



# Silver ion doped hydroxyapatite-coated titanium pins prevent bacterial colonization

Adnan Sevensan, MD<sup>1</sup>, Elif Kartal Doyuk, MD<sup>2</sup>, Nusret Köse, MD<sup>1</sup>

<sup>1</sup>Department of Orthopedics and Traumatology, Osmangazi University, Medical Faculty, Eskişehir, Turkey

<sup>2</sup>Department of Infectious Diseases and Clinical Microbiology, Osmangazi University, Medical Faculty, Eskişehir, Turkey

Orthopedic implants are used in many patients. However, infection associated with medical implants remains one of the most important complications in modern trauma and orthopedic surgery. In addition to the economic burden, it may cause implant failure and multiple operations.<sup>[1]</sup> The systemic treatment of infected implant with antibiotics is often poor, as the access of antibiotics to the infection site is inadequate. Adhesion of bacteria to the implant surface and biofilm formation are very critical stages in the progression of the infection. Bacteria colonize metal implants and create biofilms which suppress the penetration of immune defense factors and antibiotics to the underlying infection.<sup>[2]</sup> Given the close relationship between infection and the implant surface, proper handling of the implant surface can help reduce the risk of infection.<sup>[3]</sup> To improve the biocompatibility of metal implants, hydroxyapatite (HA) coatings are widely used.<sup>[4-6]</sup> Ideally, the next step is to add antimicrobials to this coating.

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**Correspondence:** Adnan Sevensan, MD, Osmangazi Üniversitesi Tıp Fakültesi Ortopedi ve Travmatoloji Anabilim Dalı, 26040 Meşelik, Eskişehir, Türkiye.

E-mail: adnansevensan@hotmail.com

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## ABSTRACT

**Objectives:** This study aims to evaluate the effectiveness of silver ion doped calcium phosphate-based ceramic nano powder-coated titanium pins in preventing bacterial colonization.

**Materials and methods:** A total of 66 titanium pins were divided into three groups of 22 implants. The first group was coated with silver ion doped calcium phosphate-based ceramic powder by using electrospray method. The second group was coated with pure hydroxyapatite (HA), and the remaining pins were used without any coating. The remaining 22 pins were used without any coating. *Staphylococcus epidermidis* clinical isolate was used for the study. Each pin was placed in  $1 \times 10^4$  CFU/mL bacterial suspension containing tube and at 24 h quantitative culture of bacteria on the broth and on the pins were performed. Free silver ions were determined by atomic absorption method. The antibacterial culture tests were repeated on Day 2 and Weeks 2, 4, 6, and 8.

**Results:** Bacterial growth was statistically higher in broth containing uncoated pins, compared to broth media containing silver ion doped HA-coated, and pure HA-coated pins at 24 h ( $p=0.036$  and  $p=0.009$ , respectively). The release of bacteria from silver doped HA-coated pins was statistically less, compared to pure HA-coated pins and uncoated pins ( $p=0.039$  and  $p=0.002$ , respectively). No significant differences were observed between the HA-coated and uncoated pin groups. Minimum inhibitory concentration levels for silver ion doped powder was  $8 \mu\text{g/mL}$  for coagulase-negative *Staphylococcus*. No free silver ions were detected in the broth media.

**Conclusion:** Silver ion doped nano size calcium phosphate-based powder-coated titanium pins reduced the bacterial colonization significantly. Using silver ion doped materials in the body can be a good option to prevent from implant related infections.

