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Evaluation of the temporary effect of physical vapor deposition silver coating on resistance to infection in transdermal skin and bone integrated pylon with deep porosity

Maxim A. Shevtsov^{1,2,3,4}, Natalia M. Yudintceva¹, Miralda I. Blinova¹, Irina V. Voronkina¹, Dmitriy N. Suslov³, Oleg V. Galibin³, Dmitriy V. Gavrilov³, Michael Akkaoui⁵, Grigoriy Raykhtsaum⁶, Andrey V. Albul⁷, Emil Pitkin⁸, and Mark Pitkin^{6,9}

¹Institute of Cytology of the Russian Academy of Sciences (RAS), St. Petersburg, Russia

²Technical University of Munich, Munich, Germany ³Pavlov First Saint Petersburg State Medical University, St. Petersburg, Russia ⁴Polenov Russian Scientific Research Institute of Neurosurgery, St. Petersburg, Russia ⁵Tanury Industries, Lincoln, Rhode Island ⁶Poly-Orth International, Sharon, Massachusetts ⁷Veterinary Clinic "Neurology, Traumatology and Intensive Therapy of Doctor Sotnikov V.V.," St. Petersburg, Russia ⁸Wharton School, University of Pennsylvania, Philadelphia, Pennsylvania ⁹Tufts University School of Medicine, Boston, Massachusetts

Abstract

Periprosthetic infection via skin-implant interface is a leading cause of failures and revisions in direct skeletal attachment of limb prostheses. Implants with deep porosity fabricated with skin and bone integrated pylons (SBIP) technology allow for skin in growth through the implant's structure creating natural barrier against infection. However, until the skin cells remodel in all pores of the implant, additional care is required to prevent from entering bacteria to the still non-occupied pores. Temporary silver coating was evaluated in this work as a means to provide protection from infection immediately after implantation followed by dissolution of silver layer in few weeks. A sputtering coating with 1 μm thickness was selected to be sufficient for fighting infection until the deep ingrowth of skin in the porous structure of the pylon is completed. *In vitro* study showed less bacterial (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*) growth on silver coated tablets compared to the control group. Analysis of cellular density of MG-63 cells, fibroblasts, and mesenchymal stem cells (MSCs) showed that silver coating did not inhibit the cell growth on the implants and did not affect cellular functional activity. The *in vivo* study did not show any postoperative complications during the 6-month observation period in the model of above-knee amputation in rabbits when SBIP implants, either silver-coated or untreated were inserted into the bone residuum. Three-phase scintigraphy demonstrated angiogenesis in the pores of the pylons. The findings suggest that a silver coating with well-chosen specifications can increase the safety of porous implants for direct skeletal attachment.

Keywords

silver coating; implants; osseointegration; prosthesis; matrixmetalloproteinases; biofilm

INTRODUCTION

Direct skeletal attachment (DSA) of limb prostheses represents an alternative technology to traditional suspension of prostheses via various socket systems, with clear indications when the sockets cannot be properly fitted. Several preclinical and clinical studies demonstrated biomechanical efficiency of the DSA in rehabilitating patients.^{1–4} However, the rate of implant-related infections is still high.⁵ For example, in the study by Branemark et al.⁴ involving 51 patients treated with DSA, the superficial infection rate was 54.9%.

A number of implant surface modifications have been suggested whose aim is to decrease bacterial adhesion and to create a bactericidal effect. The purpose is to minimize the risk of infection complications. Romano et al.⁶ divided antibacterial coatings of the implants into three groups: (1) passive surface modification that prevents biofilm formation; (2) active surface modification that contains pharmacologically active bactericidal agents (that is, metals or non-metal elements, organic substances); and (3) local carriers or coatings that are applied at the time of the surgical procedure. Antibacterial coatings employing metals (i.e., silver, copper, zinc) proved to be an effective and cost-effective strategy in reducing the risk of infectious complications^{7,8}

The antimicrobial effects of the silver are non-specific and influence many bacterial structures and metabolic processes including the disruption of the metabolic pathways^{9,10} inactivation of bacterial enzymes,¹⁰ disruption of the bacterial cell wall,¹¹ increase in the permeability of the cytoplasmic membrane,¹² generation of the reactive oxygen species (ROS),¹³ and damage of the DNA¹⁰.

