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Cobalt–Chromium Alloy Has Superior Antibacterial Effect Than Titanium Alloy

In Vitro *and* In Vivo *Studies*

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Study Design. *In vitro* and *in vivo* laboratory studies.

Objective. This study aimed to compare bacterial survival on titanium alloy (Ti) and cobalt–chromium alloy (CC) using *in vitro* and *in vivo* experiments.

Summary of Background Data. Spinal implants are frequently manufactured from Ti and CC. These foreign materials are thought to be susceptible to biofilm formation that contributes to the development of surgical site infections. Certain metals (*i.e.*, silver, cobalt) are known to have antibacterial properties.

Methods. In the *in vitro* study, discs made of Ti or CC were incubated with one of two common bacteria: *Staphylococcus aureus* (*S. aureus*) and *Propionibacterium acnes* (*P. acnes*). After incubation, discs were assessed to determine the number of viable bacterial cells. In the *in vivo* study, the discs that were made of CC or Ti were implanted into the subcutaneous layer of BALB/c mice. After skin closure, a suspension including either *S. aureus* or *P. acnes* was directly inoculated on the implanted discs. The discs were retrieved and analyzed to determine the number of viable bacteria at 0.5, 1, and 3 days after inoculation.

Results. The number of viable *S. aureus* cultured from the CC discs was $0.9 \pm 0.2 \times 10^3$ CFU/disc, which was significantly lower than the cultured Ti discs ($114.8 \pm 18.3 \times 10^3$ CFU/disc). Moreover, a significantly lower mean number of *P. acnes* were cultured with CC ($1.9 \pm 1.2 \times 10^3$ CFU/disc) compared with the Ti ($180.0 \pm 72.1 \times 10^3$ CFU/disc). The *in vivo* infection model testing against *S. aureus* or *P. acnes* showed a significantly lower number of viable *S. aureus* or *P. acnes* on CC discs than Ti discs. The result was seen at all measured time points.

Conclusion. CC suppressed *S. aureus* and *P. acnes* proliferation compared with Ti *in vitro* and in an *in vivo* infection model.

Key words: antibacterial, antibacterial testing, biomaterial, cobalt–chromium alloy, infection model, *Propionibacterium acnes*, spinal instrumentation, *Staphylococcus aureus*, surgical site infection.

Level of Evidence: N/A

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Spinal instrumentation, such as pedicle screws or rods made of titanium (Ti) alloy or cobalt–chromium (CC) alloy, is an important tool for correcting adult deformity and adolescent idiopathic scoliosis and for providing stabilization until a definitive fusion for many spinal disorders. Although successful clinical results of instrumented spinal surgery have been extensively published, the incidence of surgical site infection (SSI) remains a concern. This concern is consistent across all orthopedic surgeries including joint arthroplasty and fracture surgery.[1,2] The incidence of SSI in spinal surgery is reported to be between 0.6% and 11.9%.[3–5] It is well documented that SSI results in prolonged hospitalization, increased morbidity, reduced outcomes, and increased economic costs for patients and hospitals.[6]

SSI risk factors are multifactorial including patient characteristics and pre-, intra-, and postoperative characteristics.[7] Among the intraoperative risk factors, foreign materials, such as suture or spinal instruments at the surgery site, are known to increase the risk of SSI in surgery.[8–10] Surface modifications to give an antibacterial effect to reduce the risk of foreign materials have been reported. Coatings including silver are common to provide materials with an antibacterial effect, and some researchers have reported experimental results.[11–13] Other promising approaches to add an antibacterial effect are polymer coatings including antibacterial agents,[14] such as an iodine-incorporated oxidized layer.[15,16] Other materials

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combined with metallic cations, such as copper, zinc, cobalt, and gallium, and silver ions have also shown an antibacterial effect.[17,18] To test the hypothesis that CC is expected to show antibacterial effect according to previous reports, we conducted a comparative study of the antibacterial effect of common materials used for spinal implants.

