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# Antibacterial effect of 317L stainless steel contained copper in prevention of implant-related infection in vitro and in vivo

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**Abstract** Bone and intramedullary bacterial infections are one of the most serious complications of the surgical repair of fractures. To reduce the incidence of implant-related infections, several biomaterial surface treatments with integrated antibiotics, antiseptics, or metal ions have been developed for implants. In this study, we evaluated the antibacterial activity and biocompatibility of 317L stainless steel containing 4.5% copper alloy (317L–Cu) in vitro and in vivo using an animal model. Common pathogens of implant-related infections are *Staphylococcus aureus* and *Escherichia coli*, which were injected into implant materials to study their antimicrobial potential. We compared antimicrobial potential of 317L–Cu with 317L stainless steel (317L) and titanium (Ti–6Al–4V) alloys as controls. Compared with controls, 317L–Cu materials inhibited colonization by both bacteria in vitro and in vivo. Compared with 317L and Ti–6Al–4V controls, 317L–Cu showed no significant difference in colony formation of osteoblast-like cells on metal surfaces after 72 h of incubation in vitro. Metal screws containing these materials were also made for our vivo study in a rabbit model. Tissue-implants were analyzed for infection and inflammatory changes by hematoxylin–eosin staining of implants in bone. The screw tract inflammation and infection of 317L–Cu was minimal, although some inflammatory cells gathered at acutely infected sites. In addition, after materials had been implanted for 14 days in vivo, the expression of insulin-like growth factor-1 (IGF-1) in osteoblasts around 317L–Cu screws tracts had increased compared with 317L and Ti–6Al–4V controls. Overall, 317L–Cu demonstrated strong antimicrobial activity and biocompatibility in vitro and in vivo and may be used as a biomaterial to reduce implant-related infections.

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## 1 Introduction

Bone and intramedullary bacterial infections are serious complications of the surgical repair of long-bone fractures [1, 2]. Common pathogens of implant infections are *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) [3–7]. It is well known that the rate of implant-associated infections rises in proportion to the length of time that the implants remain in the body [8]. Once biofilms form on implant surfaces, infection is difficult to treat because the bacteria are resistant to phagocytosis and antibiotics [9]. Current treatment for implant-related infection includes a meticulous debridement of the open fracture, and high does of antibiotics [10, 11], which can often result in toxic side effects. In addition, systemic antibiotics are not often effective for implant-related infections, as local antibacterial activity is more effective [2].

To reduce the incidence of implant-associated infections, implant biomaterial surface treatments containing antibiotics, antiseptics, or metal ions have been developed [12–14]. In particular, sliver-coated materials have been studied to prevent local infections [15, 16]. However, silver has toxic effects in eukaryotic cells and is not used as an antibacterial material due to this toxicity but also due to a low biocompatibility. Stainless steel and titanium alloys have been used for orthopedic surgery [1] and have been

certified as biomaterials but it is not known if they possess antibacterial activity in vivo. Copper has been proposed as an antibacterial metal. In vivo, cooper has demonstrated excellent antibacterial properties against numerous bacteria such as *S. aureus* [17], *E. coli* [18], *Salmonella enterica* and *Campylobacter jejuni* [19], *Clostridium difficile* [20], and *Mycobacterium tuberculosis* [21].Copper is also a trace metal in living organisms, which need low level copper as cofactors for metalloproteins and enzymes [19]. Insulin-like growth factor-1 (IGF-1) is a single-chain polypeptide hormone composed of 70 amino acids and secreted by human liver cells. It plays an important role in regulating growth and development in man. IGF-1 stimulates has growth-promoting effects on almost every cell in the body, especially bone and cartilage. As a major cartilage anabolic factor, IGF-1 can keep cartilage cells in dynamic balance during normal metabolism of cartilage, but also promote cartilage healing [22]. In addition, IGF-1 can direct adipose stem cells to develop into cartilage cell sand can significantly stimulate the synthesis of DNA and type-1 collagen of tendon cells to regulate the function of bone cells both by cell signaling (paracrine) and autocrine (local) mechanisms. IGF-1 promotes the production, differentiation, and gathering of osteoblasts and inhibits cell apoptosis [23]; it also increases bone matrix deposition by stimulating DNA transcription and collagen synthesis while inhibiting collagen degradation. Studies have shown that the expression of IGF-1 in osteoblast-like cells are mediated and enhanced by $Cu^{2+}$ [23]. However, there has been little published data on antimicrobial efficacy and tissue toxic effects of copper in animal infection models.

