0/4	0/4	3/4
HA granules	0/4	0/4	4/4
Debridement	0/4	0/4	2/4

Fig. 8 A scatter plot of the area of inflammatory lesions in the debrided area after 4-week treatment.
* $p<0.05$, Mann-Whitney test

Fig. 9 A scatter plot of the bone formation area in the debrided area after 4-week treatment.
* $p<0.05$, Mann-Whitney test

Table 2 GFLX concentrations in serum ($\mu\text{g/mL}$) and tissue ($\mu\text{g/mg}$) at 4 weeks after implantation of the GFLX/HA composite ($n=4$)

Tissue	Average			
	Side	GFLX concentration	S.D.	Serum ratio
Serum	—	0.126	0.067	1.000
Gingiva	(I)*	0.122	0.041	0.968
	(U)*	0.153	0.106	1.217
Paleta	—	0.222	0.137	1.758
Tongue	—	0.139	0.056	1.106
Submandibular lymph node	(I)	0.218	0.147	1.731
	(U)	0.241	0.083	1.916
Parotid gland	(I)	0.295	0.266	2.345
	(U)	0.04	0.016	0.319
Submandibular gland	(I)	0.38	0.132	3.014
	(U)	0.266	0.108	2.111
Buccal mucosa	(I)	0.19	0.029	1.509
	(U)	0.176	0.004	1.401
Neck skin	—	0.170	0.027	1.347
Dorsum skin	—	0.160	0.030	1.267

(I)*: Implanted side, (U)*: Un-implanted side

GFLX concentrations in tissues and serum

Table 2 shows the GFLX concentrations in tissues and serum at 4 weeks after implantation of the GFLX/HA composite. High mean GFLX concentrations were recorded in the submandibular gland on the implanted side, whereas low mean GFLX concentrations were recorded in the parotid gland on the unimplanted side. GFLX concentrations were higher in the gland and lymphoid tissues than in the lower gingiva, tongue and skin and also tended to be higher on the implanted side. The GFLX serum ratio was lower than 1.0 in the lower gingiva on the implanted side and parotid gland on the unimplanted side, but 1.0 or higher in other sites. Systemic side effects of the GFLX/HA composite, such as diarrhea and loss of appetite, were not observed in any rabbits in this study.

DISCUSSION

The cure rate in osteomyelitis treatment using the conventional method of administering antimicrobial agents is low mainly because the concentration of the agents reaching the affected area is insufficient due to ischemia⁷. In this study, we used a new quinolone antimicrobial agent, GFLX, at 10 wt%, which has antibacterial activity against gram-positive bacteria, gram-negative bacteria, anaerobic bacteria, and mycoplasma. We increased the GFLX concentration in the affected area using a DDS (PLGA) as the sustained-release carrier. In addition, we used non-bioabsorbable

HA granules and fabricated a GFLX/HA composite that rapidly repairs bone defects following surgical treatment.

HA granules have porosities as high as 75%. This high porosity is achieved by macropores (average diameter of 150 μm), which are three-dimensionally interconnected to one another through micropores of 40- μm diameter or less²¹. These three-dimensionally interconnected macropores were observed by SEM in this study, and appeared to contribute to efficient loading of GFLX-containing PLGA deep into the pores of the HA granules. In addition, the large surface area resulting from high porosity allows a large amount of GFLX-containing PLGA to be loaded into the HA granules. We also found that the pores of the HA granules were filled with GFLX-containing PLGA. Furthermore, PLGA implanted into the rabbits was easily degraded and absorbed due to the presence of interconnected pores, leading to the sustained release of GFLX.

In pH analyses, changes in the pHs of Hank's solution containing the GFLX/HA composite and PLGA were closely related to one another, however, the pHs of Hank's solution containing the HA granules and GFLX remained nearly unchanged. These findings indicate that the decrease in pH was caused by the elution of PLGA. Similarly, the decrease in the pH of Hank's solution containing the GFLX/HA composite suggests that GFLX-containing PLGA was eluted from the GFLX/HA composite. In addition, it has been reported

that GFLX was sustainably released along with the elution of GFLX-containing PLGA in Hank's balanced salt solution^{16,17}. Correspondingly, GFLX sustainably released from the GFLX/HA composite was also observed in this study. From day 1 to day 7, the release speed of GFLX was very slow. However, bactericidal activity against *S. milleri* and *B. fragilis* was observed starting from 12 and 3 h after the beginning of the test, respectively. This sustained release of GFLX was continued until day 21 in Hank's balanced salt solution.

