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Prevention of Pin Tract Infection with Titanium-Copper Alloys

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Abstract: The most frequent complication in external fixation is pin tract infection. In order to reduce the incidence of implant-associated infection, many published reports have looked at preventing bacterial adhesion by treating the pin surface. This study aimed to evaluate the antibacterial activity of a Titanium-Copper (Ti-Cu) alloy on implant infection, and to determine the potential use of the Ti-Cu alloy as a biomaterial. Two forms of Ti-Cu alloys were synthesized: one with 1% Cu and the other with 5% Cu. For analyzing infectious behavior, the implants were exposed to *Staphylococcus aureus* and *Escherichia coli*. The reaction of pathogens to the Ti-Cu alloys was compared with their reaction to stainless steel and pure titanium as controls. Both Ti-Cu alloys evidently inhibited colonization by both bacteria. Conversely, cytocompatibility studies were performed using fibroblasts and colony formation on the metals was assessed by counting the number of colonies. Ti-1% Cu alloy showed no difference in the number of colonies compared with the control. External fixator pins made of Ti-Cu alloys were evaluated in a rabbit model. The tissue-implant interactions were analyzed for the presence of infection, inflammatory changes and osteoid-formation. Ti-1% Cu alloy significantly inhibited inflammation and infection, and had excellent osteoid-formation.. Copper blood levels were measured prior to surgery and at 14 days post-operatively. Pre-operative and post-operative blood copper values were not statistically different. Overall, it was concluded that Ti-Cu alloys have antimicrobial activity and substantially reduce the incidence of pin tract infection. Ti-1% Cu alloy shows particular promise as a biomaterial.

Key words: Titanium-Copper alloy; antibacterial; biocompatibility; pin tract infection; cytotoxicity

INTRODUCTION

The most serious complication in implantation surgery is bacterial infection, especially osteomyelitis. Common causes of implant-associated infections are *Staphylococcus aureus (S. aureus)* and

Staphylococcus epidermidis (S. epidermidis).[1-5] Infection rate in external fixation occurs in 2-30% of cases found in the literature.[6-10] In a report of pin tract infection, Mahan described 75% of screw tips cultured positive for infection at removal.[8] Although colonization may be present without being clinically evident, it is known that the rate of pin tract infection rises proportionally with the length of time that the fixator pins are left in place.[11] Infections are especially common when the external fixation is used for limb-saving surgery after excision of skeletal tumors as patients have low immunity due to post-operative chemotherapy. Once adhered, *S. aureus* and *S. epidermidis* form biofilms which can be difficult to clinically treat because the bacteria are protected from phagocytosis and antibiotics,[12] hence the need to prevent initial bacterial adhesion. In order to reduce the incidence of implant-associated infections, several biomaterial surface treatments have been proposed. Previous studies on conferring antibacterial properties to biomaterials have relied on surface functionalization techniques such as covalently attaching polycationic groups,[13] impregnating or loading chitosan nanoparticles with antimicrobial agents,[14,15] and coating implant surfaces with either quaternary ammonium compounds, iodine, human serum albumin, or silver ions.[16-21] In recent years, many protein-resistant coatings have been developed for implants, such as polyethylene glycol, hyaluronic acid or the Arg-Gly-Asp peptide.[22-25] In particular, many silver-coated medical materials have been reported, such as cardiac and urinary catheters, heart valves, dialysis units, and orthopedic devices.[26-31] However, silver has been found to have toxic effects towards human cells.[32,33] Therefore, we hypothesized that copper implants may be a useful alternative to silver. There are few reports concerning orthopedic implants treated with copper.

Like silver, copper has long been known to be a potent antibacterial agent with a very broad spectrum of activity against numerous bacteria. Copper is a trace metal and an essential component of several enzymes. Copper insufficiency is associated with often severe pathologic alterations including impairment of blood, liver, and immune systems.[34] However, when the amount of copper increases, toxicity is occurred.[35] Cellular actions of copper include destabilization of membranes via superoxide and hydroxyl radicals, affinity to RNA and DNA and inhibition of the transcription process,[36] inhibition of calmodulin, a regulator of intracellular Ca^{2+},[37] and induction of apoptosis.[38] It has been reported that Ag^{+} and Zn^{+} ions exhibit very strong cytotoxicity to cells at low concentrations ($LD_{50} = 3.5 \times 10^{-3}$ mmol/l) in growth inhibition tests, whereas tissue cells tolerated relatively high concentrations ($LD_{50} = 2.3 \times 10^{-1}$ mmol/l) of Cu^{2+}.[39] We designed titanium-copper alloy pins because Ti is a material with biocompatibility properties.[40] In the medical field, Ti-Cu alloys are used in odontology.[41]

This study focused on evaluating the antibacterial activity of Ti-Cu alloys and their impact on implant infection, as well as determining the potential suitability of Ti-Cu alloys as biomaterials.

MATERIALS AND METHODS

Implants

Circular implant Ti-Cu alloy, pure titanium and stainless steel disks (diameter 20 mm; thickness 2 mm) were used for *in vitro* antimicrobial tests. Semi-disks, 48-49 mm in diameter and 1-2 mm in thickness, of these metals were used for *in vitro* cytocompatibility tests. External fixation pins of Ti-Cu alloys, pure titanium and stainless steel (diameter 2 mm; length 45 mm) were used *in vivo*. The stainless steel material used in this study was SUS316. The titanium was commercially pure titanium. Two forms of Ti-Cu alloys were synthesized: one with 1% Cu and the other with 5% Cu. All Ti-Cu alloy implants were specifically produced by Kusaka Rare Metal Products (Tokyo, Japan). All the metals were processed by Koshiya Medical Instruments Company (Kanazawa, Japan).

