Evaluation of a silver-impregnated coating to inhibit colonization of orthopaedic implants by biofilm forming methicillin-resistant \textit{Staphylococcus pseudintermedius}

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\begin{abstract}
Objectives: To evaluate the \textit{in vitro} antibacterial activity of a silver-impregnated coating against a biofilm-forming strain of methicillin-resistant \textit{Staphylococcus pseudintermedius} (MRSP).

Methods: A clinical MRSP isolate sourced from a failed canine knee implant was evaluated for biofilm production and used in the present study. Using a standard test method and a clinically approved titanium substrate, the antimicrobial activity of a novel silver plasma coating was determined at two times: five minutes after inoculation of the specimens (T0) and after 24 hours of incubation (T24). Scanning electron microscopy was used to evaluate the biofilm formation on specimens.

Results: The tested clinical MRSP isolate was classified as a strong biofilm producer. The silver coating significantly reduced the MRSP growth more than four log steps compared to the non-coated specimens and showed more than 99.98\% reduction in the number of colony forming units after 24 hours. Scanning electron microscopy images revealed that silver-coated surfaces did not manifest detectable biofilm, while biofilm formation was readily observed on the control specimens.

Clinical significance: The silver coating exhibited excellent activity against the multidrug-resistant biofilm-forming MRSP isolate. The next stage of this work will involve testing in an animal model of orthopaedic infection. Positive results from animal studies would support the introduction of the silver plasma coating as a new strategy for preventing implant contamination, biofilm formation, and surgical infection in dogs undergoing orthopaedic surgery.

Introduction

Surgical site infection (SSI) after an orthopaedic procedure is a major complication that is associated with increased morbidity, mortality, and financial expense. Implant-related SSI can be difficult or impossible to resolve with routine antimicrobial therapy alone due to the formation of biofilm on the orthopaedic implants (1). Staphylococci are the most frequent causes of biofilm-associated infections as they are common opportunistic bacteria that reside on the skin and mucous surfaces (2). The most clinically relevant are the coagulase positive Staphylococci, and in dogs specifically: \textit{Staphylococcus pseudintermedius}. Recently, methicillin-resistant \textit{Staphylococcus pseudintermedius} (MRSP) has emerged as an important cause of SSI in dogs (3). Methicillin-resistant \textit{Staphylococcus pseudintermedius} isolates are often not only resistant to \(\beta\)-lactam antibiotics, but also to several other classes of antimicrobial drugs (4). The large increase in antimicrobial-resistant microorganisms clearly shows that new control strategies are required (5). Antimicrobial coatings of implant surfaces, such as coatings containing or releasing antimicrobial agents, have a great potential in this context. Coatings containing inorganic antimicrobial agents are very attractive alternatives from the perspective of coating of biomaterials, and have advantages including good antibacterial activity, biocompatibility, and stability (6).

Silver coated orthopaedic implants have
been widely used to prevent the growth of bacterial biofilms due to silver’s broad-spectrum of activity against gram-positive and gram-negative bacteria (7-9).

As the use of silver and silver-based products increases, it is becoming important to clarify the efficacy and efficiency of silver against different microorganisms and biofilms. Accordingly, the objective of this study was to evaluate the in vitro antibacterial activity of new ultrathin plasma coating with polysiloxan embedded silver particles against a strong biofilm-forming MRSP strain.

Materials and methods

An MRSP isolate (OSU 12–2910), originally sourced from an infected canine total knee replacement in a nine-year-old male neutered Kuvasz dog was evaluated for biofilm production using a microtitre plate assay (MPA) (10). The average optical density at a wavelength of 570 nm (OD\textsubscript{570}) of the triplicates of isolate and negative controls and the cut-off value (OD\textsubscript{c}) were established where OD\textsubscript{c} = average OD\textsubscript{570} of the negative control + 3×SD (where SD = standard deviation) of the negative control. According to this model the tested clinical MRSP isolate was classified as a strong biofilm producer where the OD\textsubscript{570} of the eluted crystal violet was greater than four times the cut-off value (4×OD\textsubscript{c}), consistent with an earlier study of *S. pseudintermedius*, which showed that the majority of isolates produced biofilm, and 96% were classified as either strong or moderate biofilm producers (11).

Silver/Siloxane chemistry (Ag/SiO\textsubscript{x}C\textsubscript{y}) plasma polymer-coated circular discs (10 mm diameter and 1 mm thickness) were manufactured from commercially pure titanium (ASTM F67), and had previously undergone cytotoxicity testing in L-929 mouse fibroblast cells with a method compliant with ISO 10993–5; 2009, with no cytotoxicity evident by 72 hours (unpublished data). Uncoated titanium discs were used as negative controls. All discs were sterilized by gamma irradiation prior to laboratory testing. The in vitro antimicrobial activity assay was performed according to the standard test method, ASTM E-2180–07, with the modification of using one log step higher inoculum (12).

Microbiological evaluation

The antimicrobial efficacies of silver-coated titanium specimens (n = 12), and controls (n = 12) were evaluated at two time points: 5 minutes after inoculation of the specimens (T\textsubscript{0}) and after 24 hours of incubation (T\textsubscript{24}). The numbers of recovered organisms were averaged as the mean colony forming units (CFU)/ml. The averaged means were then transformed and expressed as mean log\textsubscript{10} CFU. The statistical significance between the mean log\textsubscript{10} CFU counts of silver-coated and control specimens at T\textsubscript{0} and T\textsubscript{24} was evaluated using an unpaired t-test; a value of p <0.05 was considered statistically significant.

