A Silver Ion-doped Calcium Phosphate-based Ceramic Nanopowder-coated Prosthesis Increased Infection Resistance

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Abstract

Background Despite progress in surgical techniques, 1% to 2% of joint arthroplasties become complicated by infection. Coating implant surfaces with antimicrobial agents have been attempted to prevent initial bacterial adhesion to implants with varying success rates. We developed a silver ion-containing calcium phosphate-based ceramic nanopowder coating to provide antibacterial activity for orthopaedic implants.

Questions/purposes We asked whether titanium prostheses coated with this nanopowder would show resistance

to bacterial colonization as compared with uncoated prostheses.

Methods We inserted titanium implants (uncoated [n = 9], hydroxyapatite-coated [n = 9], silver-coated [n = 9]) simulating knee prostheses into 27 rabbits' knees. Before implantation, 5×10^2 colony-forming units of *Staphylococcus aureus* were inoculated into the femoral canal. Radiology, microbiology, and histology findings were quantified at Week 6 to define the infection, microbiologically by increased rate of implant colonization/positive cultures, histologically by leukocyte infiltration, necrosis, foreign-body granuloma, and devitalized bone, and radiographically by periosteal reaction, osteolysis, or sequestrum formation.

Results Swab samples taken from medullary canals and implants revealed a lower proportion of positive culture in silver-coated implants (one of nine) than in uncoated (eight of nine) or hydroxyapatite-coated (five of nine) implants. Silver-coated implants also had a lower rate of colonization. No cellular inflammation or foreign-body granuloma was observed around the silver-coated prostheses.

Conclusions Silver ion-doped ceramic nanopowder coating of titanium implants led to an increase in resistance to bacterial colonization compared to uncoated implants.

Clinical Relevance Silver-coated orthopaedic implants may be useful for resistance to local infection but will require in vivo confirmation.

Each author certifies that he or she, or a member of his or her immediate family, has no commercial associations (eg, consultancies, stock ownership, equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article.

All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research* editors and board members are on file with the publication and can be viewed on request. Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research. This work was performed at Osmangazi University, Eskisehir, Turkey.

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Introduction

Long-term survival and favorable outcome of orthopaedic implant use are determined by bone-implant osseointegration and absence of infection near the implants [13, 18, 19, 22, 26]. Bacteria colonize metallic orthopaedic implants

and form adherent biofilms that retard the penetration of antibiotics to the underlying infection [15]. Deep infection of implants cannot be resolved by systemic administration of antibiotics. Many of these infected implants require removal [13, 22]. Prevention of bacterial colonization and biofilm formation is an important consideration for prevention of infection.

To enhance resistance to colonization, implant materials may be modified with antimicrobial coatings. Such materials include antibiotics [2, 3, 11, 12], such as gentamicin and vancomycin, or silver and silver-containing compounds [4–7, 9–11, 16, 27]. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups [20]. There are conflicting results from reports on implanted medical devices coated with silver [9, 10]. This may be due to factors such as minimal release or nonrelease of silver-coated surfaces and limitations dictated by potential silver toxicity. Although direct coating methods of applying metallic silver to implant surfaces have been described [14, 17], silver is biologically more active when it is in ionic form (Ag^+). We developed silver ion-doped calcium phosphate-based ceramic nanopowder to provide both biocompatibility and antibacterial activity to orthopaedic implants.

We determined whether silver ion-containing calcium phosphate-based ceramic nanopowder-coated implants prevented implant-related infection by comparing silver-coated, hydroxyapatite (HA)-coated, and uncoated titanium implants in vivo using radiology, histology, and microbiology.

Materials and Methods

We implanted 27 titanium alloy (Ti6Al4 V) pins in 27 healthy adult New Zealand White rabbits, weighing 3000 to 3500 g. The pins were 25 mm long and 2.5 mm in diameter; the lower ends were bent to mimic knee prostheses. The 27 titanium implants were divided into three groups of nine implants each: (1) uncoated pins, (2) pins coated with HA, and (3) pins coated with silver ion-doped nanosized calcium phosphate-based ceramic powder. After inducing experimental bacteria, we examined the animals by radiology, microbiology, and histology. The study was approved by the institutional review board of the Animal Care and Use Committee (50-07.05.2008) and was conducted with reference to the Organization for Economic Cooperation and Development Principles of Good Laboratory Practice.

