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BASIC SCIENCE

Antimicrobial Effect of Polymer-Based Silver Nanoparticle Coated Pedicle Screws: Experimental Research on Biofilm Inhibition in Rabbits

Derya Burcu Hazer, MD,* Mustafa Sakar, MD,† Yelda Dere, MD,‡ Gülşen Altınkanat, MD,§ M. Ibrahim Ziyal, MD,¶ and Baki Hazer, PhD||

Study Design. Antimicrobial effect of a novel silver-impregnated pedicle screw in rabbits.

Objective. A novel spine implant model was designed to study the antimicrobial effect of a modified Titanium (Ti) pedicle screws with methicillin-resistant *Staphylococcus aureus* (MRSA) in multiple surgical sites in the lumbar spine of a rabbit.

Summary of Background Data. Infection in spinal implant is of great concern. Anti-infection strategies must be tested in relevant animal models that will lead to appropriate clinical studies.

Methods. Fourteen New Zealand white rabbits were divided into 2 groups: group 1: infected unmodified Ti screw group ($n=6$), and group 2: infected polyethylene glycol grafted, polypropylene-based silver nanoparticle (PP-g-PEG-Ag) covered Ti screw group ($n=6$), and 2 rabbits as sterile (sham-operated and control) group. In all groups, left L4–right L6 vertebra levels were exposed and screws were drilled to transverse processes after contamination of burr holes and surrounding tissue with 0.1 mL of 10^6 colony forming units (CFU) MRSA solutions in groups 1 and 2. After 21 days, samples were collected and infection was analyzed via light and scanning electron microscopy and culturing. Silver nanoparticles (Ag-NP) on the screws and tissues were assayed pre and postoperatively.

Results. The bacterial colony count for modified-Ti screw group was lower than for unmodified Ti screw (17.2 versus 200×10^3 CFU/mL, $P=0.029$) with less biofilm formation. There was no difference in duration of surgery among groups and within the surgical sites. Ag-NPs were detected on the screw surface postoperatively.

Conclusion. This novel experimental design of implantation in rabbits is easy to apply and resembles human stabilization technique. Modified Ti screws were shown to have antimicrobial effect especially inhibiting the biofilm formation. This anchored Ag NPs that remained after 21st day of implantation shows that it is resistant to tapping forces of the screw.

Key words: Ag nanoparticles, antimicrobial, biofilm, infection, polyethylene glycol, polypropylene, rabbit, spine, spine implant model.

Level of Evidence: N/A

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From the *Muğla SıtkıKoçman University, Faculty of Medicine, Department of Neurosurgery, Muğla, Turkey; †Marmara University, Institute of Neurological Sciences, Istanbul, Turkey; ‡Muğla SıtkıKoçman University, Faculty of Medicine, Department of Pathology, Muğla, Turkey; §Marmara University, Faculty of Medicine, Department of Microbiology, Istanbul, Turkey; ¶Marmara University, Faculty of Medicine, Department of Neurosurgery, Institute of Neurological Sciences, Istanbul, Turkey; and ||Bülent Ecevit University, Department of Chemistry, Engineering of Nanotechnology, Zonguldak, Turkey.

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Address correspondence and reprint requests to Derya Burcu Hazer, Muğla SıtkıKoçman University, Faculty of Medicine, Department of Neurosurgery, 48000 Muğla, Turkey;
E-mail: burcuhazer@hotmail.com; burcuhazer@mu.edu.tr

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Posterolateral lumbar fusion is the standard surgical treatment for many spinal disorders, including spondylolisthesis, disc degeneration, and traumatic instability.[1,2] Postoperative spine implant infection is the most common threatening event in spine surgery.[3] Although modern spinal surgical techniques have decreased the incidence of this complication, postoperative spinal implant infection still occurs at a significant rate.[4] This "unwanted" complication occurs due to adhesive properties of the bacteria and the biofilm formation.[5] The most common organism isolated from postoperative spinal wound infections is *Staphylococcus aureus*.[6]