Scanning electron microscopy evaluation

In vitro antibacterial efficiency of the silver coating and biofilm structure was secondarily evaluated by scanning electron microscopy (SEM). Three titanium specimens (one silver-coated and two control uncoated) were incubated separately, each in a petri dish containing 10 ml of MRSP suspension in tryptic soy broth of OD\textsubscript{600} = 0.5 for 24 hours aerobically at 37°C to initiate biofilm formation. Following incubation,
each specimen was washed by immersion in 10 ml of phosphate-buffered saline and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer; pH 7.4 at 4°C until time of SEM imaging.

Results

At T₀, there was no significant difference in MRSP growth between control uncoated discs (3.83 ± 0.51 log₁₀ CFU/ml, mean ± SD) and silver-coated discs (3.59 ± 0.33 log₁₀ CFU/ml) (p = 0.36) (Figure 1). This demonstrates that the initial bacterial challenge was similar in test and control specimens. At T₂₄, the silver coated discs had significantly reduced growth (0.64 ± 0.99 log₁₀ CFU/ml) which resulted in a difference of more than four log steps as compared to the non-coated discs (4.60 ± 0.91 log₁₀ CFU/ml) (p <0.0001). Uncoated discs did not show any reduction in the number of bacteria while the silver coating demonstrated a significant antimicrobial efficacy and showed more than 99.98% reduction in the number of CFU/ml after 24-hour incubation.

The results obtained by SEM revealed no bacterial growth or biofilm formation on the silver-coated specimen (n = 1) after 24 hours of incubation in strong biofilm forming MRSP suspension, while biofilm formation was observed on the control uncoated specimens (n = 2). The biofilm was characterized by microcolonies of bacteria along with large amounts of irregularly extracellular polymeric substances (EPS) (Figure 2).

These SEM images correlated with the lower number of CFU recovered on the silver-coated specimens after 24 hours of incubation compared to uncoated specimens. Similar findings were observed in another study (11).

Discussion

Previous studies that have evaluated clinically relevant Staphylococcus spp. affecting human beings rather than dogs, have shown excellent in vitro antimicrobial activity of different formulations of silver coatings against Staphylococcus aureus, Staphylococcus epidermidis, methicillin-resistant Staphylococcus aureus (MRSA), and methicillin-resistant Staphylococcus epidermidis (MRSE) (7, 13). The Ag/SiO₂/C₆ coating showed a significant in vitro antimicrobial activity against MRSA and ex vivo suppression of more than 99.9% of bacterial growth by the coating compared to non-coated samples after 28 days (7). Similar results were observed during a study on Staphylococcus epidermidis where they demonstrated that fixation pins coated with silver showed a three-log step reduction in the number of biofilm-forming bacteria compared to a non-coated stainless steel or titanium implant (8). The present study demonstrated that the new silver plasma coating was highly effective against biofilm-forming MRSP and showed more than 99.98% reduction in the number of CFU compared with the non-coated specimens. This work is the first report of successful application of silver coating technology to a MSRP isolate that is a direct isolate from an infected canine implant.

The antimicrobial activity of silver is dependent on the availability of free silver ions (SI). In the presence of moisture, the embedded metallic silver particles (Ag⁰) generate silver ions which diffuse through the siloxane top layer to create an antimicrobial surface. The pure metallic silver particles act as a depot of silver and provide a continuous and long-term generation of silver ions. Silver ions strongly bind to bacterial cellular components such as enzymes and structural proteins leading to altered function (9, 14, 15). Free SI interfere with bacterial cell metabolism and disturb the integrity of the bacterial cell membrane (14, 16). Furthermore, SI can interact with the DNA of bacteria, preventing bacterial replication (16).

Antimicrobial activity of surfaces coated with silver seems to be dependent on the size of the silver particles that are used.
Colloidal silver for coating of fixation pins resulted in deficient antimicrobial effect. In contrast, nanoparticulate silver provided a larger active surface area and a more homogeneous distribution of silver on biomaterials (17). The titanium specimens used in the present study were coated with a plasma polymer in which silver nanoparticles (5–50 nm) were embedded. Our findings are similar to those demonstrated in a previous study where smaller particles with a larger surface area available for interaction provided a more efficient means of antibacterial activity than larger particles (14). It has been reported that impregnation of silver into a coating can be more effective than direct surface coating alone as surface silver can be deactivated by protein anions (15).

In conclusion, the results from this study confirm the in vitro antimicrobial activity of the silver impregnated coating against a strong biofilm-forming MRSP strain that was isolated from a dog with an infected total knee replacement. Our findings suggest that this silver plasma coating may represent a potentially valuable strategy for reducing adhesion of MRSP and preventing implant-associated infections in dogs undergoing orthopaedic surgery.

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Conflict of interest

Dr. Allen is a paid consultant to BioMedtrix, LLC, for their canine total knee replacement program. He received the coated materials and controls from BioMedtrix for this study, however, no compensation was provided for performing the study.

References