In vitro antimicrobial properties

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We examined the antibacterial activity of the Ti-Cu alloys referring to the method established by Japanese Industrial Standards. For analyzing infectious behavior, the implants were exposed to gram positive *Staphylococcus aureus* (*S. aureus*), strain ATCC25923 or gram negative *Escherichia coli* (*E. coli*), strain MG1455. About one million colony forming units were inoculated on the circular implants, which had been previously autoclaved, and then the implants were covered by glass in a sterile dish and incubated at 37^{o}C for 2 hours, 6 hours, and 24 hours. Each implant was washed using 5ml phosphate-buffered saline (PBS). This was diluted 1 to 50 with PBS and 100 μl was spread onto the following broths: *S. aureus* was grown in brain-heart infusion broth and *E. coli* was grown in LB broth (1% w/v Tryptone, 0.5% w/v Yeast extract, 0.5% w/v NaCl) at 37 °C. After 24 hours, the number of colonies was counted. If all the pathogens were alive, about two thousand colonies were counted (Fig. 1). This method was repeated fifteen times for both *S. aureus* and *E. coli*. The reaction of pathogens to the Ti-Cu alloys was compared with their reaction to pure titanium and stainless steel as controls. The difference of the number of bacteria on each metal was statistically analyzed.

***In vitro* cytocompatibility properties**

The V79 cell line (Chinese hamster fibroblasts), provided by the RIKEN BIORESOURCE CENTER CELL BANK (Tsukuba, Japan), was used for the cytotoxicity tests. Culture medium consisted of alpha-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FBS), 100 U/ml penicillin and 100μg/ml Streptomycin sulfate, respectively. Experiments were conducted in an incubator at 37 °C with a humidified atmosphere of 95% air and 5% CO_2, and cells were allowed to grow for 24 h. Semi-disks made of stainless steel, titanium and Ti-Cu alloy, sterilized by heating at 180 °C for 1h, were placed in plastic petri dishes 60 mm in diameter. A cell suspension of trypsinized subcultured V79 cells was diluted from 10^6 cells/ml to 10^2 cells/ml. Next, 6 ml medium and

2 ml cell suspension were seeded on the semi-disks in dishes so as to provide 200 cells per dish. Control dishes without metals were also made. After seeding, the dishes were gently shaken and cultured in the incubator. After 1 week, the medium was extracted, and the cells were fixed with 5 ml 10% formalin for 30 min, stained with 8 ml 0.15% methylene blue for an additional 30 min, washed thoroughly, and then dried. In the case of the semi-disks, differences in colony formation between areas covered by metal and plastic areas of dishes were qualitatively examined. Subsequently, colony formation in the dishes was compared with control dishes by counting the number of colonies.[42]

***In vivo* non-infected and non-inflammatory effects**

We performed pin insertion on 8 mature female Japanese white rabbits weighing from 2.5 to 3.0 kg. The rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight; Warner-Lambert, Morris Plains, NJ) and an intravenous injection of pentobarbital sodium (40-50 mg/kg body-weight; Abbott Laboratories, North Chicago, IL). A longitudinal skin incision was made on the lateral side of the right thigh, and the muscle and fascia were carefully split. Three stainless steel and pure titanium half pins, 2 mm in diameter (Howmedica, Geneva, Switzerland), were inserted into the lateral aspect of the right femur of each of four rabbits. Subsequently, we inserted three Ti-1% Cu alloy half pins, 2 mm in diameter, in the left side of four rabbits (two stainless steel and two pure titanium in the right side), and three Ti-5% Cu alloy pins in the left side of another four rabbits (two stainless steel and two pure titanium in the right side).

On post-operative day 14, the animals were euthanized and histology of the pin tract site was studied. Heparinized physiologic saline was perfused through the aorta, followed by perfusion with 4% paraformaldehyde solution in phosphate buffer (pH 7.4). The femurs were fixed for 48 h in the same solution. Next, all pins were removed and the femurs were decalcified with 10% EDTA solution and embedded in paraffin. A section was chosen as a representative section for each pin tract site. The specimens were sectioned at 5 μm thickness parallel to the bone axis and stained with hematoxylin-eosin stain. We inspected and graded for the presence of pin tract inflammation, abscess of pin tract, osteomyelitis, and inflammation around the tip. Inflammation of the pin tract and around the tip was scored from 0 to 2, where 0 = no, 1 = mild, 2 = severe. Pin tract abscess was scored from 0 to 2, where 0 = no, 1 = surface, 2 = deep. Pyogenic osteomyelitis was scored from 0 to 2, where 0 = no, 1 = mild infection, 2 = abscess formation (Table 1). For the Ti-Cu Alloys, stainless steel, and pure titanium, the average score of each category and total scores were calculated. When inflammation and infection were severe, the score became higher. Each metal was evaluated for a total of 12 pins.

In vivo biocompatibility

The biocompatibility of the titanium copper alloy was evaluated by comparing osteoid formation on the surface of the external fixation pin with a pin made of pure titanium. It was judged that bone conduction had occurred normally if the osteoid formation was similar to that observed for pure titanium.

Systemic toxicity

Blood samples were taken prior to surgery and at 14 days. Copper levels were measured in the whole body. We evaluated changes in copper ion concentration.

Statistical analysis

Statistical analysis was performed using Stat View 5.0 software. The transition of the number of bacilli between each metal was analyzed by repeated measured ANOVA. The scores of inflammation and infection were compared using Fisher exact tests. The blood levels of copper between pre-operative and post-operative samples were analyzed by *t* test.

RESULTS

In vitro antimicrobial properties

The titanium copper alloys remarkably inhibited colony formation of both *S. aureus* and *E. coli* compared with stainless steel and titanium. Figures 2 show the colonization of each bacillus at twenty-four hours. The number of colonies of Ti-Cu alloys obviously decreased at all time points (Fig. 3 and 4). *S. aureus* showed markedly less adhesion compared with *E. coli*. The Ti-5% Cu alloy significantly decreased microorganism adhesion. These results were statistically significant ($P < 0.05$). However, a significant difference was not observed for *E. coli* between titanium and Ti-1% Cu alloy.

