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Research Article

Silver-Containing Hydroxyapatite Coating Reduces Biofilm Formation by Methicillin-Resistant *Staphylococcus aureus* In Vitro and In Vivo

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Biofilm-producing bacteria are the principal causes of infections associated with orthopaedic implants. We previously reported that silver-containing hydroxyapatite (Ag-HA) coatings exhibit high antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). In the present study, we evaluated the effects of Ag-HA coating of implant surfaces on biofilm formation. Titanium disks (14-mm diameter, 1-mm thickness), one surface of which was coated with HA or 0.5%–3.0% Ag-HA with a thermal spraying technique, were used. In vitro, the disks were inoculated with an MRSA suspension containing 4×10^5 CFU and incubated for 1-2 weeks. In vivo, MRSA-inoculated HA and 3% Ag-HA disks ($8.8–10.0 \times 10^8$ CFU) were implanted subcutaneously on the back of rats for 1–7 days. All disks were subsequently stained with a biofilm dye and observed under a fluorescence microscope, and biofilm coverage rates (BCRs) were calculated. The BCRs on the Ag-HA coating were significantly lower than those on the HA coating at all time points in vitro ($p < 0.05$). Similar results were observed in vivo ($p < 0.001$) without argyria. Ag-HA coating reduced biofilm formation by MRSA in vitro and in vivo; therefore, Ag-HA coating might be effective for reducing implant-associated infections.

1. Introduction

Numerous types of orthopaedic implants are used to repair bone fractures, tumour resections, and artificial joint replacements. However, implants are prone to bacterial infections, which can lead to implant-associated infections at the surgical sites. If a bacterial implant-associated infection occurs, long-term treatment is required, which can affect the patient's quality of life and pose a burden on the surgeon [1, 2].

Several recent articles have described the relationship between biofilms and orthopaedic infections [3–5]. When bacteria form a biofilm on the implant, the biofilm layer protects the bacteria from antibiotic agents [6, 7]. Nishimura et al. demonstrated that sessile *Staphylococcus aureus* in biofilms were ≥1,000 times more resistant to antibiotics than planktonic bacteria isolated from infected total hip arthroplasty patients [8]. Biofilm clusters from methicillin-sensitive *S. aureus* exhibited an antibiotic resistance normally associated with methicillin-resistant *S. aureus* (MRSA) strains [5]. Moreover, the biofilm structure can inhibit the adaptive and innate immune responses of the host [9]. Treatment becomes even more challenging if the bacteria are resistant to antibacterial drugs. Unfortunately, MRSA causes the majority of implant-associated infections [1, 10].

Multiple approaches to imparting antibacterial properties to implants to prevent infections have been reported [3, 4, 11–13]. Several studies have reported the merits of implants with antibacterial surface coatings such as gentamicin and magnesium [11–13]. Silver has strong, broad-spectrum antibacterial activity and low toxicity. For these reasons, silver has a long history of use as an antimicrobial agent, as in medical device coatings [14]. The mechanism underlying the antibacterial effect of silver was elucidated recently. Silver releases ions from its surface, which can bind to a number of bacterial

cell structures, including the peptidoglycan cell wall and plasma membrane, DNA, and proteins [15, 16]. One study reported that ion-binding to the cell wall damaged the outer cell layers, causing loss of cell contents and creating structural abnormalities [17]. These mechanisms of action result in disorganization of surface species such as proteins, of cells, and of their resultant biofilms. Although we previously reported that an excessive dose of silver inhibits bone formation, the dose-dependent influence of silver on osteoconductivity remains to be elucidated [18].

Hydroxyapatite (HA) has good biocompatibility and osteoconductivity. Therefore, it is used to coat implants to improve bone-implant attachment strength [19, 20]. There have been various reports on the use of plasma-sprayed silver-doped HA coatings [21–23]. We developed a silver-containing HA (Ag-HA) coating, with the combined properties of both silver and HA, using a thermal spraying technique [24, 25]. We previously reported that Ag-HA exhibited antibacterial activity against MRSA in vivo. In that study, the number of viable MRSAs on Ag-HA-coated subcutaneous implants was significantly lower than that on HA-coated subcutaneous implants in rats [26]. Akiyama et al. reported similar results in the medullary cavity [27]. Ag-HA can release silver ions [28] and, thus, has antibacterial activity. However, its antibiofilm properties have not been evaluated. We hypothesised that Ag-HA coatings would reduce bacterial biofilm formation and help to manage refractory orthopaedic infections. Therefore, we designed in vitro and in vivo infection models to evaluate the effects of these coatings on biofilm formation.

