

Enduring Protection Against *S. aureus*: Evaluating the Antimicrobial Efficacy of Ag-HA Coated Implants in Orthopedic Applications

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INTRODUCTION: Silver-containing hydroxyapatite (Ag-HA) coatings have been previously studied for their potential to prevent periprosthetic joint infections (PJI) – a prevalent complication following joint replacement surgeries. Despite these studies, questions persist regarding the efficacy of Ag-HA coatings against infections that manifest later post-implantation. Addressing this gap, our study delves into the antibacterial properties of Ag-HA coatings, utilizing a rat model designed specifically to simulate late-onset orthopedic implant-associated infections (LOIAI).

METHODS: Titanium disc specimens from Kobe Steel, Kobe, Japan, were coated using two processes: Ag-HA and HA-only. The Ag-HA coating was achieved by thermally spraying HA powder enriched with silver oxide followed by crystallization through vacuum heat treatment. Simultaneously, a pure HA coating, absent of Ag, was produced following the identical protocol, serving as our control group. We then prepared twenty disc specimens for each category, both HA and Ag-HA, each measuring 14 mm in width and 1 mm in thickness. For the *in vitro* experiments, both sets of specimens—HA and Ag-HA—were immersed in fetal bovine serum for a duration of 3 months, ensuring the serum's replacement twice a week. Post this 3-month incubation, discs were set on individual dishes, and exposed to 10 µL of a methicillin-resistant *Staphylococcus aureus* (MRSA) suspension with a density of 1.1×10^5 CFU/mL (equating to 1.1×10^3 CFU per disc). They were then incubated at 37°C for 48 hours. After this, we utilized calcein red-orange staining to highlight the presence of biofilms on these discs. Detailed biofilm visualization was carried out using the CLSM LSM880 microscope (Carl Zeiss AG) with a 20x air objective lens. This involved capturing 625 individual tiled images (25 × 25), assuming the total surface of a 14-mm diameter disc could be approximated by a 10 mm × 10 mm square. In the *in vivo* segment, we utilized a LOIAI rat model, achieved by subcutaneously implanting either HA or Ag-HA coated discs into Sprague-Dawley rats for 3 months. During our first *in vivo* experiment, both sets of specimens (26 from HA group and 26 from Ag-HA group) were exposed to MRSA. Post a 48-hour period, colony numbers were quantified using the plate dilution method. For our second *in vivo* experiment, the same number of specimens from both groups were inoculated using bioluminescent *S. aureus* Xen36. The progression of bioluminescence was systematically recorded over a duration using the IVIS (Lumina III version 4.4). This study was approved by an Ethics Committee. Throughout our study, all continuous variables were reported as means and accompanied by the standard error of the mean (SEM). For data comparisons between the HA and Ag-HA groups, we employed the Mann–Whitney U-test, setting our statistical significance threshold at $p < 0.05$.

RESULTS: The mean total biofilm volume per disc in the Ag-HA group ($2.4 [0.9] \times 10^5 \mu\text{m}^3$) was significantly lower than that in the HA group ($7.5 [4.0] \times 10^6 \mu\text{m}^3$; $p = 0.023$; Fig. 1). The mean bacterial count in the Ag-HA group ($1.3 [0.5] \times 10^6$ CFU/disc) was significantly lower than that in the HA group ($2.5 [0.9] \times 10^7$ CFU/disc; $p < 0.0001$; Fig. 2). The mean bioluminescence, expressed as the average radiance [photons/s/cm²/sr], in the Ag-HA group was significantly lower than that in the HA group on days 1, 2, 3, 4, and 7 post inoculation ($p = 0.0113$, $p < 0.0001$, $p = 0.0001$, $p = 0.0016$, and $p = 0.0268$, respectively; Fig. 3).

DISCUSSION: The consensus on the definition of a timeframe for LOIAI including late-onset PJI remains debatable. Nevertheless, a significant proportion of PJIs manifest within the initial 90 days following surgical intervention. To achieve an accurate estimate of surgical site infections consequent to joint replacement, surveillance extending to a minimum of 3 months post-surgery is recommended. Guided by this reasoning, our study equated LOIAI with occurrences 3 months subsequent to arthroplasty. Consequently, we devised a model that set the time post immersion and implantation at 3 months. Our investigation led to two pivotal revelations. Firstly, our Ag-HA-coated discs impeded the biofilm development of MRSA in *in vitro* experiments. Secondly, our Ag-HA-coated discs displayed sustained antibacterial efficacy against *S. aureus* in *in vivo* experiments. These observations underscore the antimicrobial prowess of Ag-HA coatings against LOIAI and spotlight its prospective utility in curbing late-onset PJIs. However, our study isn't without constraints. For one, while our *in vivo* experiments carried out subcutaneously present the merits of accommodating a variety of implant specimens through straightforward surgical techniques, it's imperative to extend this research to bone contexts, particularly in the realm of PJI research. Secondly, our assessment was exclusively centered on infections from MRSA and methicillin-susceptible *S. aureus* strains. Comprehensive evaluations encapsulating other pathogens, such as *S. epidermidis*, *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and certain fungi, warrant future consideration.

SIGNIFICANCE/CLINICAL RELEVANCE: This research underscores the potential of Ag-HA-coated implants in offering enduring protection against *S. aureus* infections. Given the challenge posed by late-onset PJIs in clinical settings, these implants may represent a promising advancement in orthopedic interventions.

Fig. 1 a) Biofilm formation measurements *in vitro*. * $p < 0.05$. b) Biofilm formation images.

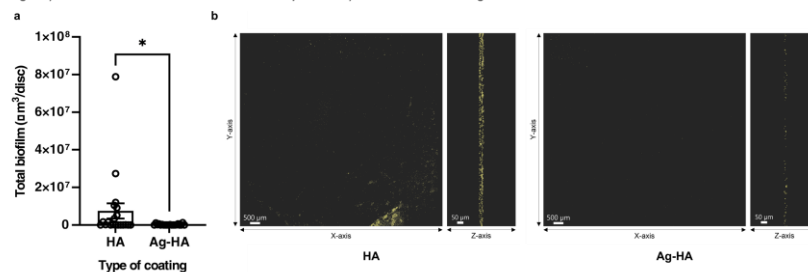


Fig. 2 Bacterial count in *in vivo* experiment 1. **** $p < 0.0001$

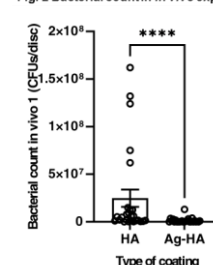


Fig. 3 a) Bioluminescence measurements in *in vivo* experiment 2 using the IVIS and Xen36. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. b) Bioluminescence images.