While many studies demonstrated clinical efficacy and safety of the implication of the silver-coated implants,^{14–19} recent reports suggest that silver may negatively affect patient health^{8,20–22}

This concern deserves special attention, since the implants for DSA are supposed to be used for the duration of a patient's life.

We developed the skin and bone integrated pylon (SBIP) technology, manufactured from medical grade titanium Ti6Al-4V-ELI (ASTM F1472),^{23,24} to optimize the DSA procedure. The patented specifications of the implant include porosity, pore size, particle size and volume fraction of the material composition for sintering and the specific silver coating.²⁵ High biointegrative and mechanical properties of the SBIP implants in various animal species including rats, mice, rabbits, and cats were demonstrated.^{24,26–28} The safety and sustainability of the skin-device and bone-device interface is provided for by the SBIP's composite porous structure, which promotes deep ingrowth of the hosting skin into the implanted pylon. The direction of ingrowth is perpendicular to its longitudinal axis and in parallel to the skin's plane. The skin ingrowth creates a natural infection barrier and fixes the

surrounding skin in place,²⁹ addressing the principal failure modes in percutaneous devices: marsupialization, permigration, and avulsion.³⁰

In the current study, silver coating using the physical vapor deposition (PVD) technique was evaluated. The purpose was to assess the feasibility of *in vivo* application of silver-coated porous titanium pylon that has an increased resistance to infection during the maximally vulnerable period after implantation. This supplementary resistance to infection is needed for about 4–6 weeks while skin remodels within the porous SBIP structure,³¹ which can also be an inviting pathway for bacteria during that period. After the ingrowth is completed, the skin is able to protect the body from infection with its natural capacity, while a continued presence of silver in the implant's structure could be a source of unwanted migrations and depositions of silver ions in tissues and organs.

In the set of *in vitro* experiments, PVD silver coated porous titanium tablets were assessed by their cytotoxic and antibacterial properties. For analysis of the cell adhesion on the SBIP silver-coated implants and formation of the cellular monolayer, the tablets were coincubated consequently with MG-63 human osteosarcoma cells, mesenchymal stem cells (MSCs), and rabbits' dermal fibroblasts. These cell types are important in general for wound healing, proliferation and tissue remodeling. Expression of the MMPs is required for the effective wound healing and later biointegration of the titanium implant. In the current study, we assessed the expression of MMP-1, -2 and -9.

A subsequent *in vivo* study in the model of above-knee amputation in New Zealand rabbits was conducted to prove the safety and efficacy of the silver-coated SBIP composite pylons.

MATERIALS AND METHODS

Implant preparation

The principal distinction between the SBIP and existing DSA systems³² is the SBIP's ability to promote the deep ingrowth of hosting tissues while still withstanding the loads associated with locomotion.^{33–35} It is manufactured with a patented combination of four parameters: *particle size*, *pore size*, *porosity* and *volume fraction*.²⁵

Volume fraction quantifies the depth of porosity in the composite device. It is defined as the ratio of the volume of the porous portion to the entire volume of the device. In totally porous devices like the tablets used in this *in vitro* study, the volume fraction is 100%. In SBIP composite pylons used for the *in vivo* study the volume fraction was between 50 and 60%. In contrast with other devices whose thin porous coating has a volume fraction under 20%,^{36,37} the SBIP's high volume fraction permits the deep ingrowth of the hosting bone and skin tissues. Despite the high degree of porosity, the durability, resistance to fatigue, safety and efficacy of the SBIP specification have been verified mechanical tests and animal studies.^{23,27,28,33}

The tablets and SBIP samples [see Figure 1(A-2,B-2)] were coated with a 1 µm silver layer with an intermittent pattern (Tanury Industries, Lincoln, RI) as specified in Ref. 25. The technology used was physical vapor deposition (PVD),³⁸ and the equipment used was the

magnetron sputtering multi-target machine, Flexicoat series (IHI Hauzer Techno Coating B.V.). Silver content of the test-items was 1.00 ± 0.02 mg/cm²; total silver content per test-item was 5.0 ± 0.1 mg.