2. Materials and Methods

2.1. Ag-HA Coating. Pure titanium disks (diameter, 14 mm; thickness, 1 mm) were used as substrates for coating deposition for both the in vitro and in vivo studies. One side of the surface was roughened using a K5 sandblasting machine (TKX Corp., Osaka, Japan) with a 180-grit aluminium oxide medium (Showa Denko K.K., Tokyo, Japan), after which the disks were washed with ethanol for 3 min under ultrasonication. Powdered silver oxide (Kanto Chemical, Tokyo, Japan) was added to powdered HA (KYOCERA Medical Materials, Osaka, Japan) to prepare the specified concentrations (w/w), and the mixtures were stirred for 5 min in plastic bags. HA powders, with or without silver oxide, were thermally sprayed onto the sandblasted surface using a flame-spraying system (Oerlikon Metco Japan Ltd., Tokyo, Japan) to coat the surface of the disks. The temperature of the flame was approximately 2,700°C. The spraying powder was carried into the flame by a dry air carrier gas during spraying, then melted through the flame, and sprayed onto the disk. The coating process was conducted under normal atmospheric pressure. The physical and chemical properties of Ag-HA, namely, a 40-μm thick layer of Ag-HA containing calcium, phosphorus, and oxygen, in which the amount of calcium/phosphorus is nearly the same as that of HA, have been reported previously [25, 27]. The disks and implants were individually packaged and sterilised using a JS-8500 gamma steriliser (MDS Nordion, Ontario, Canada). All disks were obtained from KYOCERA Medical Corporation (Osaka, Japan).

2.2. Bacteria and Culture Conditions. The MRSA strain used in this study was UOEH6 (University of Occupational and Environmental Health Hospital, Fukuoka, Japan). This strain had been isolated from a blood sample of a septic patient and was characterised as a biofilm-producing strain. Bacteria were cultured overnight in Tryptic Soy Broth (Eiken Chemical, Tokyo, Japan) at 37°C. Immediately after inoculation, serial dilutions of the residual suspension were incubated on agar plates for 48 h at 37°C, and colony-forming units (CFU)/mL were calculated.

2.3. In Vitro Experiment. Four types of implants were prepared: Ti with HA coating (HA), Ti with 0.5% Ag-HA coating (0.5% Ag-HA), Ti with 1.0% Ag-HA coating (1% Ag-HA), and Ti with 3.0% Ag-HA coating (3% Ag-HA). One implant was aseptically placed into each well of 24-well sterile polystyrene tissue culture plates (Corning, Corning, NY, USA), and 500 μL of an MRSA suspension containing 4×10^5 CFU/mL was inoculated onto each implant. The disks were incubated at 37°C for 1 h and then rinsed twice with 500 μL sterile phosphate buffered saline (PBS) to eliminate nonadherent bacteria. The disks were transferred to Petri dishes containing 20 mL heat-inactivated 100% foetal bovine serum (Thermo Fisher Scientific, Wilmington, DE, USA). The dishes were incubated at 37°C with continuous slow stirring on magnetic stirrers for 7 or 14 days. The stir bar was spun at 60 revolutions per minute. The medium was changed every 3 days. In the 7-day experiment, 7 disks with HA coating, including 2 disks of 0.5% Ag-HA, 2 disks of 1% Ag-HA, and 3 disks of 3% Ag-HA, were used. In the 14-day experiment, 9 disks with HA and 3 disks of each group of Ag-HA were used.

2.4. In Vivo Experiment

2.4.1. Animals. We used ten 8-week-old male Sprague-Dawley rats, weighing on average 331.6 g (range 316.5–355.1 g), from Kyudo (Kumamoto, Japan). The rats were housed in pairs with 12-h light-dark cycles and acclimated for 5 days prior to use in a room in which a suitable environment was maintained. All animal procedures were conducted with the approval of the Animal Research Ethics Committee at Saga University (Approval Number 27-018-0). According to their recommendation, the number of experimental animals was minimised.