This study aimed to compare the *Staphylococcus aureus* (*S. aureus*) and *Propionibacterium acnes* (*P. acnes*) survival on Ti and CC used as common spinal implant materials in *in vitro* and *in vivo* experiments. *S. aureus* and *P. acnes* are common SSI-causing pathogens and have been linked to late-onset screw loosening.[19–21]

MATERIALS AND METHODS

Specimen

For the *in vitro* antibacterial testing, discs (15 mm in diameter and 3 mm in thickness) made of either Ti (TI-6Al-4 V, ASTM F136) or CC (Co-28Cr-6Mo, ASTM F1537) were used. A disc (10 mm in diameter) made of polyethylene (Organo Corp, Tokyo, Japan) was used as a control. For *in vivo* testing, discs (7 mm in diameter and 1 mm in thickness) made from either from Ti or CC were used. To reproduce and validate the widely used implant materials, discs were provided from Medtronic Sofamor Danek Co, Ltd, in similar alloys to currently available spinal implants.

In Vitro Antibacterial Testing

Two types of bacteria, *S. aureus* (NBRC 12732, National Institute of Technology and Evaluation, Tokyo, Japan) and *P. acnes* (NBRC 107605, National Institute of Technology and Evaluation, Tokyo, Japan), were used in the *in vitro* experiments. *S. aureus* was cultured in a liquid nutrient broth (NB) medium (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) at 35°C for 48 hours under aerobic conditions. *P. acnes* was cultured in a liquid Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) at 35°C for 5 days under anaerobic conditions. The cells harvested by centrifugation were washed three times with 0.9% NaCl solution and then suspended in 0.9% NaCl solution. Cell suspensions were diluted with 1/100 NB medium or 1/100 GAM at appropriate dilution and used as the test inoculums.

The *in vitro* antibacterial testing was conducted according to the Japanese Industrial Method Z 2801:2010 "Antibacterial products–Test for antibacterial activity and efficacy" with minor modifications. To spread the inoculum on the surface and ensure close contact with the material surface, 40 μL of cell suspension was injected onto the discs and then covered with polyethylene film. The discs contacted with *S. aureus* and *P. acnes* were incubated at 35°C for 24 hours and then washed out with 4 mL of 0.9% NaCl solution containing 0.7% Tween 80 (Kanto Chemical Co, Inc, Tokyo, Japan). To determine the number of viable cells, serial decimal dilutions of 0.9% NaCl solution were prepared, and 0.1 mL was uniformly spread on an agar plate including appropriate culture mediums. The plates for *S. aureus* and *P. acnes* were incubated at 35°C for 24 hours and 5 days under aerobic and anaerobic conditions, respectively. The data of viable cell numbers were counted as colony-forming units per disc (CFU/disc) and calculated antibacterial activity as a log reduction by comparing the number of viable cells on polyethylene with that on each alloy. Mann–Whitney *U* test with a 5% significance threshold was used to determine the difference of viable cells between CC and Ti. A sample size of 5 was used in the experiments.

In Vivo Infection Model

Two types of bacteria, *S. aureus* (ATCC 25923, American Type Culture Collection, VA) and *P. acnes* (NBRC 107605), were used in the *in vivo* experiments. *S. aureus* was cultured on *Staphylococcus* agar (Becton, Dickinson and Company, NJ) at 37°C for 2 days under aerobic condition. *P. acnes* was cultured on GAM agar (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) at 37°C for 3 days under the anaerobic condition. Then, the prepared cell suspensions were diluted with a concentration of 2×10^7 CFU/mL by using physiological saline (Otsuka Pharmaceutical Factory, Inc, Tokyo, Japan).

Six-week-old, specific-pathogen-free, female BALB/c mice (Japan SLC Inc, Shizuoka, Japan) were used. A sample size of 10 animals was measured at each time period and study arms. The mice were anesthetized by an intramuscular injection of the mixture of 80 mg/kg ketamine (Daiichi Sankyo Propharma Co, Ltd, Tokyo, Japan) and 10 mg/kg xylazine (Bayer Yakuhin, Ltd, Osaka, Japan). After the back of each animal was shaved and disinfected with alcohol, an approximately 10-mm skin incision was made at the midline of the back, followed by the creation of a subcutaneous pocket for the discs. The discs made of CC and Ti were implanted onto the pocket, and then the surgical site was sutured. Then, 0.05 mL of each of cell suspensions (1×10^6 CFU) was directly injected on the surface of implanted disc. The mice were sacrificed at 0.5, 1, or 3 days with direct inoculation of excessive isoflurane anesthesia of isoflurane. Each removed disc was transferred to a test tube containing 1 mL of Hank's balanced salt solution (Thermo Fisher Scientific, MA), sonicated, and vigorously mixed to collect the *S. aureus* or *P. acnes*. The serial diluted suspension of *S. aureus* was then cultured on *Staphylococcus* agar plates at 37°C for 2 days under aerobic condition. The suspension of *P. acnes* was cultured on GAM agar plates at 37°C for 3 days under anaerobic conditions. The number of colonies on the plates was measured. Wilcoxon rank-sum test with a 1% significance threshold was used to determine the difference of viable cells between CC and Ti. A sample size of 10 was examined at each time point for the examined alloys. All experiments were approved by the Committee for the Ethical Treatment of Animals of Nihon Bioresearch Inc (Gifu, Japan) and were conducted in accordance with the guidelines published from the Ministry of Health, Labor and Welfare and Nihon Bioresearch Inc.