To overcome implant infection, many attempts have been made to improve the implant technology and experimental

implant model. Silver nanoparticles have been demonstrated to have antimicrobial effects.[7–10] Bone implants together with pedicle screws have been modified in several methods using silver nanoparticle to enhance the antimicrobial effect.[11,12] We have investigated the antimicrobial effects of polyethylene glycol grafted-polypropylene (PP-PEG) based Ag-nanoparticle (Ag-NP) coated Titanium (Ti) [Ag-PP-g-PEG-Ti] pedicle screws in a novel spine implant model in rabbits.

MATERIALS AND METHODS

We used 29 standard titanium (Ti) bone screws (2.3 mm diameter and 8 mm length) in this study. Fifteen screws were coated with Ag-PP-g-PEG, and the remaining 14 were left uncoated. One of the coated screws was used for *in vitro* study. For inoculation, 1 mL of 10^6 colony forming units (CFU)/mL methicillin-resistant *Staphylococcus aureus* ATCC 43300 was used.

Fourteen germ-free New Zealand rabbits (14 weeks old; weight 2500–3000 g; mean weight: 2750 g) were randomly divided into 3 groups: Group 1: 1 rabbit as a sterile-control group with modified-Ti screw, 1 rabbit as a sham-operated sterile group with Ti screw; Group 2: 6 rabbits as a Ti screw implanted infected group; and Group 3: 6 rabbits as a modified Ti screw implanted infected group. Surgical procedure for all rabbits was identical, each bearing 2 screws of the same type. The study was approved by local Ethics Committee. The rabbits were housed individually in standard cages (60 cm width x 40 cm depth x 50 cm height) and were daily fed with 80 to 100 g antibiotic-free rabbit chaw.

Modification of Ti Screw

The amphiphilic polymer was synthesized by a procedure previously reported.[13] A series of silver nanoparticles embedded PP-g-PEG amphiphilic graft copolymers were prepared by modifying a previously reported procedure developed by Poelstra KA et al.[14] The PP-g-PEG2000 graft copolymer (0.2 g) was dissolved in 20 mL of tetrahydrofurane. About 0.01 mL of the $AgNO_3$ (0.1 M) aqueous solution was added to this solution and a pink colored solution was prepared. Then, Ti screws were dipped into this solution, and dried in open air for 5 minutes. This procedure was repeated 20 times until a fine nanocomposite film layer was achieved on the surface of the screws. These nanocomposite film layered screws were dried under vacuum and then put in a furnace at 450°C for 2 hours to diminish the polymer and therefore silver nanoparticles are adhered to the surface of the screw. All screws were sterilized individually using ethylene oxide gas with an exposure time of 8 hours.

Evaluation of Ag NPs on Screws (*In Vitro* Studies)

Three screws out of 15 screws were randomly chosen and scanning electron microscopy (SEM) and energy-dispersive X-ray spectrometry (EDS) analysis of the screws were done to document the amount of Ag nanoparticle present on the screw. One coated screws was randomly chosen and EDS analysis of the surface of the screw before and after it was tapped into bovine vertebra was performed for validation if silver nanoparticles will slip off with tapping forces. EDS analyses were performed on selected areas; the screw was divided into 4 with imaginary vertical lines as continuation of the socket on the head of the screw, and 4 spots on this line in clockwise direction were analyzed, and the mean values were recorded. Remaining 2 screws were sterilized and implanted into 1 of the rabbits for future SEM analysis.