317L stainless steel containing 3–4% molybdenum is preferred to type 316L, which contain 2–3% molybdenum in applications requiring enhanced pitting and general corrosion resistance. In this study, we characterized the antimicrobial properties and biocompatibility of an austenitic antibacterial stainless steel containing 4.5% copper (317L–Cu alloy) in vitro and in vivo using a surgically implanted animal model, comparing it to two standard non-copper alloys used for implants.

## 2 Materials and methods

### 2.1 Implants

In this study, we used three implant materials: (1) austenitic stainless steel (00Cr19Ni13Mo3, 317L), (2) austenitic antibacterial stainless steel containing 4.5% copper (00Cr19Ni13Mo3-4.5 wt pct Cu, 317L–Cu), and (3) a titanium alloy (Ti–6Al–4V). All implant materials were prepared by the Institute of Metal Research, Chinese Academy of Sciences, Shenyang, China. For in vitro antimicrobial research and cell toxicity studies, samples were cut into slices (length 10 mm, thickness 1 mm). For in vivo studies, implant screws, 3 mm (diameter) by 10 mm (length), were implanted into male Japanese white rabbit (from China Medical University) femurs. The samples were mechanically polished with wet silicon carbide (SiC) paper up to 1,200 grit and then finely polished with 1.5 ml aluminum oxide ($Al_2O_3$) powder paste for optical observation.

### 2.2 Bacteria culture and bacteriological properties

Gram-positive *S. aureus*, strain ATCC25923 and gram-negative *E. coli*, strain ATCC25922 were used for analyzing infectious behavior. Bacterial inocula were prepared in cultures grown overnight in Luria–Bertani (LB) medium containing 10 g/l peptone, 5 g/l NaCl, and 5.0 g/l beef extract. The pH was adjusted to between 7.0 and 7.2 using 0.5 M NaOH solution. Bacteria were resuspended to a final density of $2 \times 10^6$ CFU/ml. The inoculum was incubated with metal materials at 37°C for 6, 12, 24, and 48 h. After incubation, half of the cultured materials were washed three times using 3 ml phosphate-buffered saline (PBS). Each incubated system was fixed in 2.5% glutaraldehyde and dehydrated in alcohol (50, 80, 90, 95, and 100%, 15 min each). Samples were then examined by scanning electron microscopy (SEM, S-3400N, HITACHI, Japan) to examine biofilm formation. Other cultures were washed using 0.9% saline solution three times and bacterial viability measured with 200 µl of LIVE/DEAD backlight bacterial viability stain containing SYTO 9 and propidium iodide stains (1:1), which was added onto each material away from light for 15 min. After staining, all samples were observed by confocal laser scanning microscope (CLSM, Olympus Fv10i from Japan) to count dead colonies on each metal piece. The difference in the numbers of dead bacteria on each metal was statistically analyzed.

### 2.3 Biocompatibility tests in vitro

Human osteoblast-like cell line MG63 was cultured in flasks in Dulbecco's Modified Eagle Medium (DMEM) without phenol red with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin at 37°C, 5% $CO_2$. We sterilized 317L, Ti–6Al–4V, and 317L–Cu materials by heating them to 180°C for 30 min, and placed in plastic petri dishes with 12 wells. A cell suspension of trypsinized subcultured MG69 cells was diluted from $10^6$ to $10^5$ cells/ml. Next, 2 ml of cell suspension ($2 \times 10^5$ cells/dish) was placed onto each material in the dishes. Dishes without metal materials were also made as controls. After seeding the cells,the dishes were incubated for 24, 48, and 72 h. Cultured cells were observed and imaged by light

microscopy to determine which materials altered the growth of MG69 cells compared with the controls. At the end of these culture experiments, we prepared additional cultures as described above for SEM examination to determine if cells adhered onto the metals. At the same time, the nutrient medium was extracted and analyzed by an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, OPTIMA3000 from USA) to determine the amount of $Cu^{2+}$.