When selecting the coating specification, we wanted to take advantage of the well-established bactericidal properties of silver³⁹ while avoiding the possible toxic consequences of a long-term exposure of silver,^{40–44} as the implantation of pylons for DSA patients is permanent. Accordingly, the impermanent silver layer is thin enough to dissolve about 4–6 weeks after implantation.⁴⁵ That time is sufficient for the skin to regenerate into the porous cladding of the SBIP pylon and establish a sustainable natural barrier against infection.

Both the porous titanium tablets used in the *in vitro* study [Figure 1(A)] and the SBIP composite pylons used in the *in vivo* study [Figure 1(B)] were developed by Poly-Orth International, Sharon, MA. The manufacturing process has been described in detail elsewhere.^{24,33} The samples were sintered (ADMA Products Group, Hudson, OH) from medical grade titanium Ti6Al-4V-ELI (ASTM F1472) in boron nitride molds (Payne Engineering & Fab. Co., Canton, MA). The 4-h sintering cycle was performed in a Vacuum Industries Super VII furnace (Centorr Vacuum Industries; Nashua, NH).

***In vitro* evaluation of the silver-coated SBIP tablets**

Cells—An MG-63 human osteosarcoma cell line was obtained from the Russian Cell Culture Collection at the Institute of Cytology of the Russian Academy of Sciences (St. Petersburg, Russia). MG-63 cells were grown in a CO₂-incubator (37°C, 6% CO₂) in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (100 units/mL Penicillin G and 100 µg/mL Streptomycin).

Mesenchymal stem cells (MSCs) were obtained from the femoral bone marrow of New Zealand rabbits as described previously.⁴⁶ Following extrusion by flushing from the femur, cells were grown in a CO₂-incubator (37°C, 5% CO₂) in αMEM medium supplemented with 10% FBS and antibiotics. After 4 days of incubation, nonadherent cells were removed by replacing the medium. Before the experiments, cells were harvested in the log phase of growth and their viability was determined by 0.4% Trypan blue exclusion. The cells were used after 3–5 passages.

Rabbits' dermal fibroblasts were obtained from rabbit skin fragments as was described in Ref. 47. The fragments of skin were cut into small 0.1 × 0.1 cm pieces that were incubated in DMEM medium supplemented by 2 mM L-glutamine, 10% FBS and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin G). After 3–4 weeks, a confluent fibroblast cell monolayer was developed on a plate surface. The cell viability was determined by 0.4% Trypan blue exclusion.

Cell coincubation on the tablets—We sought to analyze the cells' adhesion and evaluate whether a cellular monolayer could successfully form. For that purpose, SBIP implants were co-incubated with MSCs (2×10^6), fibroblasts (2×10^6) or MG-63 (2×10^6) cells in collagen type I gel (2 mg/mL) at 37°C in a humidified 5% CO₂/95% air atmosphere for 30 min. Following the polymerization of the collagen gel, implants were harvested in

α MEM medium supplemented with 10% FBS, 2 mM of L-glutamine and antibiotics in a CO₂ incubator for 7 days. For the control, uncoated SBIP and silver-coated implants filled with collagen gel but containing no cells were used.

Following coincubation, the implants were washed with phosphate buffer solution (PBS), fixed in 4% paraformaldehyde and analyzed using a scanning electron microscope.