In vitro cytocompatibility properties

In the experiment with metal, about 200 cells were equally and uniformly distributed on the surface of each dish. As to colony formation in the dish, stainless steel, titanium and the Ti-1% Cu alloy showed no difference in the number of colonies compared with control. No differences in colony formation between the metal area and plastic areas were observed. However, in the experiment with Ti-5% Cu alloy, colony formation on the semi-disk decreased slightly compared with the control suggesting surface toxicity of the metal (Fig. 5).

In vivo non-infected and non-inflammatory effects

Macroscopically, the reactive tissues around the pin were evaluated and 12 metal pins were scored. The average score for total points showed that Ti-1% Cu alloy accumulated the least points for all metals and the scores for "Pin tract inflammation" and "Abscesses of pin tract" were especially small (Table 2). This means that the inflammation and infection of Ti-1% Cu alloy was minimal. Statistical analysis showed that Ti-1% Cu alloy significantly inhibited inflammation and infection ($P < 0.05$). For the other metals, there was no difference of the degree of inflammation and infection.

In vivo biocompatibility

All inserted pins were evaluated histologically for osteoid formation. There were excellent osteoid formations on the surface of the Ti-1% Cu alloy pins as well as titanium pins suggesting that Ti-1% Cu alloys are a good osteoconductive material. The bone grew into the ditch of the screw and the osteoid formations continued to the opposite cortical layer from the front cortical layer. Conversely, osteoid formation was diminished in Ti-5% Cu alloy, with only partial osteoid formation (Fig. 6). Bone conduction was not possible on Ti-5% Cu alloy.

Toxicological results

Copper concentrations were determined in 8 rabbits with Ti-Cu alloy pins. The blood level of copper ions was 58.3 μg/ dl (range, 41-82) previous to surgery and 69.25 μg/ dl (range, 49-110) at the 14th day after the operation. The difference between the pre-operative and the post-operative blood copper values was not statistically significant (P=0.26).

DISCUSSION

Combined, these *in vitro* and *in vivo* results showed that Ti-Cu alloys had antibacterial activity. In particular, the Ti-1% Cu alloy showed promise as a biomaterial as there was no cytotoxic activity and it showed biocompatibility.

Post-operative infection is a severe complication in orthopedic surgery. When external fixation is used for surgery, implant-related infection can occur by the "contiguous spread route,"[43] that is, commensal skin bacteria pass subcutaneously along the surface of the pin towards the internal tissues. Because systemic antibiotics do not often provide effective treatment for infection of implanted pins, the coating of the implant that locally shows antibacterial activity is important. In order to reduce the incidence of implant-associated infections, several biomaterial surface treatments have been proposed. Many silver-coated medical materials have been reported, such as cardiac and urinary catheters, heart valves, dialysis units, and orthopedic devices.[26-31] However, it is ethically unacceptable to use silver-coated screws because this results in a significant increase in silver serum levels.[33]

In this study, pins with antibacterial activity were accomplished by making alloys of titanium and copper. Like silver, copper is a metal with known antibacterial activity against a broad spectrum of bacteria and a low incidence of resistance. Copper is a trace metal and an essential component of several enzymes. Because copper can be metabolized, it may be safer for the human body than silver. In fact, the copper can be excreted in bile and the chelating agent is used to treat even if becoming excessive. On the other hand, it is uncertain how silver is excreted from the body.

A number of authors have carried out *in vitro* studies to investigate the antibacterial effects of cooper ions against planktonic oral bacteria including Drake et al.[44] and Leonhardt and Dahlen.[45] However, there are few reports about orthopedic implants treated with copper. We made implants where copper coated the titanium pins as a pre-experiment and examined the antibacterial activity and cell toxicity. These copper-coated pins caused toxicity not only for the bacillus, but also for healthy cells suggesting that the copper-coated implants might elute copper ions voluminously. We were convinced that copper-coated implants were improper for use as a biomaterial. Next, we designed alloys of titanium and copper as a biomaterial, which did not show cytotoxic activity and therefore made the best use of the antibiotic properties of copper. These titanium copper alloys had significant antibacterial activity in this study.

Significant differences in bacterial adhesion on stainless steel, titanium and Ti-Cu alloy surfaces were observed in this study. The Ti-Cu alloy surfaces were observed to have significantly lower adhesion of *S. aureus* and *E. coli*. *S. aureus* showed markedly less adhesion compared with *E. coli*. Because orthopedic implants are commonly associated with *S. aureus* infections,[1-5] these results suggest that Ti-Cu alloys will be very effective for postoperative infections. It was reported that the biological performance of copper ions is dose-dependent just like silver.[46] In our results, the Ti-5% Cu alloy significantly decreased microorganism adhesion compared with Ti-1% Cu alloy. However, antimicrobial activity was also well obtained with only Ti-1% Cu alloy.

The present toxicological evaluation method for biomaterials by colony formation of V79 cells is suitable as a screening test for biomaterials. It has the advantages of: (1) yielding accurate and reproducible survival rates, (2) allowing direct contact between materials and cells, even with solid opaque materials, (3) allowing general observation of whether the cytotoxicity is caused by chemical or physical factors, and (4) being easy to perform and to evaluate.[42] Stainless steel and titanium have clinical applications in the field of orthopedic surgery. In our experiment, these materials showed no difference from controls in colony formation or cytotoxicity. The Ti-1% Cu alloy also had good biocompatibility because colony formation was observed in either the semi-disk metal area or the plastic part of the dishes. If both areas were colony-free, some cytotoxic chemical substance must have been released. If the physical properties, such as roughness or surface energy of the materials, affected colony formation then one would observe no colonies on the material itself, but normal colonies on the plastic part of the dishes. This was found for the Ti-5% Cu alloy as decreased colony formation was observed only on the semi-disk area. The cytotoxicity of the copper becomes visible due to direct contact of the cell with the copper surface. Therefore, it seems that the action of copper is concentration-dependent and only exerts its effect when the bacillus and the cell come into contact with the surface of the Ti-Cu alloy. It was reported that the antibiotic properties of copper only appear after contact of a fungus body with the surface of copper.[47] Therefore, Ti-1% Cu alloy can become an excellent biomaterial as it is less biologically toxic than Ti-5% Cu alloy and shows excellent antibacterial activity.