2.4.2. Surgical Technique. All rats were anaesthetised using a mixture of anaesthetic agents (0.375 mg/kg medetomidine, 2 mg/kg midazolam, and 2.5 mg/kg butorphanol) administered by intraperitoneal injection [28]. The infection model, using the back of the rat, was previously described by Shimazaki et al. [26] and was generated with slight modifications in our study.

Disks coated with HA or 3% Ag-HA were prepared. The back of the rat was shaved, cleaned with povidone-iodine, and dried. Four sagittal 1-cm incisions were made on the dorsum; two were at the level of the scapula and two were at the level of the lower rib cage, each 2 cm lateral to midline, and pockets were made not to connect to another pocket. One disk was aseptically implanted into each

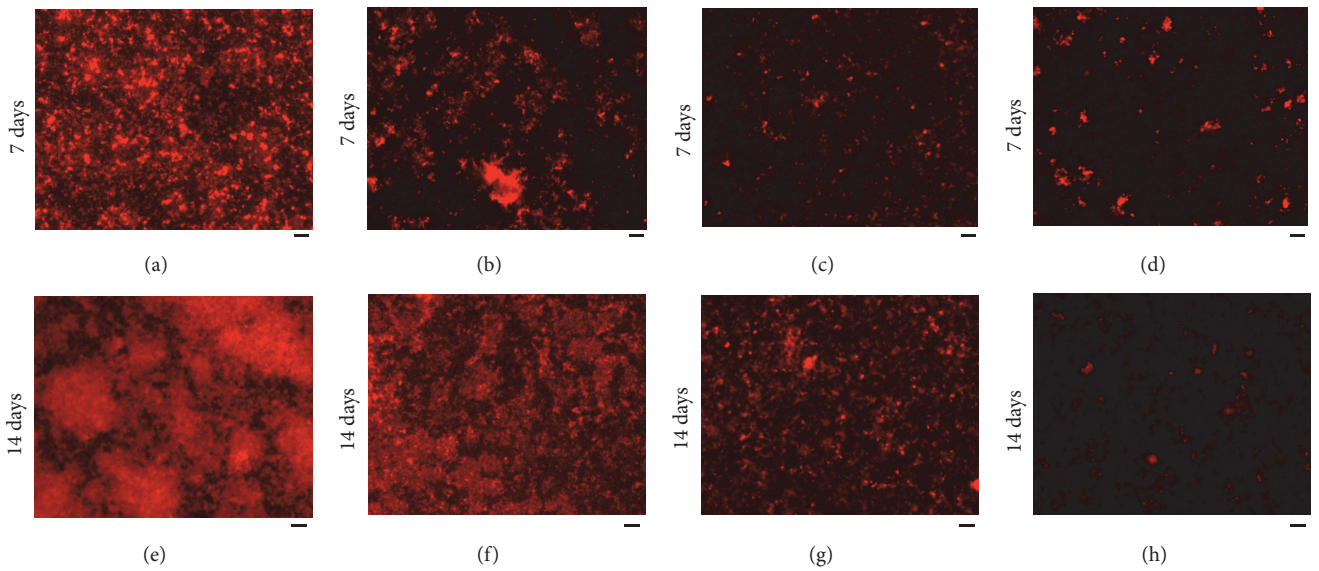

Figure 1: Representative fluorescence microscopic images of implants after bacterial culture for 7 and 14 days in vitro. The bar indicates 100 μm. (a) Hydroxyapatite (HA) coating at 7 days, (b) 0.5% silver-containing HA (Ag-HA) coating at 7 days, (c) 1% Ag-HA coating at 7 days, (d) 3% Ag-HA coating at 7 days, (e) HA coating at 14 days, (f) 0.5% Ag-HA coating at 14 days, (g) 1% Ag-HA coating at 14 days, and (h) 3% Ag-HA coating at 14 days. Calculated BCRs: (a) 19.8%, (b) 9.7%, (c) 6.1%, (d) 3.3%, (e) 43.7%, (f) (36.3%), (g) 29.1%, and (h) 8.4%.

subcutaneous pocket; two HA-coated disks were implanted on the right side of the rats and two Ag-HA-coated disks on the left side. MRSA suspension (8.8–10.0 $\times$ 10^8 CFU, 20 μL per disk) was inoculated onto the disks in the pockets. Incisions were closed using interrupted 3-0 nylon suture. After the surgery, atipamezole (0.75 mg/kg) was used to induce recovery of animals from the anaesthesia. No analgesia was used after the operation. After 1 (3 rats), 3 (3 rats), or 7 days (4 rats), the animals were euthanised and the disks removed.