Additionally, we characterized the structure of the produced silver-coated titanium tablets before and after coincubation with fibroblasts by X-ray photoelectron spectroscopy (XPS). XPS spectra of the investigated samples was assessed employing “Thermo Fisher Scientific Escalab 250Xi” spectrometer. The samples were excited by Al K α (1486.7 eV) X-rays in a base pressure of 7×10^{-8} Pa. High-resolution spectra were automatically charge compensated by setting the binding energy of C 1s carbon line to 284.8 eV.

Analysis of the matrix metalloproteinases production—The activity of matrix metalloproteinases (MMP-1, MMP-2, and MMP-9) in the culture medium following co-incubation with uncoated and silver coated titanium samples was determined on days 1, 4 and 9 by the zymography method described elsewhere.⁴⁸ Gelatin and casein were used as substrates to evaluate the activity of gelatinases (MMP-2, MMP-9) and collagenases (MMP-1), respectively. A gel (10% acrylamide) contained 1.0 mg/mL gelatin or 0.5 mg/mL casein. Ten micrograms of protein per lane were loaded into the gel. The protein content in the probes was measured by Bradford protein assay. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min and then incubated in a buffer solution (50 mM Tris-HCl pH 7.6, 0.15 M NaCl, 10 mM CaCl₂, 0.05% Brij 35) for 12 h. Then, the gel was stained with Coomassie Blue R-250 (0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 h) and, after destaining (destained with 7% acetic acid for 1 h), the bands containing MMP were developed as nonstained bands. A medium conditioned by HT-1080 fibroblasts was used as a marker to determine MMP zones (obtained from the Culture Collections of Institute of Cytology of the Russian Academy of Sciences [RAS], St Petersburg, Russia). For the quantitative assay, gels were scanned and images were processed with QuantiScan 2.1 software. MMP activity, presented in arbitrary units, was analyzed with the program QuantiScan calculating the number of colored pixels according to band the intensity of color. The results of densitometry were presented as histograms.

Bacterial strains and growth conditions—To assess surface and planktonic growth, bacterial strains selected in the study included *Staphylococcus epidermidis* (ATCC35984), *Staphylococcus aureus* (ATCC25923) and *Pseudomonas aeruginosa* (ATCC 27835). The strains were routinely cultured overnight in Columbia Agar with 5% sheep’s blood (Becton–Dickinson, Heidelberg, Germany) at 37 °C before experiments. Bacteria were then harvested by centrifugation, rinsed, suspended, diluted in PBS and adjusted by densitometry to a MacFarland 0.5 standard (MacFarland Densimat™, BioMérieux, Marcy l’Etoile, France) up to a CFU count of 1.5×10^8 CFU/mL. The colony-forming units (CFU) were counted and colony numbers calculated accordingly. For the study, every suspension with its known bacterial concentration was diluted with DMEM supplemented by 10% FBS to reach the targeted value for bacterial concentration (10^8 CFU/mL). Sample plates were placed in 24-

well culture plates and 1 mL of 10^8 CFU/mL bacterial suspensions were added. Incubation of the well plates was conducted for 24 h at 37°C.

Determination of antibacterial activity of the silver-coated implants—We sought to analyze the antibacterial properties of the PVD silver coated implants. For that purpose, two parameters were assessed: (1) bacterial biofilm formation and (2) bacterial planktonic growth in the culture medium. Uncoated porous titanium samples were used as a reference control. In the first step, bacterial adhesion was evaluated by determining bacterial concentration on the surface of the titanium implants. To determine bacterial concentration, after 24, 48, and 72 h of coincubation with *S. epidermidis*, *S. aureus* and *P. aeruginosa*, colonized titanium samples were removed from the plate, washed with PBS and transferred to vials containing 3 mL of sterile PBS and sonicated for 7 min with frequency 40 ± 2 kHz (Elmasonic S60H, Elma, Singen, Germany) to remove adhering bacteria. One hundred microliters of the suspension were aspirated, plated on Colombia Agar at 37°C for 24 h and quantified after incubation (CFU/mL). In the second step, the bacterial planktonic growth was measured in the growth medium following 24, 48 and 72 h of coincubation with titanium samples. The medium containing bacteria was plated on Columbia Agar after serial dilutions and incubated at 37°C for 24 h. Thereafter, CFU were quantified and extrapolated to CFU/mL. For every time-point group three different samples were collected and measured.