We synthesized the antimicrobial powder using the wet chemical method. Final pH was set to 5.5. After the reaction was completed, the solution was filtered and dried at 80° C. The electrospray method was used for coating the

Fig. 1 A schematic diagram illustrates the EDS setup used to coat the prostheses in this study.

endoprostheses. The coating process was performed at room temperature. A vertical configuration of the electro-spray deposition (ESD) setup was used to deposit antimicrobial powder on the implants (Fig. 1). The configuration and the working principles of the ESD have been described in detail elsewhere [28]. HA and antimicrobial powder suspensions of concentration 1 wt%, suitable for coating application, were then obtained by the addition of methanol. The precursor solutions were pumped simultaneously toward the spraying nozzle using a silicon rubber tube. The tube was connected to a stainless steel needle with an inner diameter of 320 μm . Solution flow rate was controlled by syringe pump (BS-8000; Braintree Scientific, Inc, Braintree, MA, USA). A power supply (SL1200; Spellman, Hauppauge, NY, USA) was used to generate a positive high potential difference between the nozzle and the grounded substrate and to obtain a stable cone jet mode of electrostatic atomization. A small motor was used to rotate the implants. The stabilized suspensions were pumped through the metal nozzle using a flow rate of 0.01 to 0.05 mL/minute on the implants. The nozzle substrate distance and applied voltage were fixed 4 cm and 8 kV, respectively. A stable cone jet mode was achieved with these electrospray parameters and a uniform coating was produced. A radiofrequency energy source was used to sinter the coated samples. After sintering, we observed the surface morphology of the pins using scanning electron microscopy performed on a Zeiss Supra VP50 microscope (Carl Zeiss AG, Oberkochen, Germany). Homogeneous, dense coating layers were obtained (Fig. 2). The HA- or silver-coated pins were sterilized with ethylene oxide. Pressurized steam was used for the sterilization of uncoated pins.

An electrospray method was used for the coating process because this technique is a new method [29] for

Fig. 2A–B The morphology of (A) an electro sprayed HA-coated pin and (B) an electro sprayed silver-doped calcium phosphate-coated pin is shown (original magnification, $\times 5000$).

preparing uniform ceramic thin films in a controllable way. The main advantages of ESD over other common deposition processes are suitability for less soluble materials, lack of solvent evaporation effects, possibility for realizing multilayered devices, and efficient usage of the materials [21, 23]. By embedding nanoparticles of silver into a ceramic coating, the active surface area is maximized while keeping the total amount of silver low. Thus, it is possible to tailor the antibacterial activity of the silver ions while remaining below the toxicity threshold for cells [30]. This method overcomes the problem of minimal release or nonrelease of silver-coated surfaces and potential silver toxicity. To evaluate Ag^+ ion release from antimicrobial powder to the aqueous system, 5.5% Ag^+ ion-doped HA powder was used. Powder was kept in simulated body fluid (lactated Ringer's solution) for 2, 3, 4, 6, 10, 14, and 21 days. At the end of each day, powders were dried and quantitative elemental analysis of Ag^+ ion was performed by using an energy dispersive x-ray microanalyzer (Oxford Instruments INCA Energy, Abingdon, UK). There was a

Fig. 3 A graph shows Ag^+ ion release from the powder to the aqueous system for 21 days. There was a controlled release of Ag^+ ion from powder to aqueous system. The starting value was 5.03 wt% and this value was decreased to 4.21 wt% after 21 days.

controlled release of Ag^+ ion from powder to aqueous system. The starting value was 5.03 wt% and this value was decreased to 4.21 wt% after 21 days (Fig. 3).

To create the experimental infection, we used methicillin-resistant *Staphylococcus aureus* (MRSA) standard strain (*S. aureus* ATCC® 43300; Oxoid Ltd, Basingstoke, UK). The bacteria were stored at -70°C in trypticase soy broth containing 15% (vol/vol) glycerol. Fresh isolate was subcultured twice on 5% blood agar plates for 24 hours at 35°C before the experiment. Standard bacterial suspension for inoculation was prepared by suspending colonies in sterile saline solution until the turbidity matched that of a McFarland 0.5 turbidity standard, containing approximately 10^8 colony-forming units (CFU)/mL. The suspension was further diluted with sterile saline solution to achieve 10^4 CFU/mL. The rabbits were inoculated with 50 μL bacterial suspension (amount of inoculum: 5×10^2 CFU) [11, 31].