When the inflammation area in the debrided area was compared according to the results obtained from rabbits with mandibular osteomyelitis, inflammation suppression was significantly greater in the GFLX/HA composite group than in the debridement group. However, in a previous study that evaluated the effectiveness of a composite consisting of 10 wt% GFLX carried by PLGA and loaded into β -TCP granules (GFLX/ β -TCP composite), all five rabbits showed cured inflammatory lesions in the debrided area, three of five rabbits showed cured inflammatory lesions outside the debrided area, and three of five rabbits showed bone formation in the debrided area¹⁷. Comparing these results with those in this study, we found that the composite consisting of β -TCP granules was superior in suppressing inflammation, whereas the composite consisting of HA granules was superior in promoting bone formation. Therefore, it appears that the elution of GFLX-containing PLGA loaded into the pores of HA granules in the GFLX/HA composite was delayed because the HA granules were hardly absorbed into the body, resulting in decreased GFLX concentration in the affected area. In the previous study using β -TCP granules, the β -TCP granules in the GFLX/ β -TCP composite were rapidly degraded from day 5 to 7, leading to a sharp increase in the GFLX sustained release rate from day 5 to 7¹⁷. These findings indicate that the presence or absence of bioresorbability of bone substitutes carrying GFLX-containing PLGA affected the GFLX sustained release rate, resulting in differences in inflammation suppression.

The HA granules were not degraded in the body, but retained their original shape, as observed in micro-CT images. In the GFLX/HA composite and HA granule groups, new bone formation was observed in the area from the HA granules and the existing bone around the HA granules to the periphery of the granules and gaps in between the HA granules in the bone cavity. However, in the other groups, the radiopacity decreased and bone defects formed on the buccal side to the bone marrow. The bone defects were particularly large in the debridement group compared with the other groups. In histopathological sections from the GFLX/HA composite and HA granule groups, the HA granules were not absorbed into the body, new bone formed around the granules and in their pores, and bone marrow-like tissues with new blood vessels were observed in the pores. Moreover, the bone area in the debrided area was significantly larger in the HA granule group than in the other groups. HA has good osteoconduction capacity^{22–25}.

In addition, the HA granules used in this study had 100–200- μ m-diameter macropores, which are ideal for guided formation of new bone in the pores^{26,27}, and the macropores were three-dimensionally interconnected to one another by 40–50- μ m-diameter micropores. It was reported that when a cylindrical block consisting of such HA granules was implanted into the femur of rabbits, new bone formation extended toward the center of the block 2 weeks after implantation. However, when a cylindrical block consisting of dense HA granules without interconnected pores was implanted, new bone formation was observed only in the surface layer even 3 weeks after implantation²¹. Namely, there was a difference in bone formation between the HA granule and GFLX/HA composite groups because, in the GFLX/HA composite group, new bone did not form in the pores unless GFLX-containing PLGA that was densely loaded into the pores was eluted. However, the mean inflammation area was larger in the HA granule group than in the GFLX/HA composite group because GFLX-containing PLGA was not used in the HA granule group.

Four weeks after implantation of the GFLX/HA composite, the GFLX concentration was slightly higher in adjacent tissues than in the serum. Additionally, the GFLX concentration tended to be higher on the implanted side (left side), particularly in the salivary glands. These findings are in agreement with those reported by Tamazawa *et al.*¹⁶ and Kimishima *et al.*¹⁷.

As described above, the GFLX/HA composite was effective in suppressing inflammation in mandibular osteomyelitis. The GFLX/HA composite used in this study appears to be superior to GFLX/ β -TCP composite in terms of bone formation, but inferior in terms of inflammation suppression. A potential measure to improve the therapeutic effects of this method would be to optimize the absorption rate of the granules by producing and using HA/ β -TCP biphasic granules.

CONCLUSION

We developed a composite consisting of 10 wt% GFLX carried by PLGA, which was loaded in HA granules with three-dimensionally interconnected pores. We then evaluated the effectiveness of the composite in terms of inflammation suppression and bone regeneration in a rabbit mandibular osteomyelitis model. The sustained release of GFLX from GFLX/HA composite in Hank's balanced salt solution began with a pH-decreasing action and the GFLX release gradually increased. The GFLX/HA composite had bactericidal activities against *S. milleri* and *B. fragilis* from 12 and 3 h, respectively, and exhibited anti-inflammatory effects as well as bone regeneration abilities in the debrided area. The local release of GFLX was confirmed by the tissue distribution of GFLX *in vivo*. The granular composite of 10 wt% GFLX-loaded PLGA and ultra-high porous HA acts as a sufficient local drug delivery carrier to control infection and is a promising scaffold for bone regeneration in osteomyelitis treatment.

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