TABLE 1. Results of the *In Vitro* Antibacterial Testing Against *Staphylococcus aureus* and *Propionibacterium acnes* on Titanium Alloy and Cobalt–Chromium Alloy

Material	*Staphylococcus aureus*		*Propionibacterium acnes*	
	Viable Cell / $\times 10^3$ CFU/Disc (Mean ± SD)	Antibacterial Activity	Viable Cell / $\times 10^3$ CFU/Disc (Mean ± SD)	Antibacterial Activity
Cobalt–chromium alloy	$0.9 \pm 0.2^*$	2.9	$1.9 \pm 1.2^*$	2.2
Titanium alloy	114.8 ± 18.3	0.8	180.0 ± 72.1	0.2
Polyethylene	660.0 ± 143.4	N/A	318.0 ± 67.6	N/A

$^*P < 0.05$ (Mann–Whitney U test). A sample size of 5 was used in the experiments. The number of viable S. aureus cultured from the cobalt–chromium (CC) discs was $0.9 \pm 0.2 \times 10^3$ CFU/disc, which was significantly lower than the cultured Ti discs ($114.8 \pm 18.3 \times 10^3$ CFU/disc). In addition, a significantly lower mean number of P. acnes were cultured with CC ($1.9 \pm 1.2 \times 10^3$ CFU/disc) compared with the Ti ($180.0 \pm 72.1 \times 10^3$ CFU/disc).

RESULTS

In Vitro Antibacterial Efficacy

The results of the *in vitro* antibacterial testing against *S. aureus* and *P. acnes* on Ti and CC are presented in Table 1. The number of viable *S. aureus* on CC ($0.9 \pm 0.2 \times 10^3$ CFU/disc) was significantly lower than that on Ti ($114.8 \pm 18.3 \times 10^3$ CFU/disc) 24 hours after inoculation ($P < 0.05$). The reduction ratio of CC alloy and Ti alloy (*vs.* polyethylene) against *S. aureus* was 99.9% and 82.6% respectively. The antibacterial activity of CC against *S. aureus* was calculated using a log reduction comparison polyethylene. The log reduction was measured at 2.9, which means a 99.9% reduction in viable *S. aureus* on CC surface. The numbers of viable *P. acnes* on CC and Ti after inoculation for 24 hours were $1.9 \pm 1.2 \times 10^3$ CFU/disc and $180.0 \pm 72.1 \times 10^3$ CFU/disc, respectively. Statistical difference in the number of viable *P. acnes* between Ti and CC was observed ($P < 0.05$). The reduction ratio of CC alloy and Ti alloy (*vs.* polyethylene) against *P. acnes* was 99.4% and 43.4% respectively. The antibacterial activity of CC against *P. acnes* was 2.2, which is able to reduce to approximately 100th of viable *P. acnes* in comparison with Ti.

In Vivo Antibacterial Efficacy

The number of viable *S. aureus* on Ti and CC in the *in vivo* infection model is presented in Figure 1. The number of viable *S. aureus* on the Ti was increased with extended implantation periods. In contrast, the number of viable *S. aureus* on the CC was significantly decreased from the 0.5 days after inoculation. The significant reduction of the viable *S. aureus* on the CC was kept for all implantation periods ($P < 0.01$).