We obtained the rabbits before surgery to allow acclimatization to the housing in the animal laboratory. The animals were caged individually and fed orally with standard chow and water ad libitum. Before surgery, rabbits were randomized to receive uncoated, HA-coated, or silver-coated pins. On the day of surgery, a cage was selected randomly from the pool of all cages containing animals by an individual other than the surgeon. Surgery was performed under aseptic conditions with the rabbit under general anesthesia. The anesthesia was introduced by an intramuscular injection of xylazine hydrochloride (0.2 mg/kg) and subcutaneous injection of ketamine hydrochloride (10 mg/kg). The left knees of the rabbits were shaved, and the rabbits were placed in a supine position on the operating table. After disinfection with 10% povidone-iodine and sterile draping, the knees were opened by a

medial parapatellar incision approximately 5 cm in length. The medullary canal of the femur was opened with an awl and reamed by a hand drill to a diameter of 2.5 mm. An inoculum of 50 μ L saline solution containing bacterial suspension was then injected into the cavity with a small pipette. Subsequently, a titanium endoprosthesis implant was press fit into the medullary canal. The joint capsule and the skin were closed with sutures.

The animals were examined daily for activity, eating, and wound healing. Body temperature was documented once a week. Of the 27 rabbits that had undergone surgery, four (two in the uncoated group and one each in the other two groups) died within the first week after surgery without evidence of sepsis. New rabbits were obtained to replace those that died.

The rabbits were killed by intracardiac injection thiopental sodium (200 mg/kg) given at the sixth week. The lower legs of the animals were disarticulated from the hips under aseptic conditions, and osteolysis around the implant was investigated on AP and lateral knee radiographs. The legs of the animals were disinfected with 10% povidone-iodine and isolated with sterile drapes. The knees were exposed and the endoprostheses were removed in a sterile fashion. The track where the implant had been placed was swabbed two to three times to obtain a specimen for culture.

We obtained radiographs to confirm the presence of implant-related infection at 6 weeks just after sacrifice. AP and lateral radiographs of the knees were obtained using the Philips Medio 50 CP x-ray generator (Philips AG, Hamburg, Germany) at a setting of 125 kV and 2 mA/second. The distance between the x-ray source and the samples was 100 cm and the Kodak 5000 RA automatic developing machine (Eastman-Kodak Co, Rochester, NY, USA) was used. Two of us (NK, AO) evaluated the radiographs for the presence of infection. Radiographs were scored according to the methods reported previously [1]. The criteria were (1) periosteal reaction, (2) diaphyseal widening, (3) osteolysis, (4) bone deformation, (5) sequestrum formation, (6) joint effusion, and (7) soft tissue swelling. Criteria 1 to 4 were graded as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. Criteria 5 to 7 were graded as follows: 0 = absent and 1 = present. The Pearson product-moment correlation coefficient test was used for the interobserver and intraobserver reliability. Interobserver and intraobserver reliabilities (r) for the presence or absence of infection were 0.95 and 0.98, respectively.

In addition, the effects of silver coating on bone tissue surrounding the implants were noted histopathologically. Infection was defined by the presence of the following: increased rate of implant colonization/positive culture results of the medullary canal, implant, and bone; intense leucocyte infiltration; and necrosis, foreign-body granuloma, and devitalized bone tissue noted on histologic sections and radiographic findings, including periosteal

reaction, diaphyseal widening, osteolysis, bone deformation, sequestrum formation, and joint effusion. Radiology, microbiology, and histology findings were quantified to define the infection.

Each implant was placed in a sterile tube containing 2 mL sterile normal saline solution, after which the tube was sonicated using an ultrasonic cleaner in a water bath for 3 minutes [11]. We inoculated 50- μ L aliquots of the original suspension and serial 10-fold dilutions in sterile saline solution (10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} dilutions) onto 5% sheep blood agar plates. All plates were incubated at 35° C for 24 to 48 hours. We counted plates containing approximately 30 to 300 colonies and then calculated the number of organisms per milliliter.

Each swab used to obtain a specimen from the medullary canal and implant was inoculated onto 5% sheep blood agar plates. Two to three bone samples surrounding the implant track were also obtained in a sterile fashion from each rabbit. The removed femoral bone fragments were weighed and homogenized in 1 mL tryptic soy broth with a homogenizer (Diox 100; Heidolph Instruments, Schwabach, Germany). Serial dilutions in sterile saline solution (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions) were performed. We inoculated 50- μ L aliquots of the original suspension and serial dilutions onto 5% sheep blood agar plates. After the plates were incubated at 35° C for 24 to 48 hours, we determined the numbers of bacterial colonies. Ratios of positive results of medullary swab, implant swab, bone culture, and implant culture were identified microbiologically from study groups to evaluate and compare resistance to bacterial colonization and infection.