**Keywords:** Bacterial colonization, coated pin, infection, silver ion.

Various local delivery systems have been developed to prevent bacterial adhesion to the implant for antimicrobial prophylaxis, such as antimicrobial irrigation of the surgical site, dipping the implant in an antimicrobial solution, application of an antibacterial added polymer coating; however, these strategies are often applied in a non-standardized fashion and without clear

evidence of clinical efficacy.<sup>[7]</sup> Antibiotic resistance is an important issue in orthopedic implant infections.<sup>[8]</sup> Implant-infecting *Staphylococcus aureus* (*S. aureus*) strains have high rates of antibiotic resistance, and there is an alarming increase of antibiotic resistance in other species, such as *Staphylococcus epidermidis* (*S. epidermidis*).<sup>[9]</sup> The increase in antibiotic-resistant pathogens, with the higher prevalence of infection leads to consideration of a material-based view of antimicrobials for implant-related infection. Silver is a good option for a metal-based therapeutic. Silver ions are well known to exhibit antimicrobial activity and have been incorporated into the surfaces of a variety of medical devices.<sup>[10-14]</sup>

As the colonization of the bacteria (adherence and initial multiplication) is the first stage of implant related-infection, prevention of bacterial attachment to the implant by surface treatment is critical. In this experimental study, we aimed to evaluate the effectiveness of silver ion doped calcium phosphate-based ceramic nano powder-coated titanium pins in preventing bacterial colonization and to compare quantitative culture of bacteria on the broth and on the pins (coated and uncoated). Also, the minimum inhibitory concentration (MIC) value of the silver ion doped HA coating was determined.

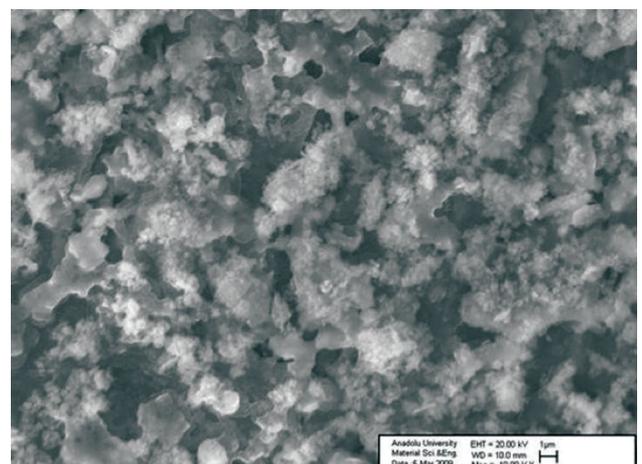
## MATERIALS AND METHODS

This experimental study was designed as a nonanimal and non-human, pure *in vitro* study and no ethic approval required. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Sixty-six titanium pins with a length of 2.5 cm and a diameter of 0.2 cm were divided into three groups of 22 implants. The first group of titanium pins was coated with silver ion doped nano size HA powder using electrospray method. Radiofrequency (RF) was used to sinter the coated samples. The second group of pins was coated with pure HA with the same process. After sintering process, surface morphologies of pins were observed with scanning electron microscope (SEM, Zeiss Supra VP50). The remaining 22 pins were used without any coating. In each group, eight implant samples were sonicated to lyse bacteria and used for bacterial count. Since the choice of sterilization methods may cause changes in the chemical structure of the coatings and their bioactivity behavior,<sup>[15]</sup> coated titanium pins were sterilized with ethylene oxide. Pressurized steam was used for the sterilization of uncoated pins.

In the study *S. epidermidis* clinical isolate, which is the major cause of foreign body (orthopedic implants) infections and which also causes the formation of slime (biofilm) virulence factor, was used. A total of  $1 \times 10^4$  CFU/mL suspension of the clinical isolate in tryptic soy broth was prepared.<sup>[16]</sup> After 1 mL of the bacteria suspension was transferred into glass tubes (0.4 cm in diameter), each pin was placed in a tube in aseptic conditions. The tubes were sealed with parafilm and incubated at 35°C/80 rpm for 24 h in a shaking incubator to allow the bacteria adhere to the pins. Following 24 h incubation, quantitative culture and determination of silver ion by atomic absorption were performed on the broth containing three different pin groups. Also, quantitative culture of bacteria on the pins taken out glass tubes in aseptic conditions was performed in addition to microscopic examination. The possible antibacterial efficacy of implant coatings was investigated for eight weeks (Day 2, Weeks 2, 4, 6, and 8). Accordingly, the implants placed in shaking incubator at 35°C/80 rpm in glass tubes were rinsed with distilled water in aseptic conditions once a week.