***In vivo* evaluation of the silver-coated SBIP pylons**

To analyze the biocompatibility and feasibility of the SBIP implants coated with silver, we applied the model of above knee amputation in rabbits as described elsewhere.³¹ Male New Zealand rabbits weighing 2.8–3.0 kg were obtained from the Rappolovo nursery of the Russian Academy of Medical Sciences (St. Petersburg, Russia). All animal experiments had been approved by the local ethical committee of Pavlov First Saint Petersburg State Medical University (St. Petersburg, Russia) and were in accordance with institutional guidelines for the welfare of animals and guidelines outlined in the NRC “Guide for the care and use of laboratory animals” (8th edition, 2011).

Following amputation, the intraosseous SBIP implants were inserted intramedullary into the rabbit’s residual femoral bone and the soft tissues and skin were closed over the residuum and SBIP implant. The animals were divided into two groups: (1) implantation of the silver-coated SBIP (five animals); (2) implantation of the nontreated control SBIP (four animals). For sedation, fentanyl/droperidol (0.2 mg/kg) mixture was administered intramuscular prior to anesthesia to avoid stress in the rabbit. For anesthesia, an intravenous injection of ketamine (10–50 mg/kg) and xylazine (1–3 mg/kg) mixture was applied. During intubation of the rabbits, diazepam (1 mg/kg, intravenously) was used for tranquilization and muscle relaxation. During surgery, the heart rate (at 130–325 beats/min) and respiratory rate (at 32–60 per min) were monitored, as well as blood pressure (at 90–130/90–60 mm) and body temperature (at 38.5–39.6°C). In the postoperative period, all rabbits were administered with the Bicillin-3 (Benzathine Benzylpenicillin) antibiotic (Sintez, Russia) 300 000 EU, one intramuscular one injection per 3 days (for a total of four injections). Additionally, animals

received CatosalTM (10% Butaphospan, Cyanocobalamin, subcutaneously) (Bayer HealthCare LLC) 1 mL for 14 days. The follow-up period was 6 months.

X-ray analysis—The rabbits' radiographs (46 kV, 200 mA, 32 ms, Trophy N800 HF, Fujifilm 24 × 30 cm² IP cassette type C, 1 m film-focus distance) were taken prior to the surgery, one month after the insertion of the intraosseous component, and then 6 months after the operation. Prior to the procedure the animals were sedated by an intramuscular injection of xylazine (1–3 mg/kg) and ketamine (10–50 mg/kg) mixture. Conventional radiography was applied for assessment of the SBIP intraosseous position in the residuum and the radiological signs of osteomyelitis (e.g., periosteal thickening, lytic lesions, osteopenia, loss of trabecular architecture, and new bone deposition).

Three-phase scintigraphy—To assess the angiogenesis in the region of the porous titanium implants, a three-phase scintigraphy was applied. The regions of interest (ROI) were set on the segment with the porous part of the pylon and on a symmetric segment on the contralateral intact limb. The parameters of the perfusion index (PI) and the uptake ratio of the blood-pool image (BPR) were calculated. Following anesthesia, 111 MBq 99mTc-methylene diphosphonate (MDP) was intravenously injected. The first-pass radionuclide angiographic data were obtained with 128 × 128 matrices in the anterior view every 2 s for 2 min. The blood-pool image (BPI) was obtained at 3 min after injection for 3 min, with 256 × 256 matrices. All data were obtained using a large field-of-view gamma camera (SPECT Infinia GE) equipped with a low-energy, high-resolution, parallel-hole collimator.

Scanning electron microscopy—The structural morphology of the cell monolayer on the scaffolds following *in vitro* coincubation on the titanium samples and the pylons extracted from the bone residuum were visualized using a scanning electron microscope (SEM; JEOL JSM-6060LV) at 10 kV.