Each 3-cm bone specimen was immediately fixed in 10% neutral-buffered formalin and smaller specimens were decalcified in 5% formic acid. Paraffin-embedded 5- μ m serial tissue sections were stained with hematoxylin and eosin and examined under a light microscope (Olympus BH-2; Olympus, Tokyo, Japan). Two examiners (CB, DB) blindly examined 10 serial bone sections from each animal and evaluated each section for degree of inflammation, type of inflammatory cells (polymorphonuclear or mononuclear), and edema. The medullary cavity (leukocytes, microabscesses, granulation tissue, fibrosis), cortex (destruction of the cortex, enlarged Haversian canals, leukocytes, microabscesses, granulation tissue, fibrosis), and periosteal reaction were scored according to methods reported previously [25, 31] (according to severity from 0 to 4) in at least 10 regions of interest. The total score was calculated and noted for the statistical analysis. The Pearson product-moment correlation coefficient test was used for the interobserver and intraobserver reliabilities. Interobserver and intraobserver reliabilities (r) for the presence or absence of infection were 0.95 and 0.98, respectively.

We compared the proportions of positive results of medullary swab, implant swab, bone culture, and implant culture among

groups using the Fisher exact test. Radiographic and histologic scores were analyzed by nonparametric tests (Kruskal-Wallis). SPSS® v13 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

Results

Radiographic, microbiologic, and histologic observations from this in vivo study suggested silver ion-doped calcium phosphate-based powder coating of titanium implants led to an increase in resistance to bacterial infection compared to uncoated implants.

Radiographic evaluation revealed no differences among groups ($p = 0.120$) (Fig. 4).

Microbiologic tests showed the proportion of infection in the medullary canal was lower ($p = 0.003$) in the silver-coated group than in the uncoated group. Swab samples taken from the medullary canal of eight rabbits in the uncoated group, five rabbits in the HA-coated group, and only one rabbit in the silver-coated group had positive culture results (Table 1). Similarly, the swab samples taken from the implants of eight rabbits in the uncoated group, five rabbits in the HA-coated group, and one rabbit in the silver-coated group had positive culture results. Silver-coated implants had a lower ($p = 0.015$ and $p = 0.131$) proportion of positive cultures than the uncoated and HA-coated implants (one of nine compared with seven of nine and five of nine, respectively). Additionally, silver-coated implants were associated with lower ($p = 0.003$) rates of osteomyelitis on bone homogenate (one of nine compared with eight of nine and five of nine, respectively). Silver-coated implants also had a lower rate of colonization. All rabbits with implant colonization (seven in the uncoated group, five in the HA-coated group, one in the silver-coated group) also had growth of *S aureus* on the culture of the swab used in the medullary canal.

Histologic evaluation of sections of the bone surrounding the silver-coated group had healthy cortical osteons and minimal or no periosteal reaction with no cellular inflammation or foreign-body granuloma. Silver particulate was not observed on surrounding bone tissue as well. Trabeculae and bone cells appeared normal (Fig. 5). The uncoated and HA-coated groups had cortical bone damage with enlarged Haversian canals and cellular inflammation around the implants and between bone trabeculae. The silver-coated group had better ($p = 0.004$) histologic scores than the uncoated and HA-coated groups at Week 6.

Discussion

Arthroplasty has become a common orthopaedic operation and is one of the most successful therapeutic procedures

Fig. 4A–C Radiographic evaluation revealed no periprosthetic osteolysis of bone suggestive of infection in (A) uncoated, (B) HA-coated, and (C) silver-coated implants.

performed by orthopaedic surgeons. Long-term survival and favorable outcome of this surgery are determined by bone-implant osseointegration and absence of infection near the implants [13, 19, 22, 26]. Despite the great progress achieved in orthopaedic surgery, 1% to 2% of implants become complicated by infection [13, 22]. The infection of the implant is one of the most important complications clinically, for both the patient and the

Table 1. Overall ratios of infection in the three groups

Swab/Culture	Number of animals with positive swab/culture			p value		
	Uncoated implants (G1)	Hydroxyapatite-coated implants (G2)	Silver-coated implants (G3)	G1 vs G2	G1 vs G3	G2 vs G3
Medullar swab	8/9	5/9	1/9	0.294	0.003	0.131
Implant swab	8/9	5/9	1/9	0.294	0.003	0.131
Implant culture	7/9	5/9	1/9	0.620	0.015	0.131
Bone culture	8/9	5/9	1/9	0.294	0.003	0.131

Fig. 5A–B (A) In an uncoated implant, intense cellular inflammation around the implant and between bone trabeculae is seen (stain, hematoxylin and eosin). (B) A microscopic section of the periprosthetic environment of a silver-coated implant shows no evidence of inflammation or foreign-body granuloma (stain, hematoxylin and eosin).

surgeon. Prevention of bacterial colonization and biofilm formation is an important consideration for prevention of infection. A possible method to reduce the incidence of bacterial colonization and biofilm formation is to use implants having antibacterial properties on their surfaces. We developed a silver ion-containing calcium phosphate-based ceramic nanopowder for implant coating to provide

both biocompatibility and antibacterial activity to orthopaedic implants and determined whether this coating led to an increase in resistance to bacterial colonization, which may be useful for prevention of local infection.