Experimental Design and Implantation of Screws

For the sake of the ethical issues, we have decided to implant the screws in 2 different lumber vertebra levels in opposite sites. This was previously proven to act as a single infection site in an animal.[15] After fasting for 8 hours, the animals were anesthetized with intramuscular injection of an anesthetic cocktail comprising 44 mg/kg ketamine HCl, 5 mg/kg xylazine, and 0.75 mg/kg acepromazine maleate. The back of the rabbit was thoroughly shaved and the positions of the desired vertebrae, L4, and L6 were marked on the back of the animal. After the back was prepared with povidone iodine, sterile drapes were used to cover the entire animal. A midline 2 cm dorsal skin incision was made followed by a single incision in the fascia to expose the spinous process. Left-sided blunt dissection of the paraverterbral muscles was carried out to expose the transverse process of L3 vertebra. Approximately 1.5 mm diameter defect is created parallel to the axis of transverse process with the aid of diamond tip (1 mm diameter) of an electrical dentistry drill. One milliliter of bacterial solution (in infected groups) or 1 mL of sterile isotonic solution (in control group) was inoculated into the burr holes and the surrounding surgical sites. Then, the designated screws were tapped parallel to the axis of transverse process. The fascia and the skin was closed immediately after the animal was uncovered; the back was again prepared with povidone iodine and covered with new, sterile cloth. With a new set of sterile instruments, the second implantation was performed on the right L6 vertebra, with approximately 4 cm separating adjacent incisions. The same surgical procedure was repeated on this site. The closed wounds were left uncovered to prevent bandage irritation of the skin. To prevent unwanted contamination, the sterile control group animals were operated first. Variability was minimized and surgical trauma was standardized by using the same surgeons (left L4 screw, MS; right L6 screw DBH) to perform all operations. No postoperative antibiotics were administered. On the postoperative first day, plain x-ray was taken from all rats to prove the screw localization (Figure 1). Animals were examined daily for activity, eating, and wound healing. Weight and temperature were measured as well. After 3 weeks, the rabbits were killed using an overdose of pentobarbital intravenously.

Harvesting the Samples

Screws were harvested with the surrounding paraspinal muscle and the transverse process it was tapped into. In the control group, 1 screw with the muscle and bone tissue in each animal was sent for microbiological analysis and

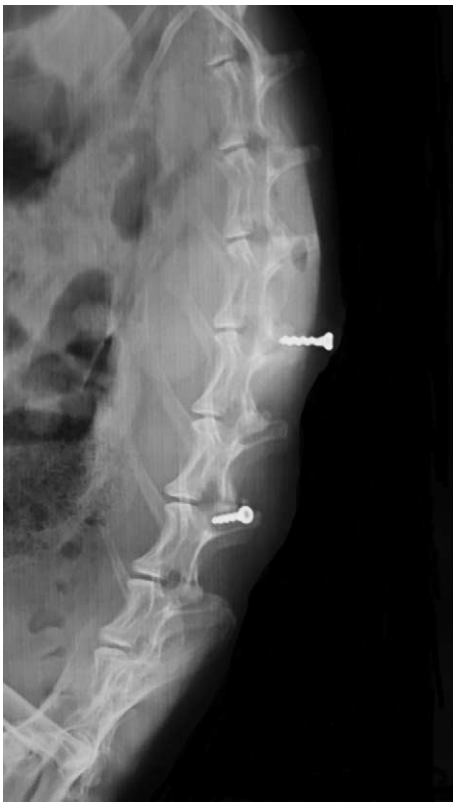

Figure 1. Lateral x-ray of screw inserted to the transverse process of the L4 and L6 vertebra.

the other set for SEM analysis. For microbiological examination, all samples were diluted 1 : 1000 [vortexed Mueller Hinton broth (MHB)] and were inoculated and then incubated at 37°C for 24 hours. To detect bacterial load, each screw was put into 2 mL of MHB and vortexed for 1 minute. Using 1 and 10 μL plastic loops, undiluted and 1 : 1000 diluted samples (vortexed MHB) were inoculated on 5% sheep blood agar by means of colony-counting method. Culture plates were incubated at 37°C for 24 hours. For each screw sample, the number of bacterial colonies counted on plates were multiplied by dilution coefficient and recorded as colony-forming unit (cfu). In the infected groups, 1 randomly chosen animal in each group was sacrificed and harvested screws with the surrounding tissues in each animal were sent for EDS and SEM analysis.