### 2.4 Cell viability assay

The 317L, Ti–6Al–4V, and 317 L–Cu alloys were placed in sterile pipes with alpha-minimal essential medium supplemented with 10% FBS with surface area of alloy samples proportionate with culture medium 3 $cm^2$/ml. Pipes were then incubated at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$, divided equally for incubation periods of 24, 48, and 72 h. Levels of viability were determined by a MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide] assay. Briefly, $1 \times 10^5$ cells in culture media were put in pipes with 317L, Ti–6Al–4V, and 317L–Cu alloys, and were plated onto 96-well tissue culture plates. MTT solution (10 μl) was added to each well and mixed by shaking briefly on an orbital shaker and then incubated at 37°C for 4 h. The MTT solution was removed and 200 μl dimethyl sulfoxide (Sigma-Aldrich Corp) was added to each well with pipetting up and down several times to dissolve the formed formazan dyes. Blank cultures (no metals) were used as a control. The absorbance (optical density, OD) was measured using a reference wavelength of 630 nm to obtain a sample signal (OD 570–630). MG63 cell proliferation was calculated by the formula:

$$\text{mean OD/blank control mean OD} \times 100.$$

### 2.5 Surgical procedure

We used male Japanese White rabbits (from China Medical University) weighing from 2.5 to 3.0 kg for all in vivo assays. All rabbits were randomly divided into five groups : (1) 10 (control) rabbits without implants but with surgical sites contaminated with bacteria, (2) 10 rabbits were implanted with 317L–Cu screws without bacteria inoculum, (3) 10 rabbits implanted with 317L–Cu screws that were immerged into bacteria inoculum ($10^5$ CFU/ml), (4) 10 rabbits implanted with 317L stainless steel screws immerged into bacteria inoculum ($10^5$ CFU/ml), and (5) 10 rabbits implanted with Ti–6Al–4V screws immerged into bacteria inoculum.

For analyzing antimicrobial effects, implants were exposed to $10^5$ CFU/ml each of gram-positive *S. aureus* (strain ATCC25923) and gram-negative *E. coli* (strain ATCC25922) for 6 min. Rabbit right flank over the femur was shaven and sterilized with iodine solution. Prior to surgery, rabbits were anesthetized by peritoneal injection of 10% chloral hydrate (0.5 ml/kg body weight), combined with an intramuscular injection of amobarbital sodium (0.2 ml/kg). A few minutes after animals were completely anesthetized, the right femur was surgically exposed, and a 2.5-mm hole was drilled into the femur using a hand-held drill. Screws of each alloy were implanted into the holes and the surgical wound was sutured in layers.

### 2.6 Bacteriological analysis and cytocompatibility in vivo

After 5 or 14 days post surgery, rabbits were sacrificed by pentobarbital injection into ear vein and the femurs exposed to remove the implanted screws. Swabs were aseptically taken from the screws, screw tracts, and screw head as well as the soft tissue in front of each screw. The swabs were placed into brain–heart infusion (BHI) culture median culture plates, which were incubated at 37°C for 24 h. The reaction of bacteria to 317L–Cu was compared with the reaction to 317L as controls. At the end of the incubation period, the number of colonies on each plate was counted and the total viable CFU load was determined and expressed as CFU/ml. All metal implants were tested using the same methods as described for in vitro experiments before the biofilms were observed by SEM.

### 2.7 In vivo noninfected and noninflammatory effects

The bone segments used for histological testing were fixed in 4% paraformaldehyde, decalcified in ethylenediaminetetraacetic acid (EDTA), dehydrated in an alcohol series, and embedded in paraffin. A section was chosen as a representative section for each screw 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 each bone section for inflammation, abscess, osteomyelitis, and inflammation in the tissues around the screw tract.

### 2.8 IGF-1 expression by immunohistochemistry

Because $Cu^{2+}$ is known to induce the release of IGF-1 [23], in our study, we used immunohistochemistry to examine the formation of IGF-1 in rabbit bone sections. For immunohistochemistry of each section, we used antibodies against IGF-1 (polyclonal rabbit anti-human, 1:100), SABC kit (Boster Biotechnical Co, Ltd, WuHan, China)

and DAB (tetrahydrochloride) detection. For each antigen, only cells that were stained brown were labelled positive. For each sample, the expression of IGF-1 was measured in at least three tissue sections taken. As a negative control, the polyclonal rabbit anti-human antibody was replaced with goat IgG. After extensive washing in PBS, the appropriate biotin labelled secondary antibody was incubated for 2 h at a concentration of 1 μg/ml in PBS containing 1% bovine serum albumin. Under microscopy, positive fields were selected at random. Positive and negative cells were counted in each field, and the number of positive cells was divided by the total number of cells (the sum of positive and negative cells) to calculate the positive ratio in each field.