In the present animal experiment, the Ti-1% Cu alloy resulted in a significantly reduced infection and inflammation rate. It is reported in the literature that inflammation was graded for the presence of erythema and purulent discharge, and mechanical anchorage was graded manually.[8,26] In our study, the pin sites were histologically inspected and graded for inflammation and infection (Table 1). If inflammation and infection were most severe, the score would be 8 points. The average score for the Ti-1% Cu alloy was 3.08, lower than that of stainless steel or pure titanium. Deep abscess formation was only observed in two of the 12 Ti-1% Cu alloy pins. For other metals, deep abscess formation was shown in the more than half of the pins. Half of the Ti-1% Cu alloy pins did show some areas of inflammation but inflammation was also found around pins made from the other metals. Osteomyelitis was seen in pins made of the Ti-5% Cu alloy. Inflammation and infection were beyond restraint in the Ti-5% Cu alloy pins. It seems that the cytotoxic activity exceeded the antibacterial activity, considering the cytotoxicity examinations of Ti-5% Cu alloy in the *in vitro* studies. Therefore, a surrounding necrotic tissue increased and infection was caused there.

In biomaterials science, osteoconduction means growth of bone on the surface of a foreign material. Osteoconduction depends not only on biological factors, but also on the response to a foreign material. The osteoconductive response is necessary for successful osteointegration.[48] The biocompatibility of the implant was evaluated by osteoconduction because bone conduction is often observed with biomaterials that have biocompatibility such as titanium. In our study, titanium also had good osteoid formation i.e. good osteoconduction. However, Albrektsson reported that bone conduction is not possible on certain materials such as copper and silver.[49] This study found that the Ti-1% Cu alloy had bone conduction because excellent osteoid formation was observed. Having combined the biocompatibility of titanium with a small amount of copper might have led to the excellent osteoid formation observed.

Excess copper within cells after *in vivo* implantation has been found in hepatocytes.[50] In Wilson's disease and Menke's syndrome, the accumulation of copper is associated with cytotoxic effects.[51,52] Unfortunately, as the bactericidal properties of copper ions increase in the blood, so do the negative effects on mammalian cells. In this study, the difference between pre-operative and post-operative blood copper levels was not statistically significant. The Ti-Cu alloy chiefly has local effects and may be also safe for the whole body. The Ti-Cu alloy is suitable as a biomaterial.

As for the Ti-Cu alloy, it is expected the strength weaken by the content of copper and the less the concentration of copper is, the less the cell toxicity is. Therefore, we try to examine whether to show the antibacterial activity in the density of 1% or less in the future. Moreover, we are going to perform a mechanical test of the alloy and to make the best titanium-copper alloy clinically.

CONCLUSION

The findings of this study suggest that Titanium-Copper alloys have antimicrobial activity and substantially reduce the incidence of pin tract infections. The Ti-1% Cu alloy has both antibacterial and biocompatible properties and shows particular promise as a biomaterial.

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Table 1

Score of inflammation and infection by histological analysis.

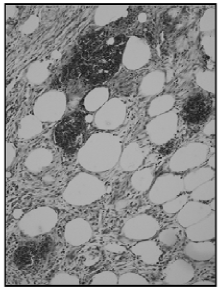
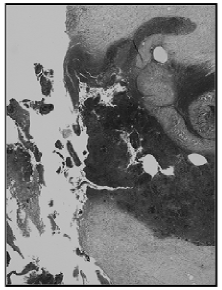
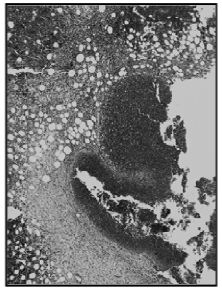
	Pin tract inflammation	Abscess of pin tract	Osteo-myelitis	Inflammation around the tip
+	Severe; 2	Deep; 2	Abscess; 2	Severe; 2
	Slight ; 1	Surface; 1	Slight ; 1	Slight ; 1
-	0	0	0	0

(points)

Table 2

Average score of inflammation and infection (n=12)

Asterisk (*) indicates significant difference at P<0.05

	Pin tract inflammation	Abscess of pin tract	Osteo myelitis	Inflammation around the tip	Total Score
					n=12
St	1.17	1.50	1.42	0.83	4.92
Ti	1.33	1.83	1.17	0.08	4.42
1% Cu	<u>0.67</u>	<u>0.75</u>	<u>1.25</u>	<u>0.42</u>	<u>3.08</u>
5% Cu	1.33	1.25	1.67	0.92	5.17

* P < 0.01

Prevention of Pin Tract Infection with Titanium-Copper Alloys

"**Figure Captions**"

Figure. 1 Antibacterial assessment; Japanese Industrial Standards modified method.

Figure. 2-a Representative photo of colonization by *S. aureus* at 24h.

