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Laboratory Study

Nanoparticle silver ion coatings inhibit biofilm formation on titanium implants

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ABSTRACT

The formation of bacterial biofilm on the surface of implanted metal objects is a major clinical problem. The antibacterial and antifungal effect of silver ions has been long known, and seems to give silver the capability to inhibit biofilm formation. To test the effect of silver ions, 20 New Zealand rabbits had bacteria applied to a screw insertion site at the iliac crest, and were then randomly divided into two groups: Group I, which had silver-coated screws applied, and Group II, which had uncoated titanium screws. After the rabbits were sacrificed on day 28, we examined the screws, the bone adjacent to the screws, and the liver, kidneys, brain and corneas of both groups under transmission (TEM) and scanning electron microscopy (SEM). We also analysed microbiological samples from the screw holes. All silver-coated screws, but only 10% of uncoated titanium screws, were sterile. All tissue samples appeared ultrastructurally normal in both groups. Biofilm formation was inhibited on all silver-coated screws, but all uncoated screws developed a biofilm on their surfaces. Our findings suggest that nanoparticle silver ion-coated implants are as safe as uncoated titanium screws and that they can help prevent both biofilm formation and infection.

1. Introduction

All implants, even temporary ones, increase the risk of postoperative infection. Implants act as passive surfaces prone to bacterial adhesion and biofilm formation. This tendency can result in implant-associated infection of the surgical site. In spinal surgery, implant-associated deep body infections are still a major problem. Some bacteria produce slime, which is responsible for bacterial adhesion and formation of biofilms on artificial surfaces. This slime is composed of proteins, hexosamines, neutral sugars and phosphorus-containing compounds.[1] If slime-forming bacteria colonize an artificial surface and develop a biofilm, this layer protects the bacteria from antibiotic agents. Thus, treatment against implant-associated infection must target the development of a biofilm. We investigated whether a silver ion coating can prevent biofilm formation on artificial surfaces.

The antibacterial effect of silver ions has been long known and silver ions are non-allergenic and non-toxic to living mammalian tissue.[2,3] However, metallic silver is not strong enough to be a load-bearing metal implant. Titanium, however, has no antibacterial properties. Therefore, silver-coated titanium implants might be useful in preventing implant-related deep body infection. The antibacterial effect of silver coating has been studied.[4,5] Thus, we aimed to investigate whether nanoparticle silver coating can inhibit biofilm formation, even in slime-forming bacteria.

2. Methods and materials

2.1. Study design, operation procedure and sampling

We obtained 40 standard titanium (Ti) bone screws (2 mm diameter and 8 mm length; M 36340; Trimed, Istanbul, Turkey) for this study. Twenty of 40 screws were coated with silver using the sol-gel method[6] (Mikron Makine, Ankara, Turkey). The other 20 screws were not coated. Coagulase-positive *Staphylococcus aureus*, capable of forming a biofilm layer, were supplied from stock solutions (Department of Microbiology, School of Medicine, Ankara University). The bacteria were kept in a 5-mL broth medium at +4 °C. Freshly cultured bacterial cells obtained from stock solutions were used during the experiment.

Twenty New Zealand rabbits (12 weeks old; weight 2000–2500 g; mean weight: 2250 g) were randomly divided into two equal groups. The study was approved by the Ethics Committee of Ankara University in accordance with the Helsinki Declaration of Animal Rights. All rabbits had bacteria applied to the surgical site: rabbits in Group I had silver-coated screws inserted whereas Group II rabbits had uncoated Ti screws inserted.

We anesthetized the animals with ketamine hydrochloride (Pfizer, New York, NY, USA) and 2% xylazine hydrochloride (Bayer, Mannheim, Germany). The animals' lower backs were shaved and

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surgically scrubbed. A 4-cm skin incision was made on the midline starting from the iliac crest. After bilateral exposure of the iliac bones, one burr hole (diameter: 2.0 mm, 8 mm deep) was drilled into each iliac bone (FF055R, FF068R; Aesculap, Tuttlingen, Germany). Each hole was irrigated with 3 mL of sterile saline. One cotton ball to which bacterial solution had been applied (0.1 mL of bacterial solution containing 10^6 colony-forming units [CFU]) was inserted into each hole, and then removed. One screw was then tightly inserted per hole. No antibiotics were used during the procedure or follow-up period.