2.4.3. Calculation of Biofilm Coverage Rates (BCRs). All disks (from in vitro and in vivo experiments) were rinsed twice with 500 μL of sterile PBS and stained with biofilm stain (FilmTracer calcein red-orange biofilm stain, Thermo Fisher Scientific) for 1 h. After staining, disks were washed twice with 500 μL of sterile PBS. Thereafter, biofilm formation on each disk was observed under a fluorescence microscope (Axioplan 2; ZEISS, Jena, Germany). All quantifications were performed at a magnification of 50x. Random areas (seven per disk) were recorded as digital images. The BCRs on the disk surfaces were calculated using the image analysis software program ImageJ (National Institutes of Health, Bethesda, MD, USA) [29].

2.4.4. Statistical Analysis. All data are expressed as the median (range). For the in vitro study, groups were compared using the Kruskal–Wallis test. For the in vivo study, groups were compared using the Mann–Whitney U test. All statistical analyses were performed using the SPSS software program version 23 for Mac (IBM Corp., Armonk, NY, USA). A p value of <0.05 was considered significant.

3. Results

3.1. In Vitro Effects of the Ag-HA Coating. Figure 1 shows representative fluorescence microscopic images after bacterial culture for 7 and 14 days. The median BCRs on HA, 0.5% Ag-HA, 1% Ag-HA, and 3% Ag-HA after 7 days were 19.1% (2.8%–34.9%; 49 images), 8.0% (2.2%–28.8%; 14 images), 5.6% (3.0%–11.7%; 14 images), and 3.9% (1.8%–6.9%; 21 images), respectively. The median BCRs after 14 days were 38.6% (13.9%–55.7%; 63 images), 27.3% (9.8%–46.1%; 21 images), 23.2% (8.6%–40.0%; 21 images), and 6.6% (3.0%–19.7%; 21 images), respectively. The BCRs on all the Ag-HA-coated disks were significantly lower than those on the HA-coated disks (7 days: 0.5%, $p = 0.011$; 1%, $p < 0.001$; 3%, $p < 0.001$; 14 days: 0.5%, $p = 0.024$; 1%, $p < 0.001$; and 3%, $p < 0.001$). At 14 days the BCRs on 3% Ag-HA-coated disks were significantly lower than those on 0.5% ($p < 0.001$) and 1% Ag-HA-coated disks ($p = 0.013$; Figure 2).

3.2. In Vivo Effects of the Ag-HA Coating. None of the rats in any of the groups died during the experiment. Figure 3 shows the skin of a representative rat at 7 days after implantation. None of the rats exhibited any skin disorders or poor wound healing, indicating no observable toxic effects of the Ag-HA. Figure 4 shows representative fluorescence microscopic images. The BCRs of Ag-HA-coated disks were significantly lower ($p < 0.001$) than those of the HA-coated disks at all time points (Figure 5).

4. Discussion

In this study, we performed a systematic evaluation of the effects of Ag-HA coating on biofilm formation by MRSA.