Histochemical and SEM Analysis

The paravertebral muscles were harvested en bloc, fixed in 10 % neutral-buffered formalin, and embedded in paraffin. Serial sagittal sections were taken and the sectioned tissues were stained sequentially with hematoxylin and eosin (H&E), and then evaluated for intensity of the infection.

For SEM analysis, the screws were fixed in 2.5% glutaraldehyde for 4 hours, washed in phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 1 hour, and dehydrated in increasing concentrations of ethanol. Following dehydration, the samples were air-dried and mounted on metal stubs with a double-sided adhesive band. The specimens were then sputter coated with gold (Emitech K550X Sputter Coating Systems, Quorum Technologies, UK). All samples were examined under a scanning electron microscope (SEM) (Jeol JSM-7600F, Joel Ltd., Tokyo, Japan) at an accelerating voltage of 15 kV.

Statistical Analysis

Comparison of the duration of surgery in between different screw types was done by the Student's *t* test. Comparison of the bacterial colony count in between different screw types and for peripheral bone and muscle tissues was done by the Mann–Whitney *U* test. The number of bacterial colonies is given as the median value with 25% and 75% percentiles. The software SPSS for Windows Version 14.01 (License number: 986964) was used in statistical analysis data. The value *P* less than 0.05 was considered statistically significant.

RESULTS

During the time of implantation, no systemic infection with mortality was detected. No leakage from the surgical wound or any fewer was detected postoperatively. However, in 4 rabbits in the infected group, there were pus formations localized to the fascia plane.

Microbiological Analysis

The duration of operation did not differ in between groups ($P = 0.855$) (Table 1). The bacteria colony count on the screw samples was found to be higher in Ti-screw group than in PP-PEG-Ag Ti screw ($P = 0.029$). The bacteria colony count in bone and muscle tissues surrounding these different types of screws was found to be similar ($P = 0.579$; $P = 0.481$) (Table 2, Figure 2).

In Vitro Studies

Preoperative SEM analysis of the screw revealed Ag nanoparticle on the screw (Figure 3). Preoperative EDS analysis of 1 of these screws revealed 30.18 % of Ag nanoparticles on the head of the screw, 20.91% in the middle and 3.73 % on the tip of the screw. After the implantation, there was almost

TABLE 1. Duration of Operation in Infected Groups is Presented

Screw Type	N	Operation Time	
		Mean ± SEM	Std. Deviation
PP-PEG-Ag Ti screw	12	10.08 ± 0.38	1.31
Ti screw	12	10.00 ± 0.25	0.85
Statistical significance (Student's *t* test *P* values)	0.855		

TABLE 2. Number of Bacteria Colonies on Samples are Presented

	Number of colonies x 10^3 (CFU/mL)		
	Ti screw	PP-g-PEG-Ag Ti screw	Statistical significance (Mann–Whitney *U* test *P*)
Sample type	Median (Percentiles 25; 75)	Median (Percentiles 25; 75)	
Ti screw	200 (31; 800)	17.2 (0; 95)	0.029*
Paraspinal muscle tissue	2.2 (0.2; 14.5)	1.6 (0; 12.5)	0.579
Bone	14 (0.8; 40)	4 (0; 30)	0.481

CFU indicates colony forming units.
$P < 0.05$, Mann Whithney U test.

a half percentage drop of Ag nanoparticles to 10.29% of Ag nanoparticles on the head of the screw, 10.24% in the middle and 1.03 % on the tip of the harvested screw (Table 3).

In Vivo EDS Analysis of Modified Screws and Surrounding Tissue

EDS analysis of the two modified Ti screws revealed drop in the Ag level postoperatively. The amount of silver nanoparticles was detected highest on the head of the screw; however, there was a decline in the amount of Ag nanoparticles percentage on the middle and on the tip of the screw (Table 3). We have also checked the Ag nanoparticle on the tissues in this animal, the muscle and bone tissue revealed negligible amount of Ag nanoparticles in the postoperative period.