### 2.9 Systemic toxicity

To measure copper levels systemically, blood samples from rabbits were taken before surgery and at 0, 5, 14 days post surgery. We evaluated changes in copper ion concentration in the blood by an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, OPTIMA 3000 from USA) to determine the amount of $Cu^{2+}$.

### 2.10 Statistical analysis

Statistical analysis was performed using SPSS software. The transition of the number of bacteria between each metal and MTT assay (by OD) were analyzed by analysis of variance (ANOVA). The blood levels of copper between preoperative and postoperative samples were analyzed by the Student's *t* test.

## 3 Results

### 3.1 In vitro antimicrobial properties

Analysis by confocal laser scanning microscopy (Fig. 1) as well as number of CFU in culture (Figs 2) showed that compared with the 317L alloy, the 317L–Cu alloy clearly inhibited colony growth of both *S. aureus* and *E. coli* at 24 h (Figs. 1, 2). Quantitative analysis of cultures containing both alloys (Fig. 2) showed that at all time points (6, 12, 24, and 48 h), 317L–Cu inhibited bacterial growth of both bacteria while cultures containing 317L continued a stable growth. By 48 h, the cultures containing 317L–Cu had decreased bacterial growth to nearly zero colonies. SEM images of bacterial colonization showed that the colonization formation and adhesion of bacterial biofilm was markedly less with some dead bacteria visible on the 317L–Cu alloy compared with 317L or Ti–gAl–4V alloys (Fig. 1).

### 3.2 In vitro biocompatibility properties

SEM images of MG63 osteoblast-like cells on different alloy surfaces are displayed in Fig. 3. 317L–Cu, 317L, and Ti–6Al–4V alloys showed no difference in the number of live cells after incubation at 24 h. Compared with controls, the MG63 cells cultured with 317L–Cu appeared to have increased growth, which was observed between the metal and plastic areas.

Cell viability was measured by the MTT assay in which increased absorbance (optical density or OD) indicates increased viability. Compared with blank control cultures (common culture media), viability of MG63 cells with 317L–Cu alloy was not significantly different ($P > 0.05$, Fig. 4), indicating that cultures with 317L–Cu alloy had significant cellular growth compared with controls.

### 3.3 In vivo antimicrobial properties of alloys

At 5 and 14 days after surgery, rabbits were sacrificed and the femur screws removed for testing. In the negative control groups (no bacterial but 317L–Cu alloy screws), all results were negative; in the positive control group (no implants but added bacteria), cultures from soft tissues around screw holes showed growth in 9/10 cultures. In tests with bacteria-impregnated screws, the swabs from soft tissues as well as swabs from screws, screw head, and screw tracts were positive for bacterial growth from 317L and Ti–6Al–4V alloys but negative for 317L–Cu, similar to the negative control. This was confirmed by direct examination of the cultures taken from screws impregnated with both *E. coli* and *S. aureus* 5 days post surgery (Fig. 5).

### 3.4 In vivo noninfected and noninflammatory effects

The tissues around the screws were evaluated macroscopically, and the soft-tissues and bone connected with the screws stained with hematoxylin–eosin were observed microscopically (Fig. 6). Macroscopic examination of bone and intramedullary tissues around 317L–Cu screws showed little to no inflammation or gross infection, whereas areas around 317L and Ti–6Al–4V screws showed exudation of inflammatory cells in intramedullary tissues. Microscopic examination of hematoxylin–eosin stained bone tissues around screws as well as intramedullary tissues around screws showed that screw tracts of 317L and Ti–6Al–4V were acutely and severely inflamed and acute infection areas had many inflammatory cells. By contrast,