Each broth sample was diluted in series up to a million times (from 10<sup>-1</sup> to 10<sup>-6</sup> times) using sterile broth. Of each dilution, 5% was, then, inoculated onto sheep blood agar. Following 24 h incubation in aerobic media at 35°C, the number of bacteria in the original broth culture was calculated by counting the colonies. *S. epidermidis* was identified using conventional methods and automated identification system (Phoneix, BD, USA). Titanium pins placed in tubes each containing 1 mL of ringer's lactate



**FIGURE 1.** Scanning electron micrographs showing the surface morphology of silver doped hydroxyapatite coating.

TABLE I						
Bacterial counts from the quantitative cultures from broth media						
	Day 1	Day 2	Day 15	Day 30	Day 45	Day 60
Silver 1	88.900					
Silver 2	13.100	100				
Silver 3	0	100	18			
Silver 4	7.600	400	23	10	7	
Silver 5	0	3.500	18	7	6	4
Silver 6	73.600	0	22	6	11	3
Silver 7	39.700	600	13	12	6	7
Silver 8	81.600	1.400	13	29	2	2
Silver 9	25.100	1.000	22	14	0	4
Silver 10	47.300	100	14	8	3	6
Silver 11	56.000	1.000	12	2	1	3
Silver 12	112.500	0	13	10	4	2
Silver 13	22.900	2.200	18	12	1	5
Silver 14	107.300	3.500	14	16	4	0
HA1	56.000					
HA2	62.400	400				
HA3	32.100	300	20			
HA4	126.400	1.500	9	7	2	
HA5	80.900	700	18	7	0	3
HA6	26.400	600	31	4	0	11
HA7	150.400	200	8	122	0	8
HA8	66.500	0	18	0	2	26
HA9	116.800	5.800	19	0	5	3
HA10	18.200	8.000	8	9	3	8
HA11	72.800	3.200	6	14	4	3
HA12	27.600	600	25	11	1	2
HA13	10.500	11.000	9	9	2	0
HA14	96.000	1.200	18	7	6	1
T1	22.900					
T2	9.500	6.200				
T3	25.800	700	15			
T4	12.500	14.000	4	10	2	
T5	6.000	0	23	9	0	0
T6	24.000	1.500	11	3	5	2
T7	12.500	5.700	10	6	11	1
T8	4.000	3.400	4	12	1	39
T9	13.500	400	7	11	5	2
T10	64.000	3.200	22	14	2	3
T11	19.500	4.800	10	9	2	3
T12	10.000	600	23	3	3	6
T13	6.500	700	14	8	5	1
T14	26.000	19.300	12	9	4	2

Silver: Silver ion-coated titanium pins, HA: Hydroxyapatite-coated titanium pins; T: Uncoated titanium pins.

were sonicated for 60 sec to allow for the lysis of the bacteria (Sonics Vibra-Cell VCX 750 ultrasonic homogeniser amplitude 20%, 3-4 Watts, 200-230 Joules, 20 kHz).

The liquid from the tubes containing titanium pins were diluted in series and subjected to quantitative culture. Following incubation, bacterial count was performed as above.

TABLE II

Bacterial counts from the quantitative cultures of bacteria lysed from the sonicated implants

	1	2	3	4	5	6	7	8	Mean
Silver	3.104	2.402	1.864	3.696	272	3.544	280	440	1.950
HA	3.360	2.272	8.192	4.640	7.936	3.552	9.571	12.740	6.532
Uncoated	17.920	7.040	2.848	4.132	9.540	8.950	8.861	10.128	8.677

Silver: Silver ion-coated titanium pins, HA: Hydroxyapatite-coated titanium pins.

The MIC value of silver ion used for coating the implants was determined by agar dilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines. For agar dilution test, a series of two-fold agar dilutions (0.03 to 32 µg/mL) of silver ion were prepared. As the quality control strains, *S. aureus* ATCC 29213 and *Escherichia coli* (*E. coli*) ATCC 25922 were used.