Statistics—The Kruskal-Wallis was employed to detect the presence of differences. Continuous variables were compared using a paired Student's test. All data were assessed using Statistica Version 9.2 for Windows. Statistical significance was determined at $p < 0.05$. For assessment of the differences in the parameters of angiogenesis (i.e., perfusion index [PI] and the uptake ratio of the blood-pool image [BPR]) between the silver-coated and control groups, we applied the Friedman rank sum test, which is the nonparametric analog to the two-way ANOVA test.

RESULTS

In vitro study

Cell adhesion—In the tested samples, we observed the formation of a cell monolayer for all analyzed cells (Figure 2).

The cytomorphology of the cells did not differ from the cells grown in the cultural flasks. The cells covered the entire surface area and formed intercellular contacts between the pores of the implants. Detection of the cells throughout the whole sample indicates the possibility that the tested cells can migrate inside the pores of the SBIP.

We additionally assessed the presence of the silver coating of implants if being co-incubated with fibroblasts. For this silver-coated tablets were co-incubated with rabbits' fibroblasts for 1 and 3 weeks with subsequent X-ray photoelectron spectroscopy (XPS) studies. Figure S1, Supporting Information shows XPS of Ag region for silver coating deposited on the surface of the titanium tablets. Thus, following 3 weeks of co-incubation the silver layer was still present on the surface of titanium implants.

Effects of silver coating on MMP production—We evaluated effects of the PVD silver-coating on normal dermal fibroblasts, MSCs and MG-63 cells by the production of the matrix metalloproteinases (MMP-1, MMP-2, and MMP-9). Following co-incubation with uncoated and coated samples for 1, 4 and 9 days the culture medium was analyzed for MMP activity using a zymography assay. The enzymatic activity of the MMPs for the tested samples was different for various cell types (Figure 3).

For the MG-63 cells, we observed a significant increase of MMP-1 production as compared to non-treated samples on days 1, 4 and 9. ($p<0.001$). At the same time, a decrease of MMP-2 activity was detected relative to the control. In dermal fibroblasts, we also observed a significant increase in MMP-1 production and reduction of MMP-2 enzymatic activity ($p<0.001$). MMP-9 levels did not significantly change for either cell type, although for MG-63 on day 9, there was an increase of the protein. For the MSCs the levels of MMP-1, MMP-2, and MMP-9 were slightly increased at day 9 as compared to the control (Figure 3) ($p<0.001$).

Antimicrobial effect of PVD silver coating—An analysis of bacterial adhesion to the porous titanium tablets fabricated with the SBIP specification [Figure 1(A)] demonstrated strain dependent differences in growth between untreated (control) and silver coated samples. After 24 h of incubation of untreated samples, the following was the average adherence for different strains: $5.00\pm0.53 \times 10^5$ CFU of *S. epidermidis*, $4.23\pm0.91 \times 10^6$ CFU of *S. aureus* and $4.17\pm0.75 \times 10^6$ CFU of *P. aeruginosa* (Figure 4; Table S1).

Following 72 h of co-incubation, a significant increase of bacteria was detected for all strains: $7.63\pm1.12 \times 10^5$ CFU of *S. epidermidis*, $7.77\pm0.61 \times 10^6$ CFU of *S. aureus* and $8.03\pm0.71 \times 10^6$ CFU of *P. aeruginosa*.

With the PVD silver-coated implants we observed a significant reduction of the bacterial adhesion for all tested strains following 24 h of co-incubation [Figure 4(A)]: $1.37\pm0.25 \times 10^5$ CFU of *S. epidermidis*, $1.47\pm0.21 \times 10^6$ CFU of *S. aureus* and $1.47\pm0.25 \times 10^6$ CFU for *P. aeruginosa* ($p<0.001$). As compared to nontreated samples, decreased levels of bacteria were observed following 48 and 72 h of incubation.