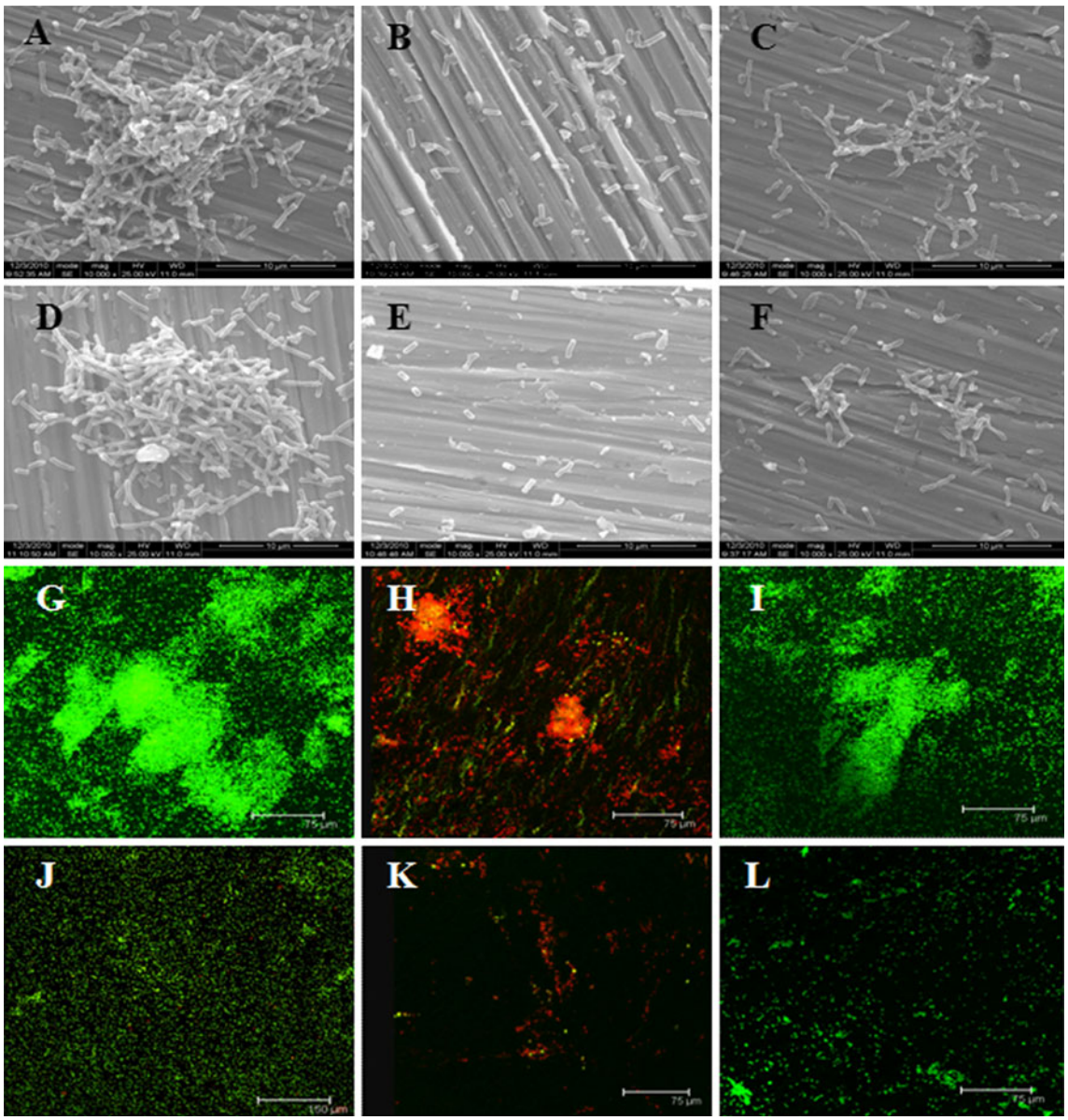


**Fig. 1** Scanning Electron Microscope (SEM) images of *E. coli* colonization on implant materials. **a** 317L cultured for 24 h; **b** 317L–Cu cultured for 24 h; **c** Ti–6Al–4V cultured for 24 h; **d** 317L cultured for 48 h; **e** 317L–Cu cultured for 48 h; **f** Ti–6Al–4V cultured for 48 h. Confocal Laser Scanning Microscopy (CLSM) images of *E. coli* and *S. aureus* cultured on implant materials for 24 h. **g** 317L cultured with *E. coli*; **h** 317L–Cu cultured with *E. coli*; **i** Ti–6Al–4V cultured with *E. coli*; **j** 317L cultured with *S. aureus*; **k** 317L–Cu cultured with *S. aureus*; **l** Ti–6Al–4V cultured with *S. aureus*. The *light grey* denotes living bacteria, while *dark grey* means dead bacteria

inflammation and infection of 317L–Cu screws was minimal (Fig. 6). In addition, the shape of the threads from 317L–Cu screws tended to be uniform and compact.