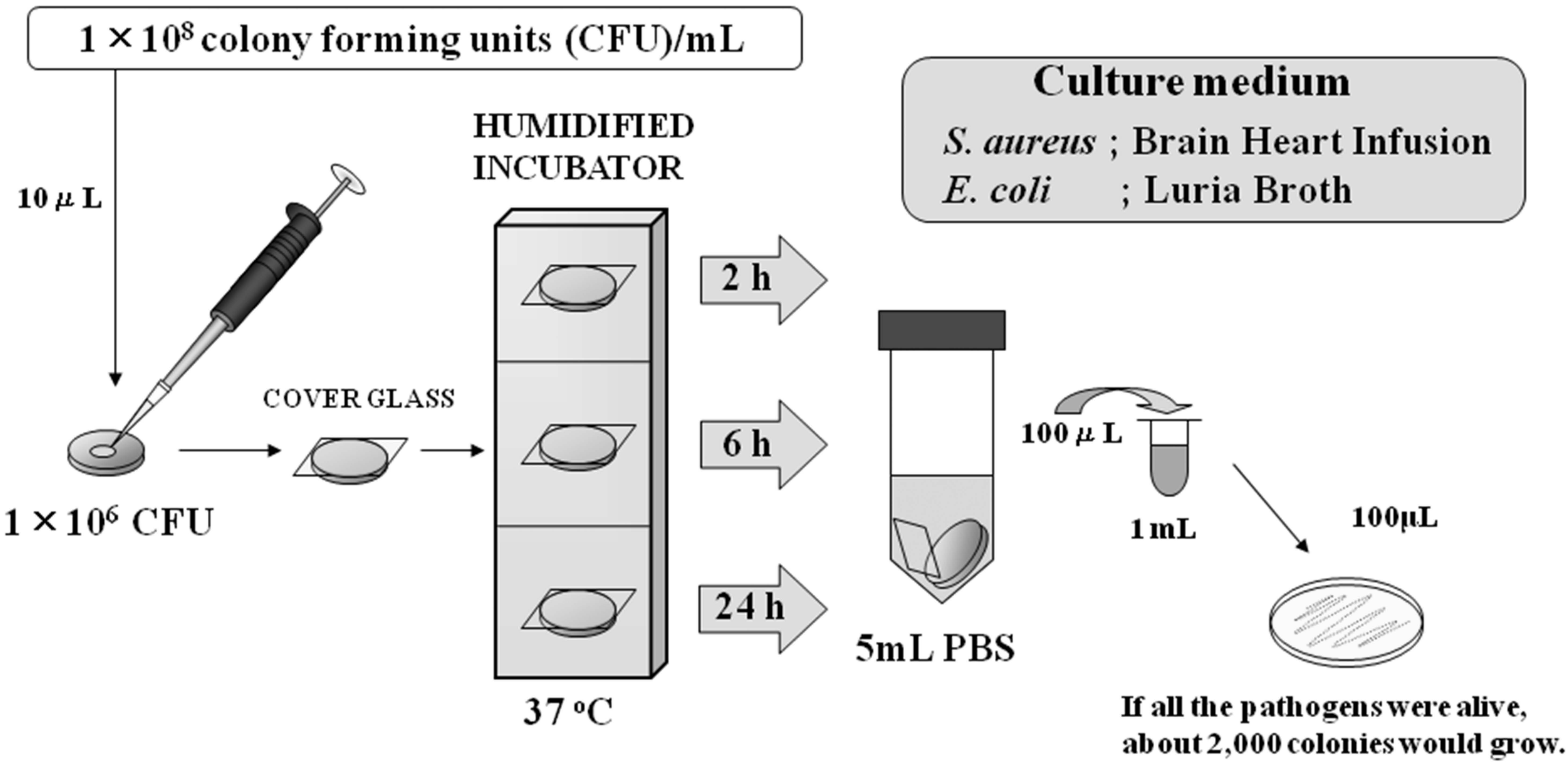
2-b Representative photo of colonization by *E.coli* at 24h.

Figure. 3 Changes in number of colonies of *S. aureus*.

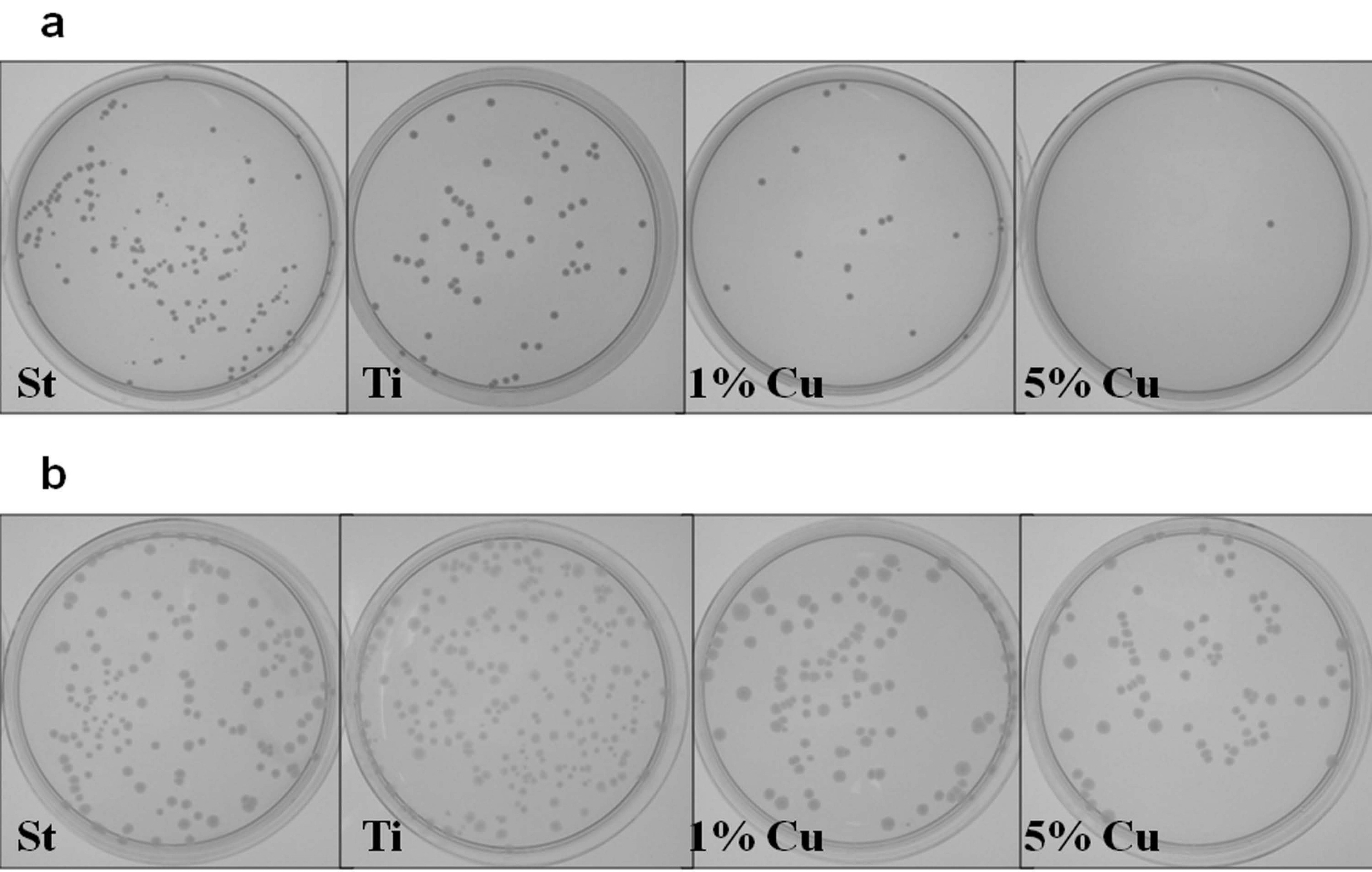
Figure. 4 Changes in number of colonies of *E. coli*.