All rabbits were sacrificed on day 28 by injection of thiopental sodium via the auricular vein. The screws were removed under sterile conditions. The bone specimens, which included the screw holes, were cut as cubes of 10-mm-length sides. One screw and bone specimen from one side of each animal was taken to the electron microscope laboratory, and the other set was taken to the microbiology laboratory for confirmation of the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) results. Samples of the cornea, liver, kidney and brain of all animals were also viewed under the TEM to detect damage caused by silver ions.

2.2. TEM of tissue samples

For examination with TEM, the tissue samples were fixed in 2.5% glutaraldehyde solution for 6 hours, washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH: 7.4) for 2 hours, and dehydrated in increasing concentrations of alcohol. The tissues were then washed with propylene oxide and embedded in epoxy-resin embedding medium. Semi-thin sections (about 2 μm thick) were cut with a glass knife on a LKB-Nova ultratome (LKB-Produkter, Bromma, Sweden). The semi-thin sections were stained with methylene blue and examined under a Nikon Optiphot light microscope (Nikon Corporation, Tokyo, Japan). Following this examination, the tissue blocks were trimmed, and ultrathin sections (about 60 nm thick) were cut using the same ultratome. Sections were stained with uranyl acetate and lead citrate. Following staining, all ultrathin sections were examined under a Jeol JEM 1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

2.3. SEM of screws

For examination under SEM, the screws were fixed in 2.5% glutaraldehyde for 6 hours, washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 2 hours, and dehydrated in increasing concentrations of acetone. Following dehydration, we performed critical point drying on the samples, and mounted them on metal stubs with a double-sided adhesive band. The specimens were then sprayed with a 100 Angstrom-thick layer of gold in a Bio-Rad sputter apparatus (Bio-Rad Laboratories, Hemel Hempstead, Hartfordshire, UK). All samples were examined under a Jeol ASID-10 scanning electron microscope (Jeol) at an accelerating voltage of 80 kV.

2.4. Microbiological confirmation of the antibacterial effect of silver coating

All samples were appropriately coded for microbiological analysis, and put in tubes containing saline. All tubes were vortexed for 1 minute (112 g [radius of rotation: 16 mm, 2500 revolutions per minute]). Pellet samples (100 μL) were diluted to 1:10, 1:100 and 1:1000 and cultured on blood agar and Mueller-Hinton agar. Culture plates were incubated at 37 °C and the number of CFU were counted after 24 hours and 48 hours. We evaluated the purity of each culture using a coagulase test and Gram staining of randomly chosen colonies on culture plates. Gram-positive, mass-forming and coagulase-positive cocci were evaluated as *S. aureus*. If we did not detect or suspect any contamination, no further identification procedure was needed.

The data on CFU numbers were analyzed statistically by applying a Kruskal–Wallis variant analysis test.[7] Comparisons of the parameters for each group were performed using a multiple comparison test. A p value of <0.05 was considered statistically significant for all paired comparisons.

3. Results

3.1. Electron microscope studies

In Group I, a biofilm was not detected on the surface of any of the screws. All coating layers were still intact, which showed that these coatings can withstand insertion into bone tissue. No bacteria were found on the surface of any silver-coated screws (Fig. 1D), and few bacteria were detected on the bone specimens from Group I rabbits (Fig. 2A). Microbiological confirmation of this group showed that there was no bacterial growth (see Section 3.2).

In Group II, a biofilm was detected on all uncoated titanium screws. This biofilm layer consisted of ample bacteria and had a web-like structure (Fig. 1A–C). The bone tissue of this group had many more bacteria than in Group I samples (Fig. 2B). Microbiological confirmation supported this conclusion.

All tissue samples appeared normal (Fig. 3A, C, D) except one kidney from a Group II sample (using uncoated screws) (Fig. 3B), in which there was mild glomerular damage in a few areas of one sample. These ultrastructural pathological changes may have been an artifact due to delayed fixation. There was no cellular swelling, necrosis, bacterial infiltration, or reaction to foreign material due to silver or titanium accumulation. There were also no metallic deposits of silver or titanium, nor was there any ultrastructural change in tissues in either of the groups (Fig. 2C, D and Fig. 3A, C, D).