### 3.5 In vivo biocompatibility

At 5 and 14 days post surgery, inserted screws were evaluated histologically for osteoid formation. SEM images of implanted screws 5, 14 days post surgery (Fig. 7) showed strong osteoid formation on the surface of 317L–Cu screws, showing that the bone matrix had grown into the screw ditch compared with the surface of 317L screws, which showed weaker osteoid growth.

### 3.6 IGF-1 expression by immunohistochemistry

Immunohistochemistry analysis showed positive staining of IGF-1 in the bone tissues around 317L–Cu screws 14 days post surgery. By comparison, less IGF-1 positive staining was seen around the tissues surrounding implanted 317L or Ti–6Al–4V screws (Fig. 8).

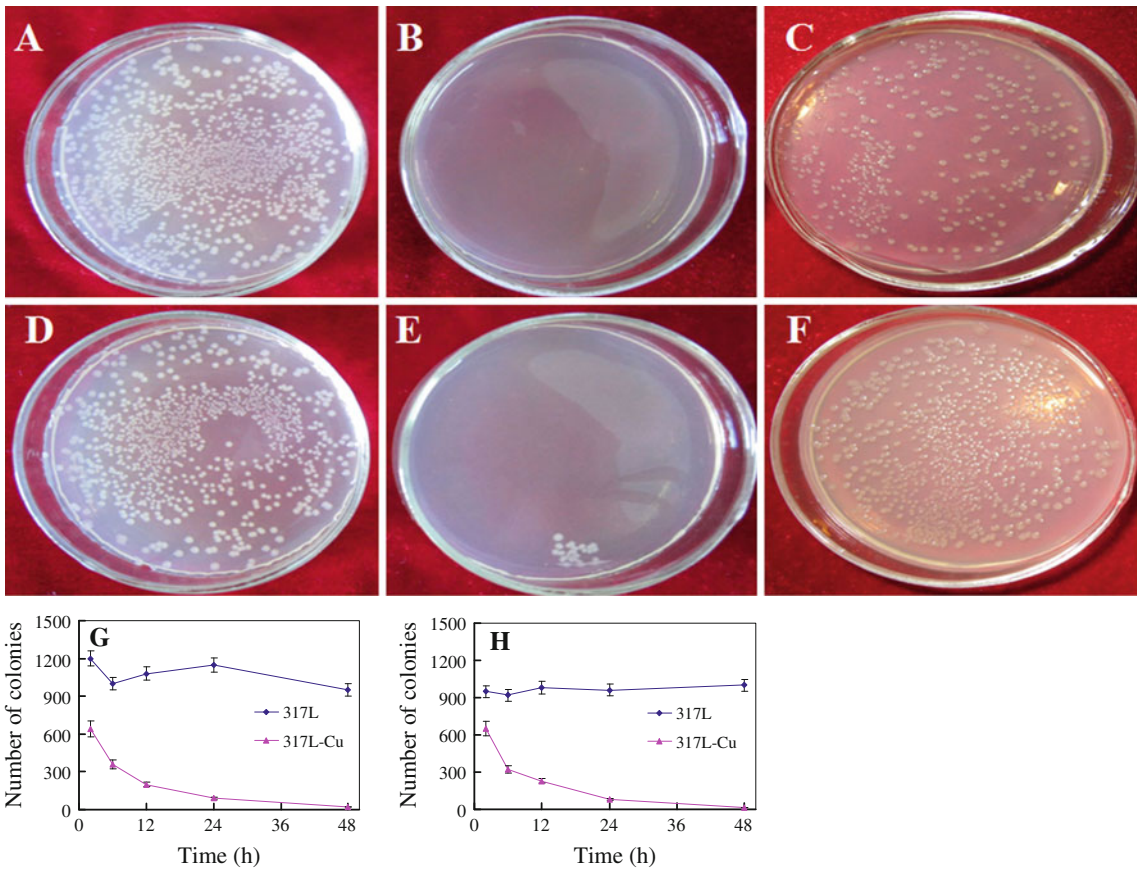


**Fig. 2** Petri dishes showing colonization of *E. coli* and *S. aureus* at 24 h. **a** 317L cultured with *E. coli*; **b** 317L–Cu cultured with *E. coli*; **c** Ti–6Al–4V cultured with *E. coli*; **d** 317L cultured with *S. aureus*; **e** 317L–Cu cultured with *S. aureus*; **f** Ti–6Al–4V cultured with *S. aureus*. Changes in number of colonies of *E. coli* (**g**) and *S. aureus* (**h**) at 6, 12, 24, and 48 h. Because 317L cannot kill bacteria, colonies of both *E. coli* and *S. aureus* continue to grow; 317L–Cu contains antimicrobial properties, and colonies decrease at all time points ($P < 0.05$)

### 3.7 $Cu^{2+}$ in media supernatant

The concentration of $Cu^{2+}$ was determined in the supernatant of media cultured with different alloys and the plasma fraction from centrifugation collected from rabbits before surgery and during sacrifice. $Cu^{2+}$ concentrations in the 317L–Cu media supernatant kept in a stable level after