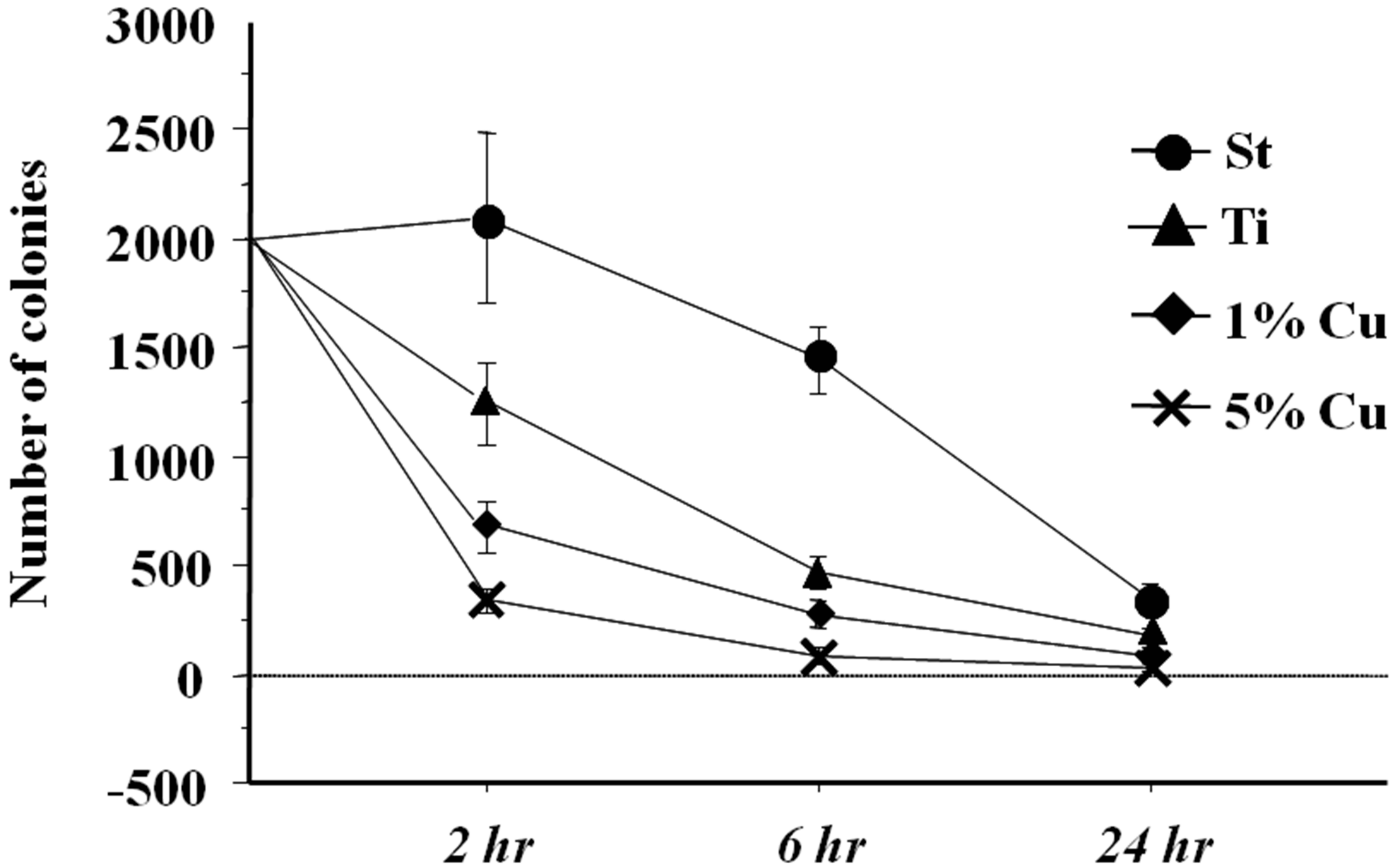
Figure. 5 Colony formation on the metal semidisks. Colony formation on the Ti-5% Cu alloy semi-disk decreased slightly compared with the control.

Figure. 6 Osteoid formation on the surface of a Ti-Cu alloys. There were excellent osteoid formations on the Ti-1% Cu alloy pin, but only partial osteoid formations on the Ti-5% Cu alloy pin.