We acknowledge limitations to our study. First, the number of rabbits in our study is not sufficient to provide a conclusive answer regarding increased infection resistance; however, the data suggest colonization is reduced by this silver coating. Second, we used only one type of bacteria in this study. For antimicrobial-coated implants to provide ideal protection, it is essential they provide activity against not only the most common causative bacteria (ie, *S aureus*) but also other potential pathogens. However, it is impractical to examine all pathogens. Third, our study is short-term and it is difficult to speculate whether the silver coating will show increased infection resistance in the long term. The length of the study was not long enough to evaluate infections with low-virulence organisms such as *S epidermidis*, which may not manifest clinically until months or years after insertion of a prosthesis [11], but it was long enough to evaluate infection due to the most common causative bacteria (ie, *S aureus*). Fourth, we did not examine blood silver level to see whether the safe limit was exceeded. We did not consider it necessary as the study time was short and massive silver material was not used [30]. Fifth, we did not establish infection first and then treat it with antibacterial-coated implants as the scope of the project was limited to examine whether silver coating of implants led to an increase in resistance to bacterial colonization. Sixth, we did not perform statistical analyses for histologic assessment because our data were semi-quantitative. We believe these data were enough to show the presence of infection on bone tissues.

A number of methods can be used to add silver onto the surfaces of medical implants. Direct coating methods of applying metallic silver to implant surfaces have been described [14]. Gosheger et al. [14], studying the antimicrobial efficacy of a direct metallic silver coating on the surface of megaprotheses in an animal model, showed silver-coated prosthesis resulted in lower infection rates than uncoated prostheses (7% versus 47%). Noda et al. [23] evaluated in vitro antibacterial activity and Ag⁺ ion release

of silver-containing calcium phosphate coating and reported the coating of silver-containing material could release markedly more silver ions than metallic silver. Silver is biologically more active when it is in ionic form (Ag^+). We used silver ion-doped calcium phosphate-based ceramic nanopowder for coating. Our results showed a substantial effect in reducing infection proportions. Swab samples taken from both medullary canals and implants revealed lower positive culture ratios in silver-coated implants (one of nine) than in uncoated implants (eight of nine). Silver use has been restricted due to the toxicity of silver ions to humans. The use of nanoparticulate silver may represent a promising new possibility. We designed silver ion-doped calcium phosphate-based ceramic nanopowder to overcome the problem of minimal release or nonrelease of silver-coated surfaces and potential silver toxicity. By embedding nanoparticles of silver in bioceramic coatings for implants, the active surface area is maximized while keeping the total amount of silver low.

Radiographs showing periosteal new bone formation, foci of osteolysis, and bone resorption are highly suggestive of infection but are typically late findings. Some samples presented bacteria and histologic evidence of infection without radiographic signs. This finding was in line with the literature [24] suggesting plain radiographs in the acute setting may demonstrate no appreciable changes. The degree of destruction of the bone architecture was not visible macroscopically. High-resolution digital and computed radiography systems can be used to evaluate structural changes of animal bones [8].

Hardes et al. [17] evaluated toxicologic side effects in silver-coated megaprotheses in humans. They reported histopathologic examination of the periprosthetic environment in two patients showed no signs of foreign-body granulomas or chronic inflammation. In our study, histologic evaluation of sections of the bone surrounding uncoated implants showed intense cellular inflammation around the implants and between bone trabeculae, suggestive of infection. In silver-coated implants, no cellular inflammation or foreign-body granuloma was observed around the implant. Silver particulate was not observed on surrounding bone tissue as well. Trabeculae and bone cells appeared normal.

Although our findings support the use of silver ion coating of implants, further in vitro and/or in vivo animal investigations are warranted. We observed swab samples taken from both the medullary canals and implants revealed the infection ratio was lower in silver-coated implants than in uncoated implants. Silver-coated implants also had a lower rate of colonization. All rabbits with implant colonization also had growth of bacteria on the culture of the swab used in the medullary canal. In addition, no cellular inflammation or foreign-body granuloma was observed

around silver-coated implants. Trabeculae and bone cells were normal. The observations suggest adding a silver ion-doped nanosized calcium phosphate-based powder coating to orthopaedic implants may prevent bacterial colonization as compared with uncoated prostheses.

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