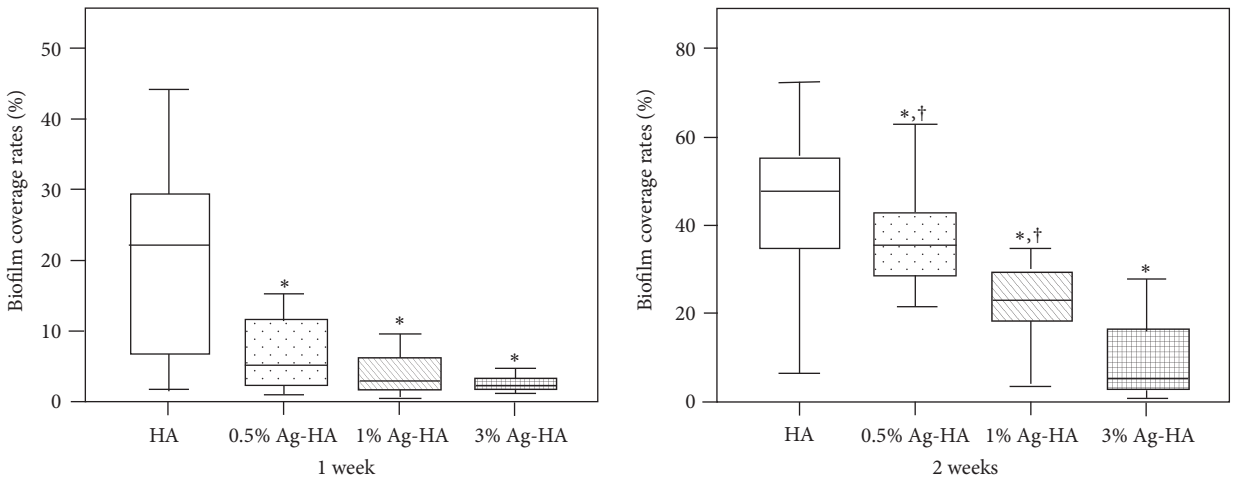

Figure 2: Box-and-whisker plots of biofilm coverage rates (BCRs) of implants after bacterial culture for 7 days and 14 days in vitro. In the 7-day experiment, 49 images of HA coating, including 14 images of 0.5% Ag-HA, 14 images of 1% Ag-HA, and 21 images of 3% Ag-HA, were used. In the 14-day experiment, 63 images of HA and 21 images of each group of Ag-HA were used. ∗ denotes significant differences between the BCRs of HA and each concentration of Ag-HA. The significance levels are as follows: 7 days: 0.5% Ag-HA, $p = 0.011$; 1% Ag-HA, $p < 0.001$; and 3% Ag-HA, $p < 0.001$; 14 days: 0.5% Ag-HA, $p = 0.024$; 1% Ag-HA, $p < 0.001$; and 3% Ag-HA, $p < 0.001$. † denotes significant differences between the BCRs of 3% Ag-HA and other concentrations (0.5%, 1%) of Ag-HA. The significance levels are 0.5% Ag-HA, $p < 0.001$, and 1% Ag-HA, $p = 0.013$.

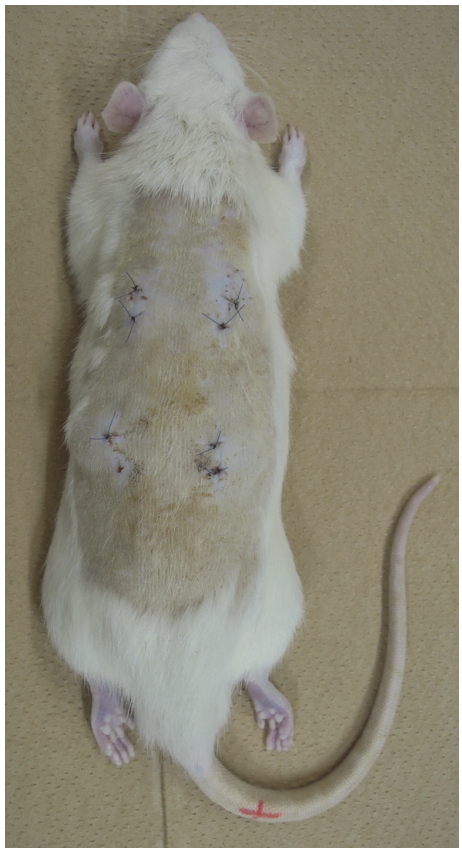

Figure 3: Representative photograph of rat dorsal skin at 7 days after implantation. None of the animals exhibited any signs of skin disorders or poor wound healing.

For evaluation of the biofilm coverage rates, the MRSA biofilm was stained with calcein red-orange. Methylene blue and SYTO 9 green fluorescent nucleic acid stain (Live/Dead staining) were not used, because these stains react not only with the MRSA biofilm but also with the debris of rat inflammatory tissues. To our knowledge, this study is the first to demonstrate that silver-containing HA reduces biofilm formation in vivo.