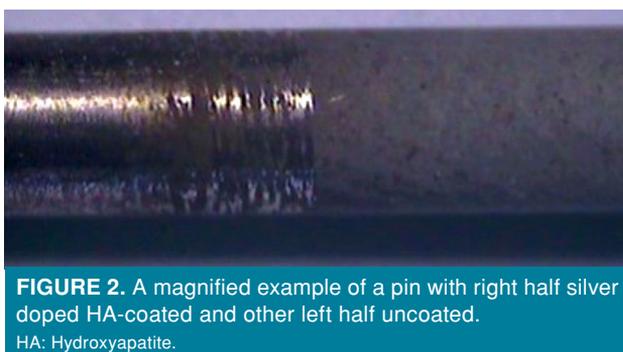
Following 24 h incubation, the presence of silver ion was investigated using atomic absorption method.

#### Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Due to the high number of data in groups, a normal distribution test was performed. One-way analysis of variance (ANOVA) was performed to compare the groups. Multiple comparisons (Tukey's honestly significant difference test) were done to identify differences among the groups. A *p* value of <0.05 was considered statistically significant.

## RESULTS

Surface morphologies of electrospray-coated and sintered silver ion doped calcium phosphate-based ceramic nano-powder coatings are shown in Figure 1. The SEM examinations of the pins showed that the coatings were homogenous with a uniform particle size.



**FIGURE 2.** A magnified example of a pin with right half silver doped HA-coated and other left half uncoated. HA: Hydroxyapatite.

The results of quantitative cultures from broth media are presented in Table I. Normal distribution of data was shown by logarithmic transformation. The difference among the groups was significant only for measurements at 24 h in the analysis results ( $p=0.008$ ). No significant differences among the groups were detected at the other time points. In the ANOVA performed to identify the difference among the groups at 24 h, the bacterial growth was statistically higher in broth containing uncoated pins, compared to broth media containing silver ion doped HA-coated and pure HA-coated titanium pins ( $p=0.036$  and  $p=0.009$ , respectively).

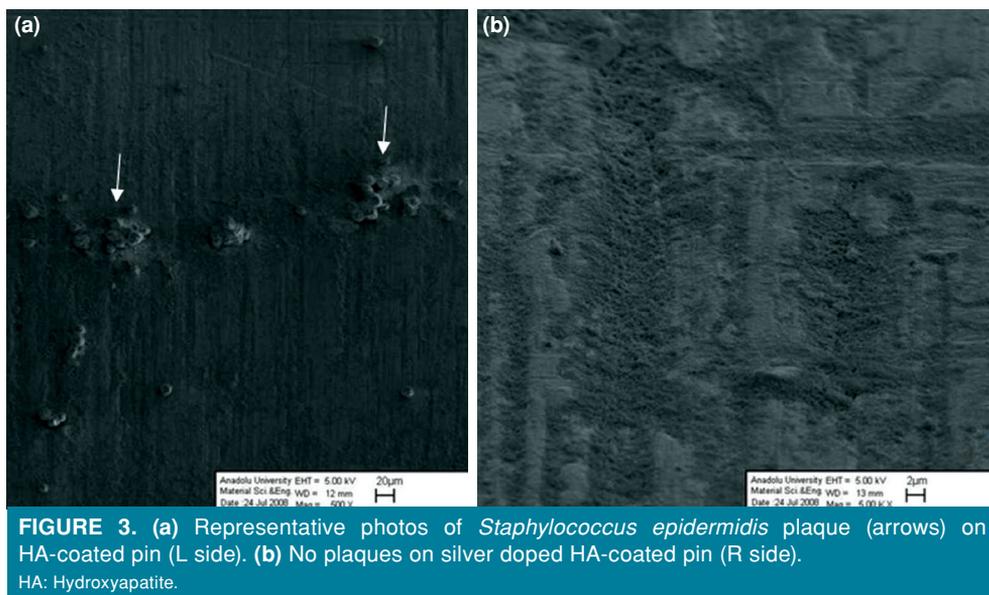
Bacterial counts obtained from quantitative culture of bacteria lysed from the sonicated titanium implants are presented in Table II. Figure 2 shows an example of silver doped HA-coated and uncoated pins. The release of bacteria from silver ion doped HA-coated pins was statistically less, compared to pure HA-coated pins and uncoated pins ( $p=0.039$  and  $p=0.002$ , respectively) (Figure 3). No significant differences were observed between the pure HA-coated pin and uncoated titanium pin groups.