The number of viable *P. acnes* on Ti and CC in the *in vivo* infection model is presented in Figure 2. The number of viable *P. acnes* on Ti and CC for 0.5 days after inoculation was $2.8 \pm 1.1 \times 10^1$ CFU/mm^2 and $0.5 \pm 0.1 \times 10^1$ CFU/mm^2, respectively. Significant differences were observed

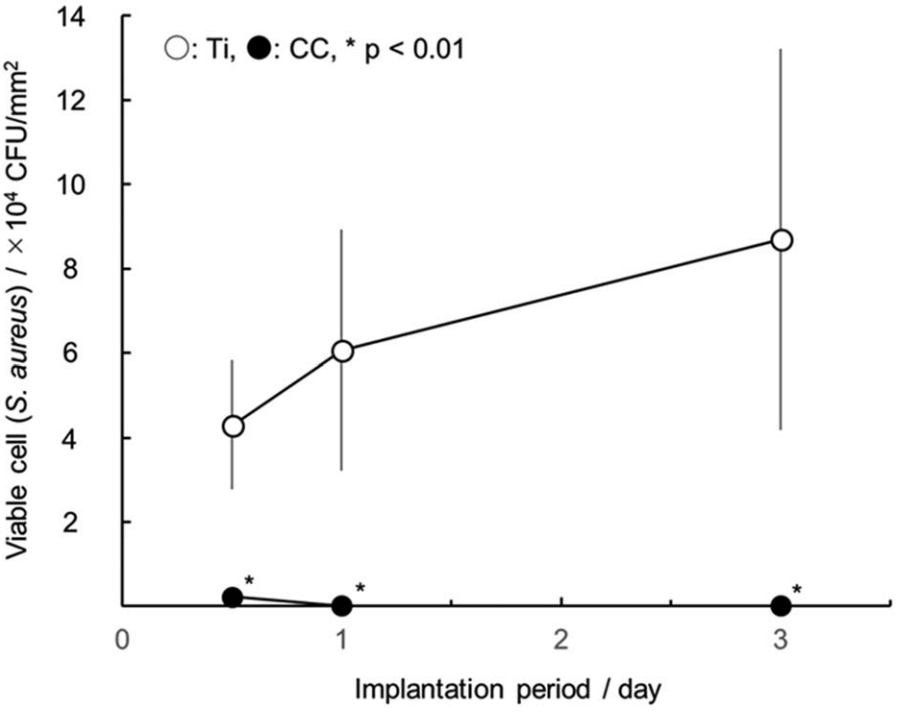

Figure 1. The number of viable *Staphylococcus aureus* on titanium alloy and cobalt–chromium alloy in the *in vivo* infection model (data shown as mean ± 2SE).

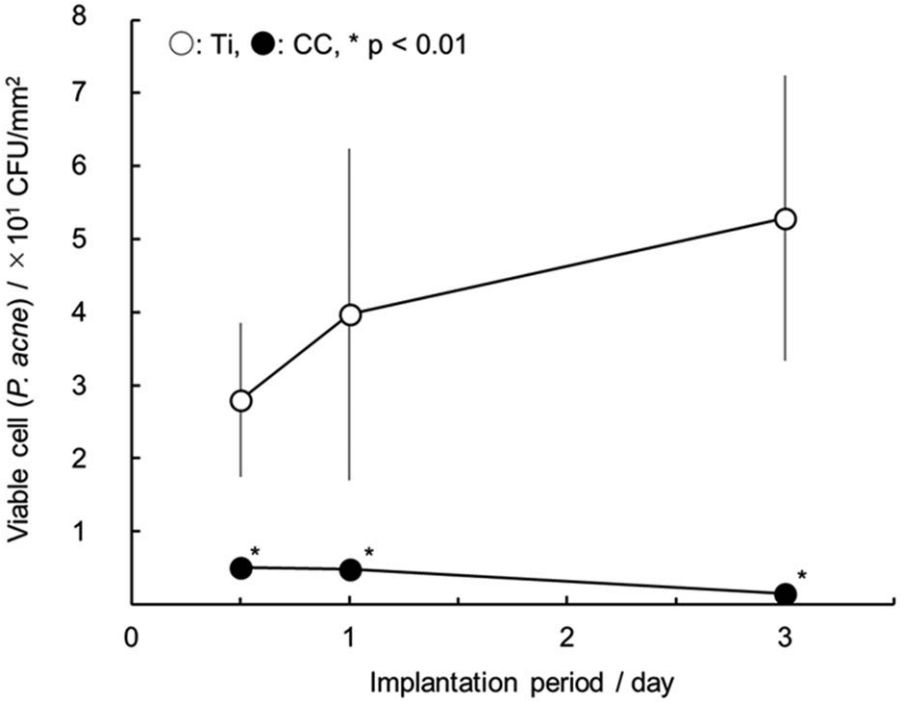

Figure 2. The number of viable *Propionibacterium acnes* on titanium alloy and cobalt–chromium alloy in the *in vivo* infection model (data shown as mean ± 2SE).