SEM Analysis

There was a great difference in between Ti screw and Ag-PP-g-PEG screw on SEM photograph. Bacterial colonization on the Ti screw was prominent with biofilm layer on the surface of the screw (Figure 4A); however, there was only few bacteria scattered on the surface of Ag-PP-g-PEG covered screw without any biofilm formation (Figure 4B). Similarly, there was prominent bacterial colonization on the muscle and bone tissue in the infected-Ti group and less in the Ag-PP-g-PEG-Ti group.

Histological Sections

The muscle tissue surrounding the Ti screw in the infected group revealed prominent infiltration of lymphocytes and polymorphonuclear leucocytes and with increased vascular infiltration (Figure 5A). On the contrary, the Ag-PP-g-PEG coated screw group revealed very few inflammatory cells and without obvious edema in the muscle tissue. There is a mild thickening of the fibrous tissue on the layer facing the screw (Figure 5B). In both sections, there were mild foreign body reactions, presented as giant cells scattered within the fibrous tissue.

DISCUSSION

We introduce a novel Ag nanoparticle coated Ti screw via PP-PEG polymer carrier system, in a new experimental spinal implant model in rabbits and investigated the viability of this new experimental model with antimicrobial studies of these novel screws. We have detected promising results with antimicrobial activity of the screws and resistance of anchored Ag NP against tapping forces.

Many experimental designs for investigating spine implant infection and testing new implant[12,15–18] designs have been presented. Implantation of a rod to the transverse process,[15] or implantations of a screw to the iliac crest[16] have been used in other studies. In both models, the implant

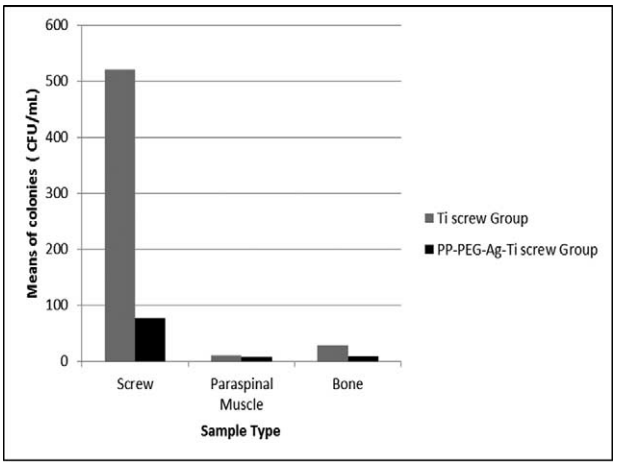

Figure 2. Diagram of bacterial colony count on harvested tissues and screws.

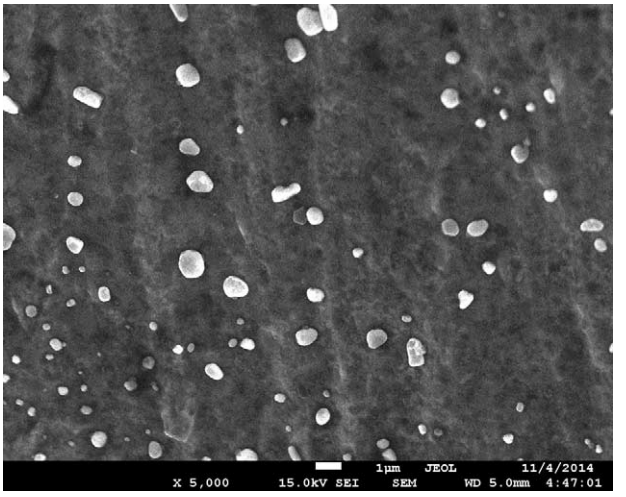

Figure 3. Ag-NP present on the Ag-PP-g-PEG-Ti screw (SEM analysis, 1 µm, x5000).