3.2. Microbiological confirmation of samples

There was no bacterial growth from Group I samples. In Group II, considerable bacterial growth was detected on 16 of 20 screws from 10 rabbits (80%), and in 18 of 20 bone specimens from 10 rabbits (90%) (60–2200 CFU/mL). These results were compatible with the findings of the SEM and TEM studies. We detected a significant difference between Group I and Group II both in screws and bone samples ($p < 0.001$).

4. Discussion

The formation of bacterial biofilm on the surface of implanted metal objects is a major clinical problem.[8,9] A biofilm is a community of microorganisms encased within an exopolysaccharide matrix, attached to an artificial surface. The extracellular polymeric matrix is the most important structural component of biofilms and is responsible for the attachment of biofilms to artificial surfaces. The matrix, composed of polysaccharides, proteins, nucleic acids and water, protects the bacteria from various environmental stressors including UV radiation, extreme pH values, osmotic pressure, dehydration and antibiotics.

Implanted artificial surfaces act as passive surfaces prone to bacterial adhesion and are prone to implant-associated infection.[10] Antibiotic treatment of infected implants is ineffective because the biofilm protects the adhering organisms. The minimal inhibitory concentration of antibiotics needed to inhibit free-floating bacteria is about 50 or 500 times lower than that

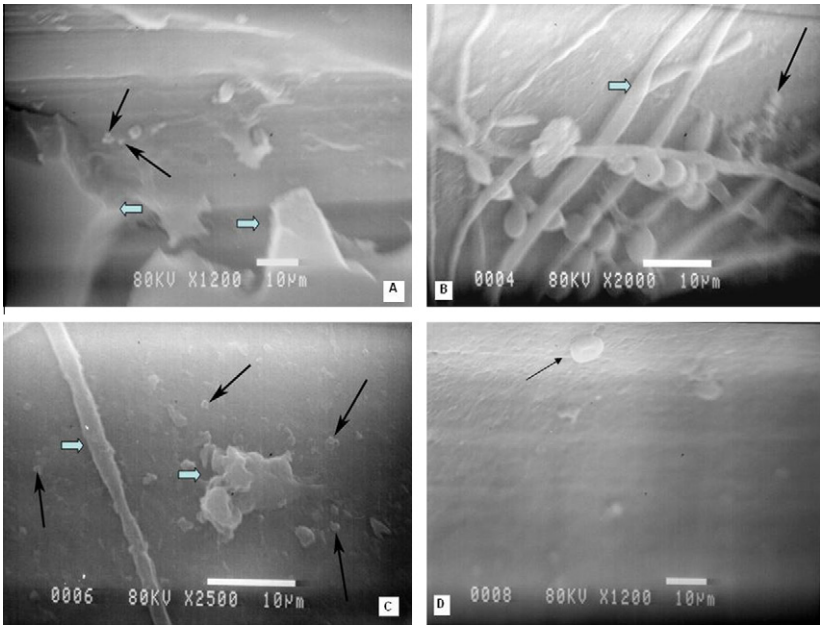

Fig. 1. Scanning electron microscopy (SEM) showing: (A) bacteria (thin arrows) and the biofilm formation (thick arrows) on the surface of uncoated screws; (B) bacteria (thin arrow) and the web-like configuration of hyphae (thick arrows) forming the biofilm layer; (C) bacteria (thin arrows) and the biofilm layer (thick arrows); and (D) the lack of bacteria or a biofilm layer on a nanoparticle silver ion-coated screw, arrow is showing the blood cell (bars represent 10 μm).