In our study, 3% Ag-HA reduced, but did not fully prevent, MRSA biofilm formation. We did not use any antibiotics in in vivo experiment. However, we usually use antibiotics to prevent surgical site infection at the time of orthopaedic surgery in humans. Morones-Ramirez et al. reported that silver ions enhanced the activity of antibiotics and broadened the spectrum of vancomycin [30]. Thus, synergism between Ag-HA and antibiotics could be expected to increase their effectiveness in treating implant-associated infection. Moreover, because silver has broad-spectrum antibacterial activity, Ag-HA can affect other bacteria as well.

In addition, we evaluated the potential toxic effects of the silver in Ag-HA in vivo. Argyria, a grey-blue tissue discoloration that can be observed in humans exposed to silver or using silver-containing medications, is the most common toxic effect of silver [31, 32]. In this study, none of the animals exhibited any signs of skin disorders or poor wound healing caused by silver toxicity, despite implantation of the disks just under the skin. Silver levels in the blood below 200 ppb are considered normal, whereas levels higher than 310 ppb are reported to cause argyria, argyrosis, and liver and kidney damage [33]. Tsukamoto et al. previously demonstrated that, in a rat tibia model with Ag-HA-coated implants, serum silver levels were sufficiently low to avoid

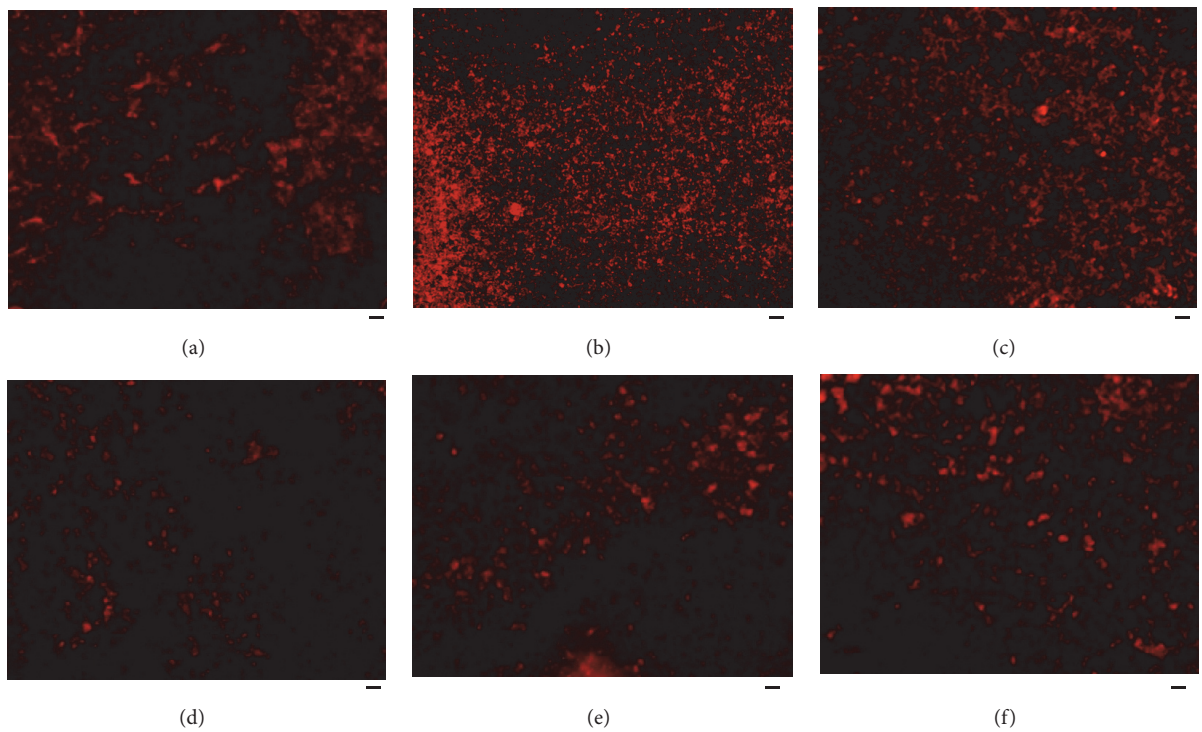

Figure 4: Representative fluorescence microscopic images of implants (50x objective) after bacterial infection in vivo. The bar indicates 100 μm. The red coloured areas in the picture correspond to the locations covered by biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA). (a) HA coating at 1 day, (b) HA coating at 3 days, (c) HA coating at 7 days, (d) 3% Ag-HA coating at 1 day, (e) 3% Ag-HA coating at 3 days, and (f) 3% Ag-HA coating at 7 days. Calculated BCRs: (a) 16.0%, (b) 26.6%, (c) 30.1%, (d) 8.9%, (e) 14.6%, and (f) 13.7%.