Subsequent assessment of the bacterial planktonic surface growth in the culture medium demonstrated strain dependent differences. With the nontreated titanium samples, the planktonic growth of bacteria after 24 h. showed $5.03\pm0.21 \times 10^5$ CFU of *S. epidermidis*, $4.50\pm0.20 \times 10^6$ CFU of *S. aureus* and $5.33\pm0.47 \times 10^6$ CFU for *P. aeruginosa* [Figure 4(B), Supporting Information Table 2]. These levels were significantly reduced with the silver-coated implants: $2.40\pm0.36 \times 10^5$ CFU of *S. epidermidis*, $2.27\pm0.35 \times 10^6$ CFU of *S. aureus*

and $2.63 \pm 0.35 \times 10^6$ CFU for *P. aeruginosa* ($p < 0.001$). Following 48 and 72 h of co-incubation the strain-dependent reduction of the planktonic growth was detected in the silver-coated samples as compared to the control, indicating the successful antimicrobial features of the implants.

***In vivo* feasibility study**

Following above-knee amputation, the silver-coated or non-treated SBIP was inserted into the bone residuum. All rabbits recovered from the procedure without complications [Figure 5(A)]. During the 6 months after the insertion of the SBIP intramedullary component, there were no complications in the femur bone (e.g., bone ulceration, bone thinning, etc.) as demonstrated by the radiographs [Figure 5(B)]. From the serial radiographs it was clear that there was no axial displacement of the titanium pylon.

Three-phase (25 μ m) scintigraphy was used to assess the angiogenesis in the intramedullary component of the SBIP. The procedure was performed 1, 2, 4, and 8 weeks after implantation [Figure 5(C)]. The silver-coated and control groups were compared via two parameters: the perfusion index (PI) and the uptake ratio of the blood-pool image (BPR). The p values equaled 0.187. For the Friedman test assessing the difference in BPR between the silver-coated and control, the p values equaled 0.5637. Therefore, the perfusion index (PI) and the uptake ratio of the blood pool image (BPR) did not differ significantly among the two groups, indicating that impregnated silver did not compromise the ingrowth of the tissues inside the pores of the implant (Figure 6).

Subsequent scanning electron microscopy (SEM) of the extracted porous intramedullary pylons on the sixth month of the follow-up period clearly demonstrated fibrovascular connective tissue as well as host cells which migrated into the pores of the pylon in both the control and the experimental groups [Figure 5(D)].

The silver coating appears to have dissolved: visual observation of the extracted implants did not reveal the presence of any silver coating. In future studies, we plan to include a more detailed evaluation of the dynamics of silver ion release into the surrounding tissues and their deposition in kidney and liver.^{15,49–51}

DISCUSSION

Staphylococci and enterococci account for most deep infectious complications associated with bone-anchored percutaneous titanium implants.^{52–54}

In the current study, we aimed to test a silver coating of the SBIP device to increase the antimicrobial resistance following implantation. PVD silver-coated SBIP tablets demonstrated increased antibacterial activity toward *S. aureus*, *P. aeruginosa*, and *S. epidermidis* as compared to the uncoated samples. These results are consistent with previously published data of the antimicrobial effect of silver coating.^{55–58} The PVD silver-coated SBIP implants were found effective in preventing biofilm formation and in decreasing the number of planktonic bacteria for the whole period of observation (i.e., 72 h) (Figure 4). Subsequent SEM analysis demonstrated that silver coating did not interfere with

cell adhesion (for MG-63 cells, fibroblasts and MSCs) or the formation of a cellular monolayer on the titanium implants (Figure 2). Measurement of MMPs' production by the MG-63 cells, fibroblasts or MSCs demonstrated that cellular functional activity was also not affected (Figure 3).