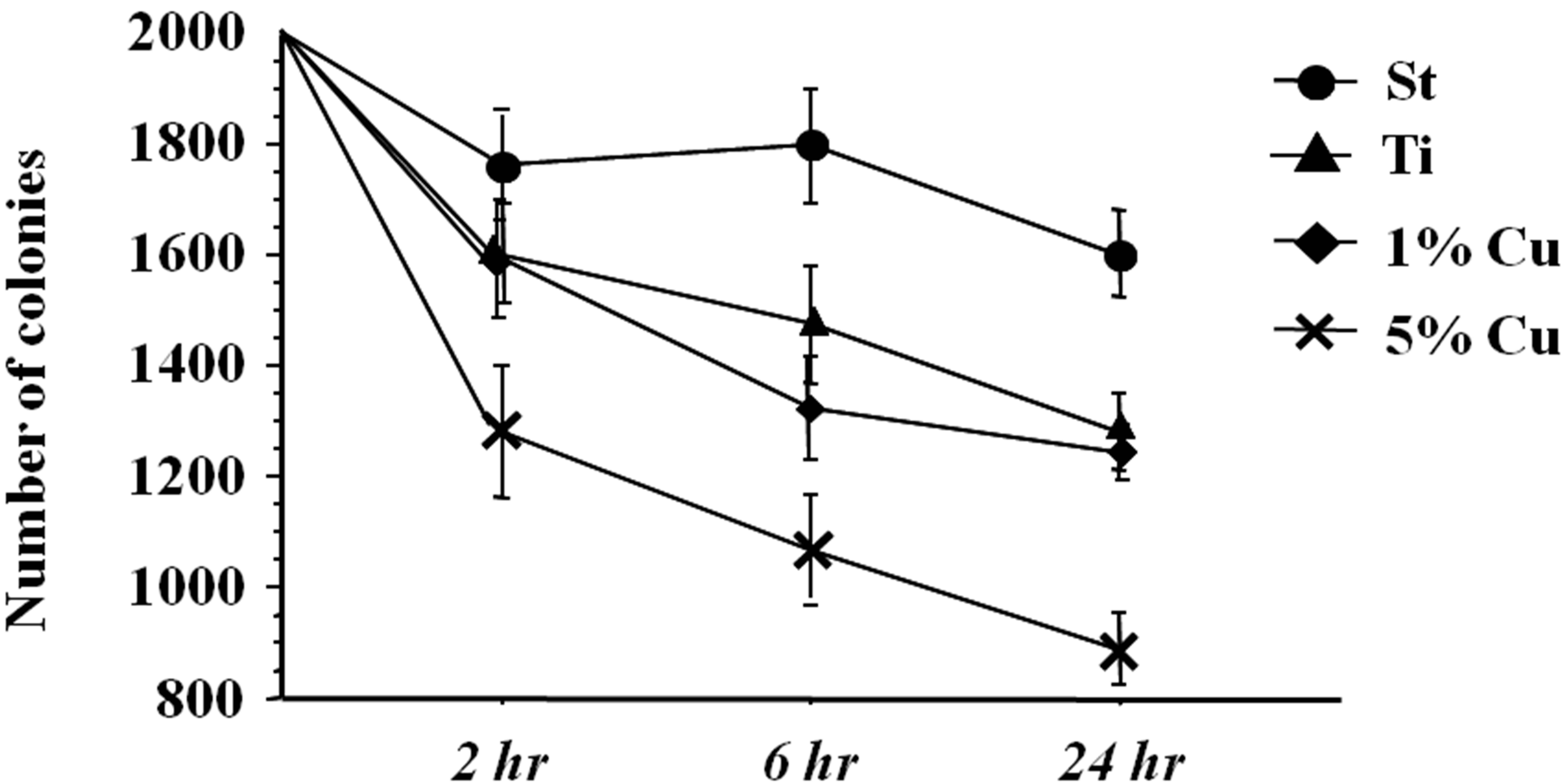


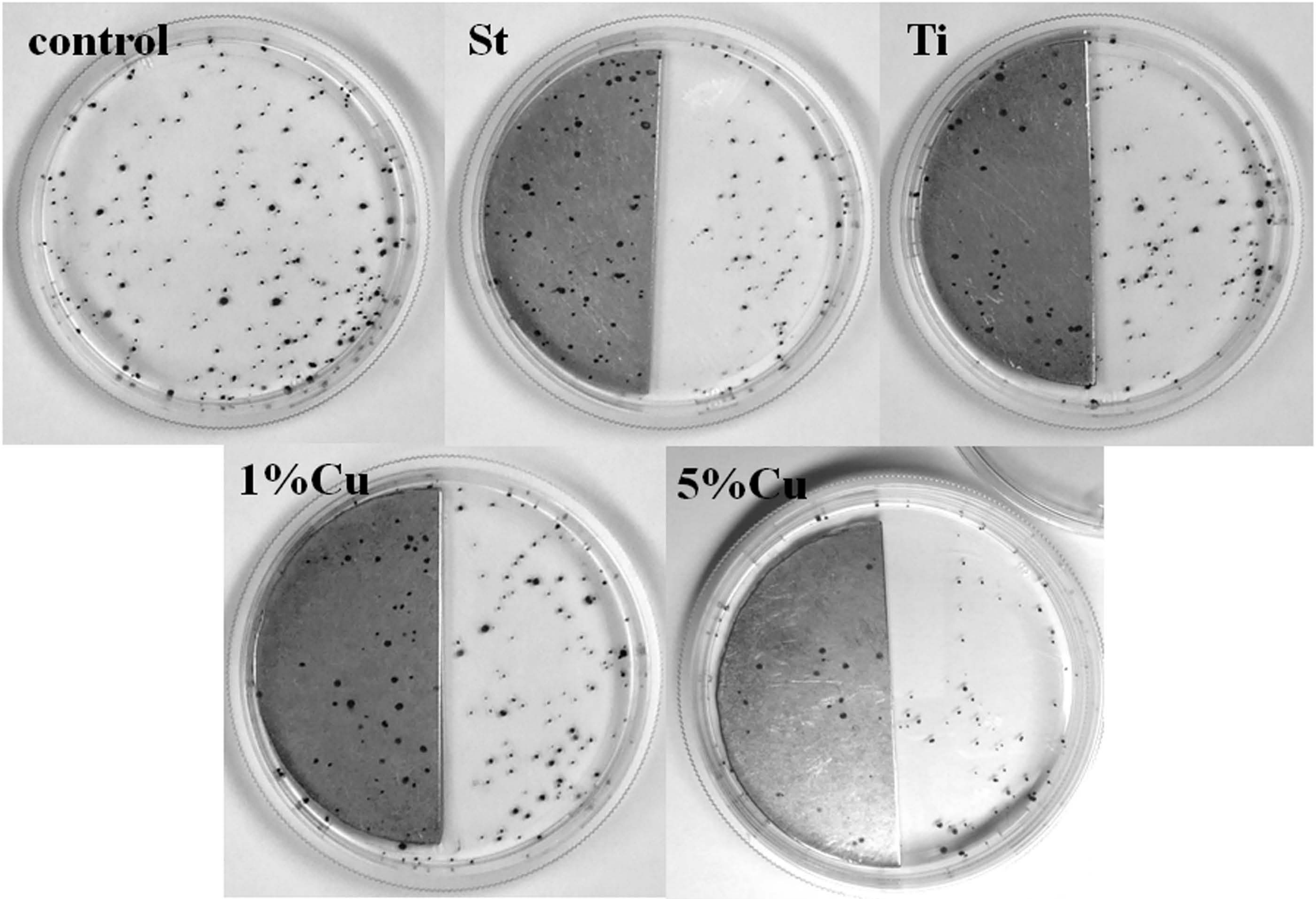
1×10^8 colony forming units (CFU)/mL
Culture medium
S. aureus ; Brain Heart Infusion
E. coli ; Luria Broth
HUMIDIFIED
INCUBATOR
10 μ L
COVER GLASS
1×10^6 CFU
2 h
6 h
24 h
5mL PBS
100 μ L
1 mL
100μL
37 °C
If all the pathogens were alive,
about 2,000 colonies would grow.