TABLE 3. The *In Vitro* and *In Vivo* Ag Nanoparticle Analysis on the Screw, Bone, and Muscle Tissues is Presented

Ag Nano-particle, mean	*In Vitro* Ti Screw, Preoperative (n = 1)			*In Vitro* Ti Screw, Postoperative (n = 1)			*In Vivo* Ti Screw, Preoperative (n = 2)			*In Vivo* Ti Screw, Postoperative (n = 2)				
	Head	Middle	Tip	Head	Middle	Tip	Head	Middle	Tip	Head	Middle	Tip	Muscle	Bone
Weight%	30.18	20.91	3.73	10.29	10.24	1.03	25.12	15.36	8.6	12.22	8.3	0.29	0.03	0.01
Atomic%	10.15	7.68	2.06	8.08	8.08	0.03	5.2	3.2	1.3	5.48	2.08	0.08	0.01	0.01

design was successful. In our novel implant infection model, we have implanted the screws directly to transverse processes mimics the surgical procedure in human in respect of spine biomechanics and close contact to paravertebral muscle. We also operated on 2 different vertebra levels with opposite sites, and evaluated them as different surgical sites. Cross-contamination was prevented in these multiple surgical sites, and also despite substantial bacterial inoculation, they remained isolated due to separate fascia plane. Therefore, this experimental spine implant model seems promising in future studies.

Many studies have recently focused on silver nanoparticles and its antimicrobial effect *in vivo*. Their antimicrobial activity is introduced to medical applications, especially metal implants used in skeletal system.[11,19,20] They have stated to have bactericidal effect[21] and biocompatibility with fibroblasts and osteoblasts.[22] The bactericidal activity of AgNPs is attributed to the release of Ag^+ ions from soluble complexes, and surface reactions that generate reactive oxygen species or promote catalytic oxidation of cellular components.[23] There are several techniques for integrating silver nanoparticles to medical devices,[24–26] such as silver plasma ion immersion technique or by physical vapor deposition process. There are also carrier systems such as chitosan or polyhydroxylated polymers[21] used in modification of the Ti surface, because they were stated to be good reducing agent and immobilize Ag nanoparticles on the Ti surface. We have used PP-PEG for a carrier system to integrate AgNP to Ti screws, which is highly soluble and has a good soft tissue response.[10]

Bacteria colony count on the surface of the Ag-PP-g-PEG-Ti screws was significantly low compared with unmodified Ti screws. Similarly, paravertebral muscle tissue of the Ti screw revealed intense inflammation compared with Ag-PP-PEG covered Ti screws. This indicates that modified screws suppress bacteria attachment to the surface of the screw, and therefore has a bactericidal effect both on the screw surface and neighboring tissue.

S. aureus, a typical Gram-positive coccus, is one of the most common pathogens that cause biofilm formation, which is a critical process in the implant infection.[27–31] Biofilm formation usually starts 24 to 48 hours and occurs in 2 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.[32] It is matured up to 14 days, and after 14th day, regression of the bacterial load occurs due to decreased blood flow.[32] After degradation of the biofilm formation, residual bacteria may cause reinfection up to 28 days. Therefore, it is stated that key point for prevention