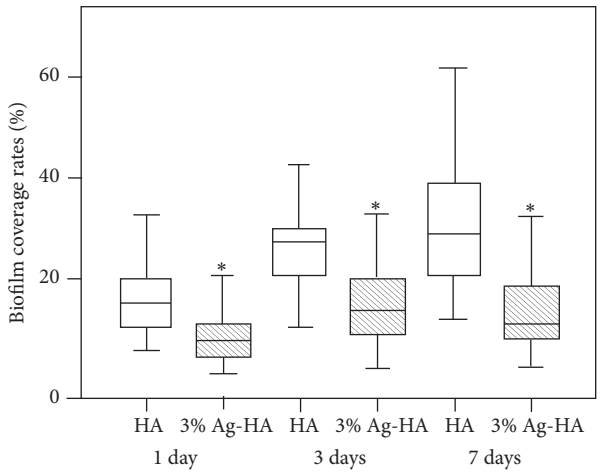

Figure 5: Box-and-whisker plots of BCRs of implants at 1, 3, and 7 days after bacterial infection. $*$ denotes significant differences ($p < 0.001$) between the BCRs of HA and Ag-HA. Calculated BCRs after 1 day: HA 14.7% (5.4%–54.7%; 42 images) and 3% Ag-HA 7.6% (0.8%–28.2%; 42 images); after 3 days: HA 27.2% (10.4%–53.3%; 42 images) and 3% Ag-HA 13.6% (2.8%–33.0%; 42 images); and after 7 days: HA 28.8% (12.0%–61.3%; 56 images) and 3% Ag-HA 11.0% (2.3%–32.2%; 56 images).

harmful effects, and no degeneration was observed in the brain, liver, kidneys, or spleen. The amount of silver required for Ag-HA coating of femoral replacements in humans is low enough to avoid argyria [34]. In fact, in a 1-year follow-up study, we found that none of the patients developed any adverse reactions to silver from Ag-HA-coated implants in total hip arthroplasties [35].

Furthermore, it is important to consider conglutination of implants to the bone. Yonekura et al. reported that 50% Ag-HA coating inhibited bone formation, while 3% Ag-HA coating showed good osteoconductivity [18]. Accordingly, Eto et al. reported that 3% Ag-HA supported viability and function of osteoblasts, as well as anchorage strength. In pull-out tests using rat femurs, there were no significant differences between 3% Ag-HA and HA, whereas 50% Ag-HA required less force [36]. Therefore, we speculate that orthopaedic implants coated with 3% Ag-HA will have low silver toxicity while maintaining antibiofilm activity of silver, combined with the good osteoconductivity of HA. In our clinical trial with Ag-HA-coated implants, there were no implant failures [35].

Despite our promising results, this study had some limitations. The experiments were performed for only 7 (in vivo) or 14 (in vitro) days, which reflect acute infection. Such a short duration is not suitable for the evaluation of antibiofilm activity in chronic infections. In a previous study using a rat tibial model, antibacterial activity was demonstrated 4 weeks after implantation, suggesting that Ag-HA could sufficiently prevent acute and subacute infections [27]. To evaluate potential resistance to chronic infection, other models are needed.

In our in vivo study, we observed differences between HA- and Ag-HA-coated disks implanted in the same rat. Thus, the released silver ions affected only the local surgical site. Usually, only the part of the implant surface that is in contact with the bone, and not the entire orthopaedic implant, is coated. However, because the released silver ions can spread, the effectiveness of the antibiofilm activities of Ag-HA on the uncoated part of the implant surface remains to be clarified.

5. Conclusion

Ag-HA coating reduced biofilm formation by MRSA in vitro and in vivo, indicating that Ag-HA coatings could help manage refractory orthopaedic infections. Coating of orthopaedic implants with Ag-HA might be expected to decrease the incidence of postoperative implant-associated infections, improve the quality of life of patients, and promote favourable outcomes in orthopaedic surgery.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

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