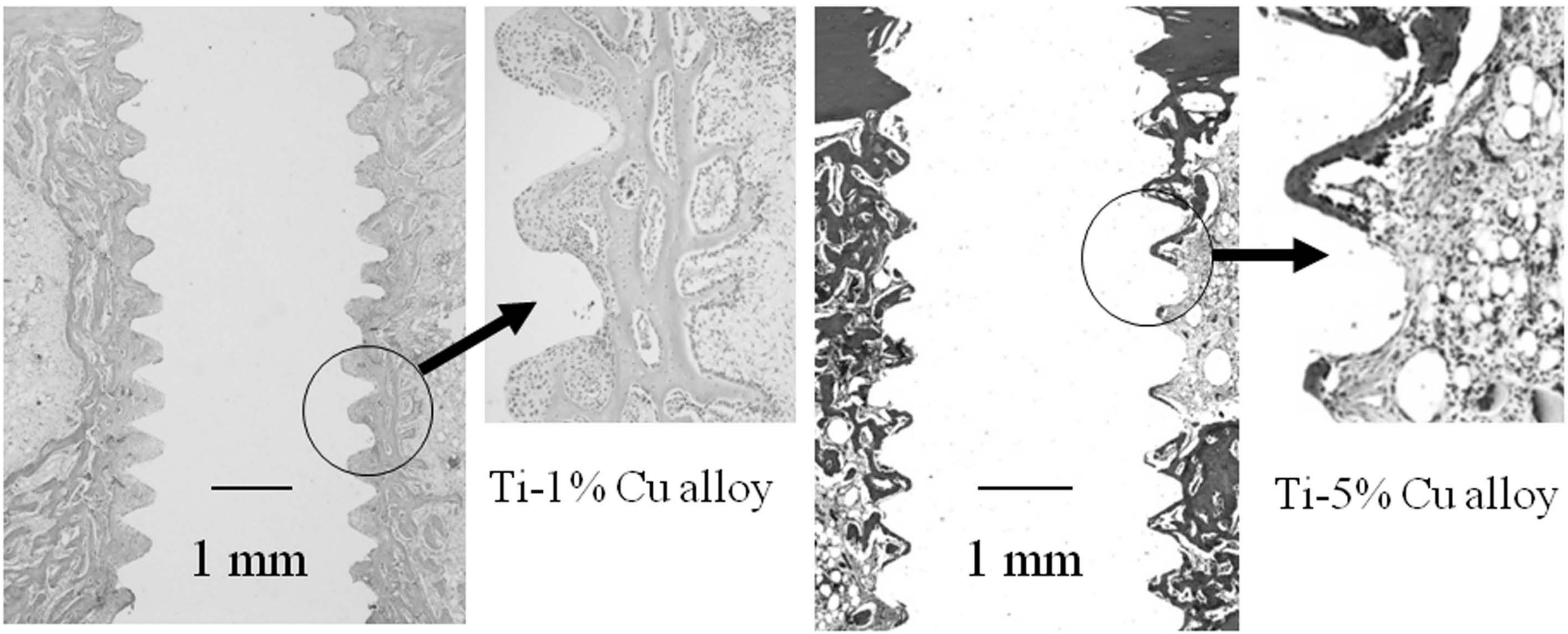

a
St
Ti
1% Cu
5% Cu
b
St
Ti
1% Cu
5% Cu



3000
2500
2000
1500
1000
500
0
-500
Number of colonies
St
Ti
1% Cu
5% Cu
2 hr
6 hr
24 hr


Number of colonies
2000
1800
1600
1400
1200
1000
800
2 hr
6 hr
24 hr
St
Ti
1% Cu
5% Cu


control
St
Ti
1%Cu
5%Cu


1 mm
Ti-1% Cu alloy
1 mm
Ti-5% Cu alloy