incubation at 24, 48, and 72 h (about 1.00 ppm). Rabbit $Cu^{2+}$ blood levels were 52.8 ± 6.8 μg/dl prior to surgery and 53.3 ± 5.6 μg/dl on the 5th day post surgery, and 52.9±4.8 μg/dl on 14th day post surgery. The difference between the preoperative and the postoperative blood copper value was not statistically significant ($P > 0.05$). Thus, $Cu^{2+}$ blood concentrations in rabbits with embedded 317L–Cu screws were physiologically stable over a 14-day period.

## 4 Discussion

Results from in vitro and in vivo assays clearly show that 317L–Cu alloys had significant antibacterial activity and exhibited good biocompatibility, as it was not cytotoxic to bone cells. Therefore, our findings support the use of 317L–Cu alloys for localized antimicrobial prophylaxis of implantation-related infections.

In our study, we characterized the antibacterial and biocompatibility of copper implants in vitro, and we also manufactured bone screws that were embedded in an animal model to study these properties in vivo. Although it is known that copper has antimicrobial properties, in high concentrations, $Cu^{2+}$ is highly toxic physiologically. We therefore needed to produce copper-containing biomaterials that suppress bacterial growth without being cytotoxic.

Swab cultures and SEM analysis from our in vitro antibacterial studies showed that the 317L–Cu surfaces had significantly lower adhesion of *E. coli* and *S. aureus*. Because implants are commonly contaminated by *S. aureus*, these results suggest that 317L–Cu alloy, which prevent bacterial adhesion or kill bacteria shortly after adhesion, would be an effective implant material to reduce the possibility of postoperative infection.