The MIC levels for silver ion doped nano size HA powder was 8 µg/mL for coagulase-negative staphylococcus and 8 µg/mL and 16 µg/mL, respectively for the comparison of *E. coli* and *S. aureus*.

No free silver ions were detected in broth media using atomic absorption method which has a detection threshold as low as 0.02 mg/L.

## DISCUSSION

The present study proved that silver ion doped HA coating of titanium pins grossly inhibited bacterial colonization in the broth media and on the pins while comparing only HA coating or pure titanium pins. No free silver ions presentation in broth media containing silver-coated pins by atomic absorption method also showed the stabilization of this coating compound by electrospray method.



Advances in orthopedic surgery have increased dependence on various medical devices and the number of patients in need of orthopedic implants has been growing rapidly.<sup>[17]</sup> Titanium alloys have been widely used as orthopedic implant materials due to their good mechanical properties, chemical stability, and biocompatibility.<sup>[18]</sup> Long-term survival and favorable outcomes of orthopedic implant use are mainly determined by bone-implant osteointegration and absence of infection neighboring the implants. To improve the biocompatibility of titanium-based implants, various physical and chemical treatments have been used to modify the structure, composition, and chemistry of the titanium surfaces. Among the various techniques, HA coatings are widely used.<sup>[4-6]</sup> Despite the great progress that has been achieved in orthopedic surgery, implant-related infection of bone and soft tissues remains as a serious problem. Although there is an interaction of various factors such as bacterial load, microorganism type, patient's immune status, surgical procedure and technique, implant type, and antibacterial prophylaxis in the emergence of implant-related infections, the most critical event is the onset of bacterial adhesion to an implant and the formation of biofilms which effectively protect microorganisms from the immune system and systemic antibiotics.<sup>[3]</sup>

The fight for implant-related infection continues, with emerging technologies targeting biofilm formation and methods to prevent adhesion and proliferation of micro-organisms to the metallic surfaces.<sup>[3,19]</sup> Local antibacterial implant protection

can be achieved in different ways. Romanò<sup>[20]</sup> classified antibacterial coatings in three groups as passive surface modification, active surface modification, and perioperative antibacterial local carriers or coatings.

In the study, *S. epidermidis* was used as the infectious organism, as delayed infections caused by *S. epidermidis* are more dangerous and more difficult to treat than early infections caused by *S. aureus*.<sup>[21]</sup> The most important findings of the study are that the bacterial growth was statistically higher in the broth containing uncoated pins, compared to the broth media containing silver ion doped HA-coated and pure HA-coated pins. No free silver ions were present in broth media containing silver-coated pins by atomic absorption method.

Bacterial counts obtained from quantitative culture of bacteria lysed from the sonicated implants showed that the release of bacteria from silver group was statistically less, compared to pure HA-coated pins and uncoated pins. No significant differences were detected between pure HA-coated and uncoated pins groups. Therefore, it can be speculated that the effect obtained depends on the silver ions in the structure and the novel coating with silver ions doped HA has antibacterial properties. It seems to be effective in reducing bacterial colonization.

In their study, Wiglusz et al.<sup>[22]</sup> showed that the HA impregnated with the silver ions had a slightly better antimicrobial activity than HA with metallic silver. The authors reported that the

minimal bactericidal concentration of Ag against Gram-positive bacteria was (*S. aureus*, 3.7 µg/mL), indicating a lower concentration than our results. The MIC levels detected in our study for silver ion doped nano HA powder was 8 µg/mL for CNS and 8 µg/mL and 16 µg/mL, respectively for the comparison of *E. coli* and *S. aureus*. No free silver ions were detected in broth media using atomic absorption method which has a detection threshold as low as 0.02 mg/L.