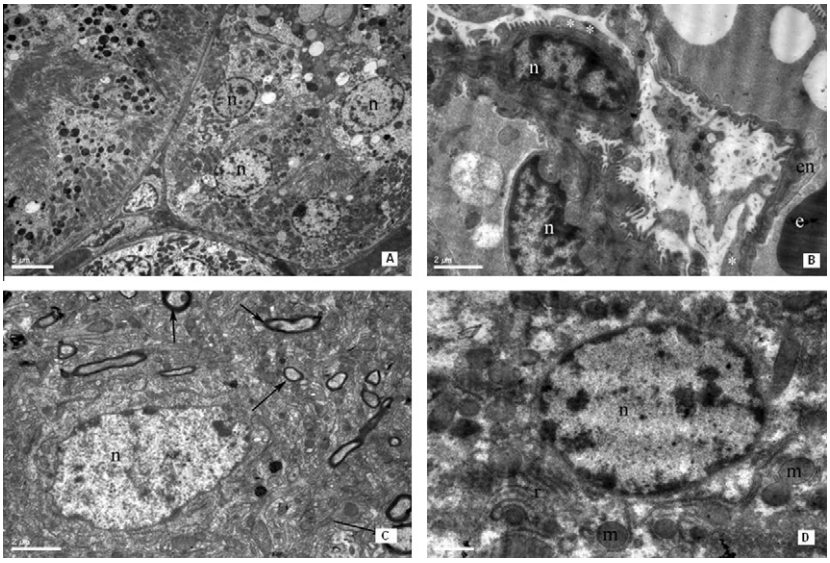

Fig. 2. Transmission electron microscopy (TEM) showing: (A) a kidney of normal appearance from a Group I rabbit implanted with silver-coated screws (n = nucleus of tubulus cell; original magnification, ×4000); (B) mild reversible glomerular damage in a focal area from a Group II rabbit implanted with an uncoated titanium screw, probably an artifact due to delayed fixation of the sample (n = nucleus of podocytes, * = degeneration and damage in pedicels, e = erythrocyte in capillary lumen, en = capillary endothelium cell; original magnification, ×12,000; (C) a liver of normal appearance in a Group I rabbit (n = hepatocyte nucleus, m = mitochondria, r = granular endoplasmic reticulum; original magnification, ×20,000); and (D) a brain of normal appearance from a Group I rabbit (n = nucleus of a neuron cell, arrow = normal myelinated axons; original magnification, ×12,000).

required for bacteria within a biofilm.[8] Infections associated with foreign materials are expensive to manage. Implant-related deep body infections are still a clinical problem in managing spinal surgeries with implants, and antibiotic therapy alone is not effective in about half of these infections. Most patients must undergo re-operation. Post-operative infection rates have increased, especially in last 10 years, which is parallel to the increased use of implants in spinal surgery. If prophylactic antibiotics are used in a operation without implants, the rate of infection is reduced from 2.1–8.5% to 1%. However, if implants were used, the percentage remains unchanged, despite the use of antibiotics.[11] There is a strong relationship between implant usage and infection.

The antibacterial and antifungal effect of silver ions has long been known. Salts of silver were used therapeutically by the Mohammedian school of physicians in the 8th century. Avicenna, in 980 AD, used and prescribed silver as a blood purifier for heart palpitations and for offensive breath. Silver was combined with arsephenamine, because of its antimicrobial properties, and was used to treat syphilis during the early part of the 20th

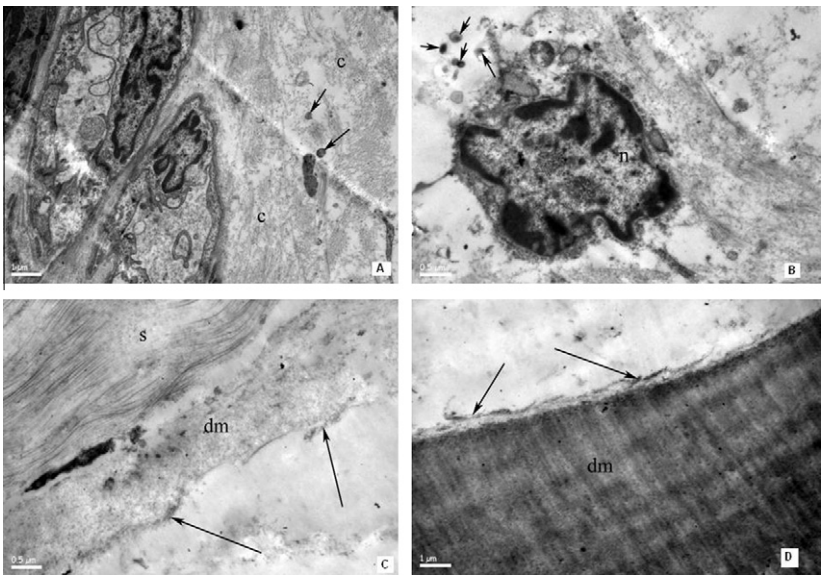

Fig. 3. Transmission electron microscopy (TEM) showing: (A) peri-implant bone tissue from a Group I rabbit implanted with silver-coated screws – bacteria were probably not living due to lethal effect of silver ions (c = collagen fibers, arrows = bacteria in neighbouring connective tissue; original magnification, ×15,000); (B) peri-implant bone tissue from a Group II rabbit implanted with uncoated titanium screws – bacteria were in cancellous bone tissue probably due to the lack of antibacterial effect of silver ions (arrows = bacteria; n = osteocyte nucleus; original magnification, ×30,000); (C, D) cornea of normal appearance from a Group I rabbit (arrow = endothelium of cornea, dm = descement membrane, s = stroma; original magnification, [C] ×15,000, [D] ×30,000).