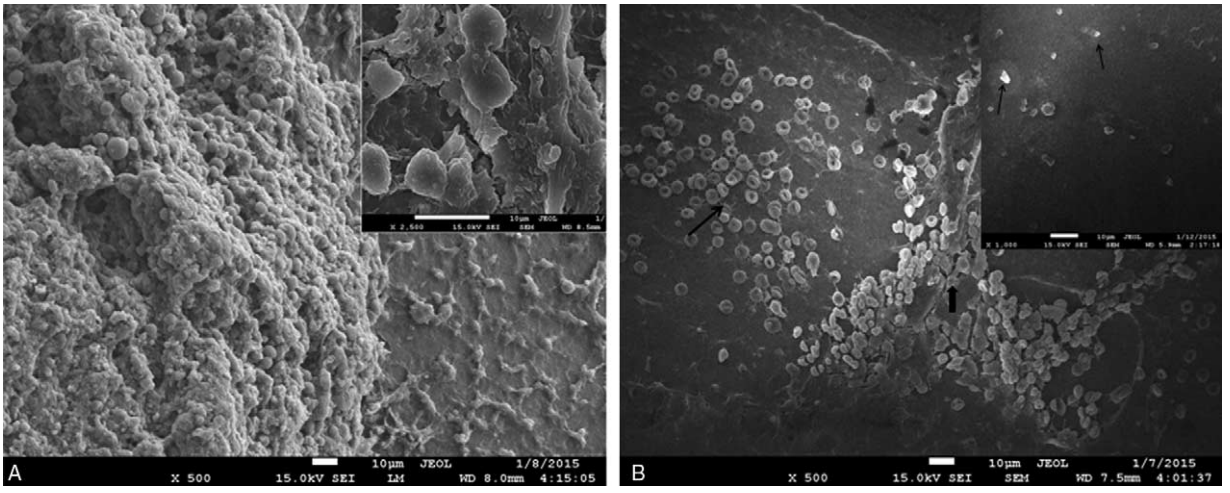

Figure 4. SEM analysis of the (A) bacteria and biofilm formation on the unmodified Ti screws in infected group with SEM analysis (10 µm, x500), closer view of the bacteria and the biofilm layer in the upper right corner (10 µm, x2500)and (B) fewer bacteria with remanants of the biofilm on modified Ti screw group (10 µm, x500) and closer view of the Ag-NP present among the bacteriain the upper right corner (10 µm, x1000). Thick black arrow: red blood cells; arrow head: bacteria and remanants of the biofilm; thin arrows: Ag-NP.

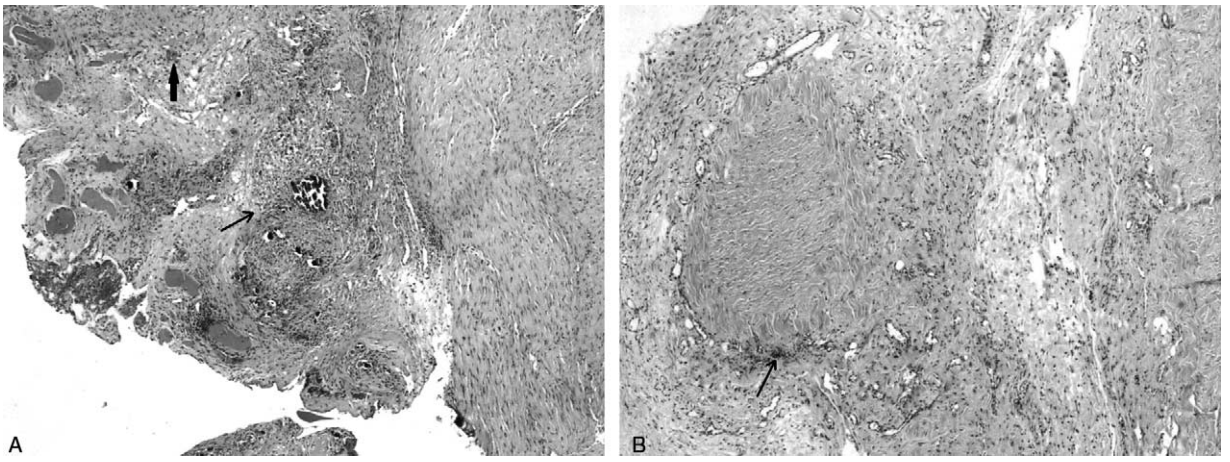

Figure 5. Light microscopic sections of the paravertebral muscle tissue. (A) High amount of lymphocytes and polymorphonuclear leucocytes together with vascular proliferation (thin arrow) and giant cells (thick black arrow) present in the thick fibrous tissue in the infected Ti screw group; H&E, x40. (B) Few lymphocytes and polymorphonuclear leucocytes (thin arrow), with few giant cells present in the infected Ag-PP-g-PEG-Ti group; H&E, x40.