in the number of viable *P. acnes* on CC in comparison with Ti for 0.5 days after inoculation ($P < 0.01$). The proliferation of *P. acnes* on the Ti was continued for all implantation time points, but not on CC. Significant difference was also observed in the number of the viable *P. acnes* on 1 and 3 days after inoculation on the CC in comparison with the Ti.

DISCUSSION

Because CC alloys has high corrosion resistance, wear resistance, fatigue strength, and biocompatibility, they have been widely used for currently available spinal implants. In this study, CC alloy significantly reduced *S. aureus* and *P. acnes* proliferation compared with Ti alloy in the both *in vitro* and *in vivo* experiments.

The experiments were conducted with two common SSI pathogens, *S. aureus* and *P. acnes*. *S. aureus*, which has been reported as the most common pathogen, is an aerobic bacterium that causes superficial SSI (64%)[19] and deep incisional SSI (63%)[4] in orthopedic and spinal surgeries, respectively, whereas *P. acnes* is an anaerobic bacterium that has been found in delayed postoperative spinal infections and late-onset screw loosening for spinal instrumentation.[19,20] Shiono *et al*. reported that *P. acnes* and *P. species* are the most frequent pathogens from intraoperative specimens collected during posterior correction surgery.[22] They also reported that delayed infection of *P. acnes* occurred only in the presence of implants in the *in vivo* experiments.[23] Bacteria are initially attached to the material surface in a reversible adhesion. After changes to the irreversible adhesion on the material surface, the bacteria start to proliferate and produce extracellular polysaccharides as biofilms. The formed biofilms prevent the penetration of antimicrobial agents and disable their efficacy. The antibacterial effect of the CC alloy might be expected to suppress the *S. aureus* and *P. acnes* proliferation on the surface during initial phases of SSI development and may reduce one of the risks of SSI and delayed postoperative spinal infection and late-onset screw loosening in the spinal instrumentation. Only one clinical report can be found that assessed the effect of implant materials on SSI in pediatric scoliosis surgery, and there are no significant differences in SSI with stainless steel, Ti, or CC alloy/Ti alloy instrumentation.[24]

Similar studies have been reported with different antibacterial testing methods and/or different species. Patel *et al*.[25] reported that the biofilm formation rate on CC against *S. aureus* was significantly higher than that on Ti. The testing methodology used small cut rods made of CC or Ti, and the rods were soaked in a culture medium with *S. aureus* for 24 hours. Optical density values and CFUs were measured to find the results. Conversely, Koseki *et al*.[26] found a lower biofilm coverage rate of *S. epidermidis* on CC than Ti after culturing for 6 hours. In their experimental method, the bacteria suspension was directly poured on the discs made from various materials. There was no statistical difference between CC and Ti after 2 and 4 hours of culture. Although the experimental methods of these two papers and our *in vitro* testing methods seem similar, the testing methods were quite different. Direct comparisons are difficult to make leading to the need for an agreed-upon testing standard.

In conclusion, this study demonstrates that CC alloy suppresses *S. aureus* and *P. acnes* growth and survival compared with Ti alloy in both standardized *in vitro* antibacterial testing and in an *in vivo* infection model. This

study has many limitations, such as a small sample size, the use of a single small animal type, the use of CFUs as a proxy for infection strength, and an absence of clinical data. Adequately powered clinical studies are required to investigate whether differences in SSI incidence and screw loosening related to the examined pathogens exist.

➢ Key Points

- ❑ The antibacterial effects of CC alloy and Ti alloy against *Staphylococcus aureus* and *Propionibacterium acnes* were measured.
- ❑ CC alloy significantly suppressed *Staphylococcus aureus* and *Propionibacterium acnes* proliferation and inhibited survival compared with Ti alloy using both *in vitro* and *in vivo* experiments.
- ❑ Clinical studies are required to determine whether differences in surgical site infections exist between Ti and CC implant materials.

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