There are two main types of coatings for effective antibacterial surface treatment as degradable and non-degradable with either “contact killing” or drug-eluting properties. Successful prophylaxis best achieved by local delivery devices, either made of degradable or non-degradable materials.<sup>[19]</sup> Local delivery of high doses of antibiotics has disadvantages such as burst release than subtherapeutic dose of antibiotics.<sup>[19,22]</sup> Also, the increase in antibiotic-resistant pathogens leads to consideration of a material-based view of antimicrobials for infection.<sup>[8,9,23]</sup> Silver is a good option for a metal-based therapeutic. As an ionic form, it exhibits cytocompatibility, efficacy against planktonic and sessile bacteria, and bactericidal activity against many strains.<sup>[23]</sup> The clinical incidence of silver-resistant bacteria remains low, as silver, unlike common antibiotics, activates multiple mechanisms and hits different targets within the bacterial cells.<sup>[24,25]</sup> In animal studies, silver-coated prosthesis and implant applications have been shown to reduce the likelihood of infection. There has been a concern, however, about the toxicity of silver ions. The research efforts have focused on the development of silver coating technologies which reduce or even eliminate toxicity, while maintaining antibacterial effects.<sup>[19]</sup>

Darouiche<sup>[12]</sup> speculated that, despite promising *in vitro* results, implanted medical devices coated with silver have not been proven to be infection-resistant in the majority of studies. The conflicting results of the reports can be due to the factors such as minimal release or non-release of silver-coated surfaces and limitations dictated by potential silver toxicities. The silver toxicity is correlated to its bioavailability and determined by its solubility, oxidation state, complexation ability toward biological targets, excretion, and detoxification routes. In the literature, the probable cause of the discrepancy between the studies on the antimicrobial activity and toxicity of silver relates to the amount of silver and the way silver is given (i.e., metallic silver, ionic silver, or nanoparticle silver). Therefore, the silver form is as important as the total amount of silver for the toxicity.<sup>[26,27]</sup>

It has been reported that intravenous administration of about 0.6 g of silver can lead to argyria which is the only known clinical picture of chronic silver intoxication.<sup>[28]</sup> The authors concluded that higher levels of silver, up to 0.1 mg/L (a concentration that gives a total dose over 70 years of half the human No Observed Adverse Effect Level [NOAEL]- of 10 g), could then be tolerated without any risk to health.<sup>[29]</sup> The co-sputtered silver-containing HA coatings (2.05 wt % Ag) exhibited no osteoblast-precursor cell cytotoxicity with an antibacterial property. A number of studies have previously reported the development of a new silver ion doped nano-size calcium phosphate-based ceramic powder.<sup>[13,14]</sup> It has been demonstrated that the powder prevents infection and does not disturb angiogenesis, which plays a key role in osseous tissue growth and, at used concentrations, it is not cytotoxic. Our microbiological results specifically proved that silver ion doped nano-size calcium phosphate-based powder-coated titanium pins reduced the bacterial colonization significantly.<sup>[30]</sup> Free silver ions were not detected in the liquid surrounding the coated implant, since the silver-added ceramic powder is not dissolved in the liquid medium. The presence of silver ions trapped in the porous material ensures the contact killing locally over a sustained time without the risk of toxicity to host and resistance of the pathogens.

Nonetheless, there are some limitations. This is an *in vitro* study, and the results may not be directly related to clinical cases. Only one type of bacteria, *S. epidermidis*, was used in this study. Although *S. epidermidis* is the most common cause of infection, it is not the only cause of implant-associated infections. The detection method used for broth silver level, atomic absorption spectroscopy, is not usually considered the most sensitive method. The levels, however, may be below the detection of atomic absorption spectroscopy.

In conclusion, this *in vitro* study demonstrated better outcomes of silver ion doped HA coating in the prevention of the bacterial colonization than the uncoated or pure HA-coated pins. Based on these results, this technique can potentially reduce implant-related infection.

#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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