of the biofilm formation, and related implant infection, is to also prevent the reinfection on the critical days between 14 and 28 days.[26] In one recent study, plasma ion immersed Ag nanoparticle covered Ti rods were found to inhibit bacterial adhesion via suppressing polysaccharide intercellular adhesion synthesis up to 4 weeks in *in vivo* studies. In our study, SEM analysis revealed very few bacteria load on the surface of Ag-PP-g-PEG-Ti screws with some reminants of polysaccharide matrix resembling the biofilm layer. This may be explained with the duration of the implantation, which is within the time period of the degradation of the biofilm and reinfection, as described above. Therefore, it is demonstrated that this novel modification technique decreases the biofilm formation and even might be successful in preventing re-infection. In addition, there was no correlation between the time the animal was under anesthesia and the colony-forming unit values found after death in infected sites randomly operated on. This states that the duration of the operation did not affect the outcome of the experiment.

One of the questions that will come up is whether or not these ''anchored'' silver nanoparticles may be swept to the top of the screw by tapping forces. With both *in vitro* and *in vivo* experiments, we have shown remaining silver nanoparticles on the screw surface, mainly on the head and also on the shaft of the screw. We had also repeated the same analysis for the screws used *in vivo*. The Ag nanoparticles on the screw after 21 days of implantation were still present, but in very low amount compared with preoperative levels. This phenomenon could be explained with the method of the analysis, as EDS can only see the present nanoparticle on the surface. The surface of the harvested screw from the infected animals is covered by the bacteria or blood cells, and therefore, the silver nanoparticles could be blocked and detected in very low amount. In the peripheral muscle and bone tissues, there was no prominent accumulation of the silver nanoparticles.

In this experimental study, we have detected Ag-PP-PEG-Ti screws that inhibit biofilm formation on the implant surface with less bacterial load. To our knowledge, this is the first study in which the screws are placed into the transverse processes of the rabbits. This is a pilot study resembling the posterior vertebral stabilization in humans. However, we are aware that there are some limitations of this study in the way the experiment was conducted. The study protocol could be rearranged with increased number of cases in control group to investigate the natural history of methicillin-resistant *S. aureus* in the posterior spine of the rabbits. In addition, another experimental group could be added to the protocol to significantly prove that there is no cross-contamination in between different surgical levels. Also, bone grafts and modified rods could be added to mimic *in vivo* conditions in spine. However, to be able to reduce the number of confounding factors, only the screws have been studied. Future studies could be planned with implantation of modified screws and rods with bone grafts bearing different amounts of Ag NP in higher species such as dogs and sheep, which will more resemble human spine stabilization and fusion.

CONCLUSION

This novel experimental design of implantation in rabbits is easy to apply and resembles human stabilization technique. Polymer-based Ag nanoparticle coated Ti screws were shown to have antimicrobial effect and especially inhibiting the biofilm formation. These modified screws, anchored with Ag nanoparticles, showed resistance to tapping forces and were specifically effective in preventing re-infection in spine implants. However, further studies could be planned to investigate the antimicrobial effect of different amounts of Ag nanoparticle anchored to the Ti screws with different time periods.

➢ Key Points

- ❑ Ag-PP-g-PEG coating on Ti screws was detected to inhibit biofilm formation on implanted screws.
- ❑ Implantation of pedicle screw into the transverse process of lumbar spine of a rabbit is presented as a novel implant model to investigate biofilm inhibition.
- ❑ PP-g-PEG polymer seems to be a good vehicle for impregnation of Ag-NP on the screw surface.
- ❑ Most of the Ag-NP was found to be present on the screws after implantation, and negligible amount was present on the neighboring tissues.

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