century.[2] Because of this history, and more recent studies,[4,5] we anticipated a lack of bacterial growth in Group I rabbit samples.

Silver ions inactivate sulfhydryl enzymes when combined with amino, imidazole, carboxyl and phosphate groups.[2] Silver ions affect DNA replication and stop mitosis in prokaryotes.[2] Silver ions also: affect the selective permeability of the cell membrane;[12] combine with tissue protein, which renders proteolytic bacteria unable to replicate;[13] and stop replication of log phase *Pseudomonas aeruginosa* by binding with DNA.[14] Silver can also inhibit oxidation of glucose, glycerol, succinate, D-lactate and L-lactate and endogenous substrates of intact cell suspensions of *Escherichia coli*, inhibiting the respiratory chain.[15] However, silver also inhibits the beta-galactosidase enzyme, causing a bactericidal effect on *E. coli*.[16]

When silver preparations were used in burn patients, silver was shown to have affected renal function and had accumulated in the cornea. Silver has been known to be eliminated via the renal system, and can be absorbed easily through the gastrointestinal system.[2] In our study, cornea, kidney, liver and brain tissues were selected to see if any accumulation or side effects of silver occurred and caused ultrastructural changes. Our results from TEM showed that silver did not accumulate in the cornea, kidney, liver or brain tissues. These results showed that using silver-coated implants had the same effect in terms of accumulation as traditional, uncoated, titanium alloy implants. We have measured the concentration of silver in the cornea, kidney, liver and brain using the atomic absorption spectrophotometer and found no accumulation of silver even 28 days after implantation (manuscript, in preparation).

The major goal of the study was to investigate whether silver coatings can inhibit biofilm formation and our results using SEM revealed that silver coatings can be used for this purpose. Biofilms are two-layered structures: the subjacent layer consists of yeasts; and the upper, thicker layer consists of hyphal-forming organisms.[17,18] Silver ions are both bacteriostatic and fungicidal, even in minute quantities,[5,19] and these properties might inhibit biofilm formation.

Biofilm formation usually takes 24 hours to 48 hours and occurs in two sequential steps: (i) initial attachment of the bacteria to a solid surface; and (ii) proliferation and accumulation of cells in multilayers, and enclosing the bacterial community in an *in vitro* polymeric matrix. In mature biofilms, the release of planktonic cells occurs from the outer layer, which might be responsible for infection re-occurrence. Therefore, we waited for 28 days for maximum infection and biofilm formation *in vivo*. This period was long enough to exclude the occurence of systemic infection. Temperature and whole blood count were recorded periodically to detect any systemic infection in the experimental animals. We had planned to exclude any sysTemically infected rabbits but this was not required.

Electric current has been used to remove biofilms from medical surfaces.[20] Poortinga et al.[21] state that it is possible to stimulate bacterial detachment from conducting indium tin oxide-coated glass using a 10 $\mu A/cm^2$ electrical current. Similarly, Borden et al.[22] have reported electrical current-induced detachment of *S. epidermidis* from surgical implants. A 100 μA direct current (DC) yielded 78% detachment, whereas a 100 μA block current under the same experimental conditions yielded only 31% detachment. The same trend was found for application of a 60 μA current, with 37% detachment for a DC and 24% detachment for a block current. These studies aimed to remove the biofilm layer; however, none of them aimed to prevent formation of biofilm on artificial surfaces. To our knowledge, this is a unique study demonstrating the prevention of biofilm formation with silver coatings.

In summary, our study revealed that nanoparticle coatings of silver ions on artificial surfaces can inhibit biofilm formation, that this method is non-toxic, and no harmful side effects were detected on the kidney, liver, brain or cornea.

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