For the fibroblasts and MG-63 cells we detected a manifold increase in the levels of MMP-1. Matrix metalloproteinase-1 (MMP-1) (collagenase-1) is a member of the family of zinc-dependent endopeptidases known to be a key regulator of epithelial cell migration indicating its role in re-epithelialization.^{59–61} MMP-1 also plays role in osteogenesis and muscle regeneration by improving the differentiation and migration of myoblasts, and promotes myogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs).^{62–65} Zhang et al. demonstrated that silver nanoparticles were efficient in promoting osteoblastic differentiation in both *in vitro* and *in vivo* studies.⁶⁶ Presumably, further modification of the implant surface would increase not only the infection control but also stimulate bone repair. Thus, Jia et al. reported the efficient *in vitro* application of the macroporous scaffolds with dual TiO₂- poly(dopamine)/Ag (nano) postmodifications for the three-in-one strategy (infection prophylaxis, infection fighting, and bone repair).⁶⁷

The obtained *in vivo* data demonstrated the biocompatibility and absence of toxic side effects of the silver-coated implants in the model of above-knee amputation in rabbits. Accordingly, the parameters of angiogenesis (PI and PBR) did not differ between non-treated and silver-coated SBIP.

The purpose of the selected coating parameters was to employ the bactericidal properties of silver, but to avoid possible negative consequences of the permanent implantation of scaffolds containing silver.^{40–44,68} While in the current study we did not analyze *in vivo* the presence of the silver coating following implantation into the bone residuum, the authors understand that this phenomenon needs to be evaluated in greater detail using objective methods (e.g., X-ray spectrometry, EDS).⁶⁹ We plan to address this limitation of the current study in the near future.

CONCLUSION

The *in vitro* bacteriological assessment of bacterial adhesion and bacterial planktonic growth revealed the effective antibacterial properties of the silver coating toward the most common pathogens (i.e., *S. epidermidis*, *S. aureus*, and *P. aeruginosa*).

The *in vivo* study in the model of the above knee amputation in rabbits did not show any side effects (i.e., clinical and radiological symptoms of inflammation) of the inserted pylons with a temporary coating that are usually associated with prolonged presence of silver in the body.

The potency of the silver coating with selected characteristics in decreasing the number of planktonic bacteria and in preventing the bacterial adhesion makes the suggested technology a promising candidate to reduce infections in DSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

(A) – Tablets (10 mm diameter and 3 mm thickness) for *in vitro* study: 1 – without silver coating; 2 – silver-coated. (B) - SBIP samples for *in vivo* study: 1 – without silver coating; 2 – silver coated.

FIGURE 2.

Representative scanning electron micrographs of the porous intramedullary silver-coated pylon after 7 days of being co-incubated with MG-63 cells, dermal fibroblasts or MSCs. Cells are pointed by the red solid arrows. Scale bar: 25 μm.

FIGURE 3.

Zymography graphs for experimental studies. Representative zymograms for gelatin zymography (MMP-9, MMP-2) and casein zymography (MMP-1) of control and silver-coated implants co-incubated with MG-63 cells, fibroblasts and MSCs.

FIGURE 4.

(A) – Bacterial growth of tested strains on the sample surfaces of non-treated and silver-coated SBIP implants after 24, 48, and 72 h incubation. (B) – Planktonic growth in the nutrient solution of non-treated and silver-coated SBIP implants after 24, 48, and 72 h co-incubation with the samples.

FIGURE 5.

(A) – Intraoperative microphotographs of intramedullary inserted silver-coated SBIP implants. (B) – Radiographs of the silver-coated and non-treated implants 6 months following operation. (C) – Perfusion scintigram for animal #3 with silver-coated SBIP implant 8 weeks after the implantation of the intramedullary component into the femur. (D) – scanning electron micrographs of the of the SBIP pylon 6 months after implantation into the rabbit's femur (scale bar: 100 μm). A higher magnification shows fibrovascular connective tissue as well as host cells which migrated into the pores of the pylon.

FIGURE 6.

Boxplots of the perfusion index (PI) and the uptake ratio of the blood-pool image (BPR) for the control and silver-coated implants.