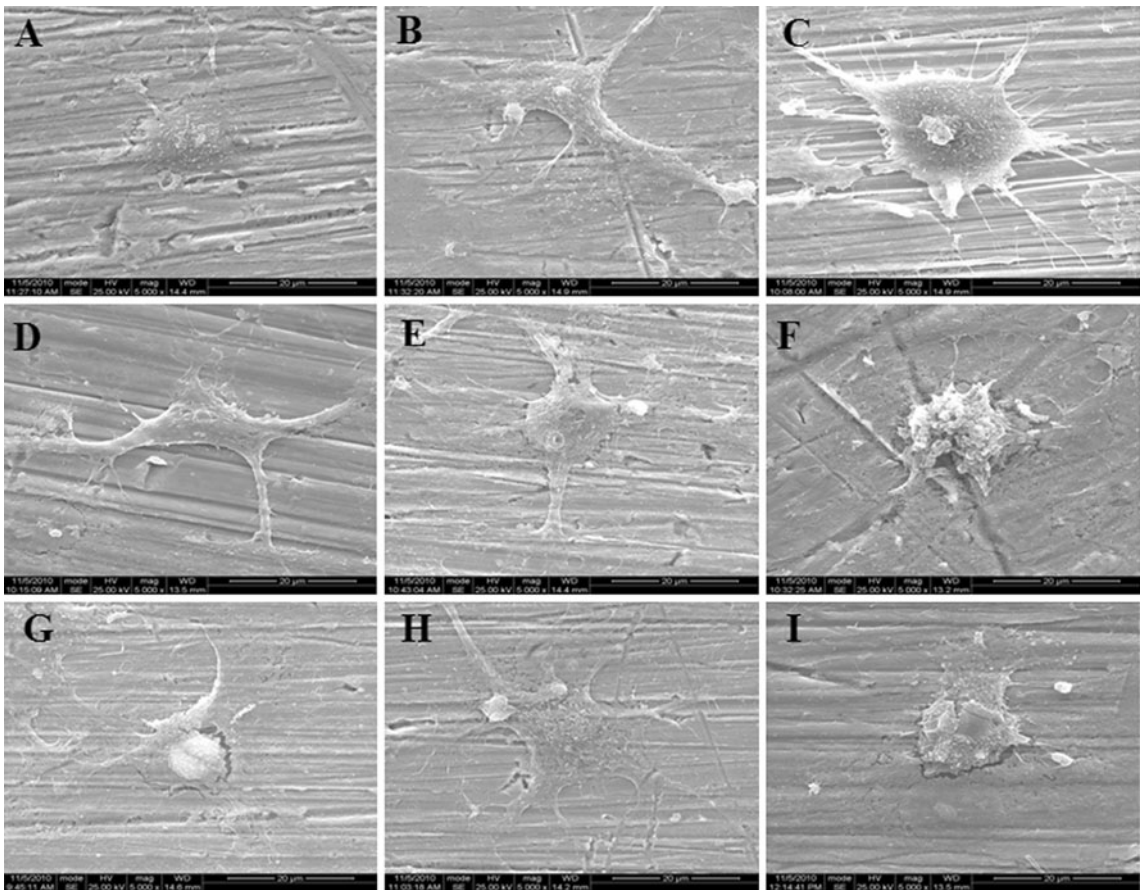


**Fig. 3** SEM image of MG63 cells cultured on implant materials. **a** 317–L for 24 h; **b** 317L–Cu for 24 h; **c** Ti–6Al–4V for 24 h; **d** 317–L for 48 h; **e** 317L–Cu for 48 h; **f** Ti–6Al–4V for 48 h; **g** 317–L for 72 h; **h** 317L–Cu for 72 h; **i** Ti–6Al–4V for 72 h. No difference in the number of cells was observed after incubation in vitro

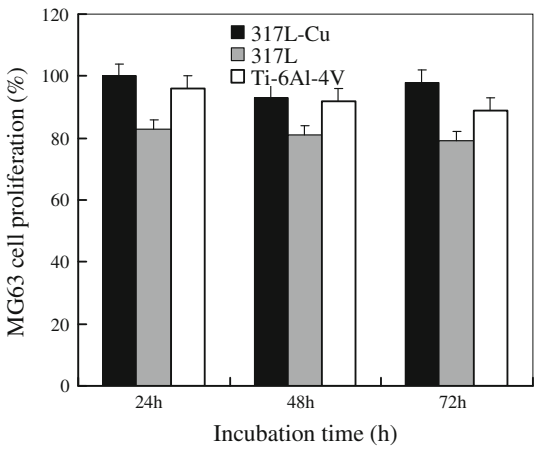


**Fig. 4** The relative growth rates of the MG63 cells assessed using MTT-based methods at different time points of incubation on the different substrates ($P > 0.05$)

To evaluate whether 317L–Cu is biocompatible in vivo, we studied the material's effect on growth of osteoblast-like MG63 cells by microscope and SEM images. In our studies, we observed no difference compared to controls in cell growth and formation by SEM images at different time points. In addition MG63 cellular formation appeared more like controls in plastic dishes and cell viability was actually significantly greater than controls in the MTT assay, indicating the alloy is highly biocompatible to osteoblast-like cells. Kishimoto et al. reported that $Cu^{2+}$ cellular toxicity was concentration dependant, like silver [24]. In our study, $Cu^{2+}$ concentration in culture supernatant from MG63 cells was dependent on the time of copper exposure: $Cu^{2+}$ concentrations increased slowly after 48 h. If excessive $Cu^{2+}$ had been released in our assay, its cytotoxicity would have been easily detectable by cellular growth changes between the plastic dishes and the 317L–Cu surfaces compared to controls. Although it can be toxic at high concentrations, copper at physiological concentrations serves as a cofactor in enzymes that modifies neuropeptides, generates cellular energy, detoxifies oxygen-derived radicals, mobilizes iron, coagulates blood, and cross links connective tissue [25]. In addition, copper binds to DNA to form new molecular species and is necessary during

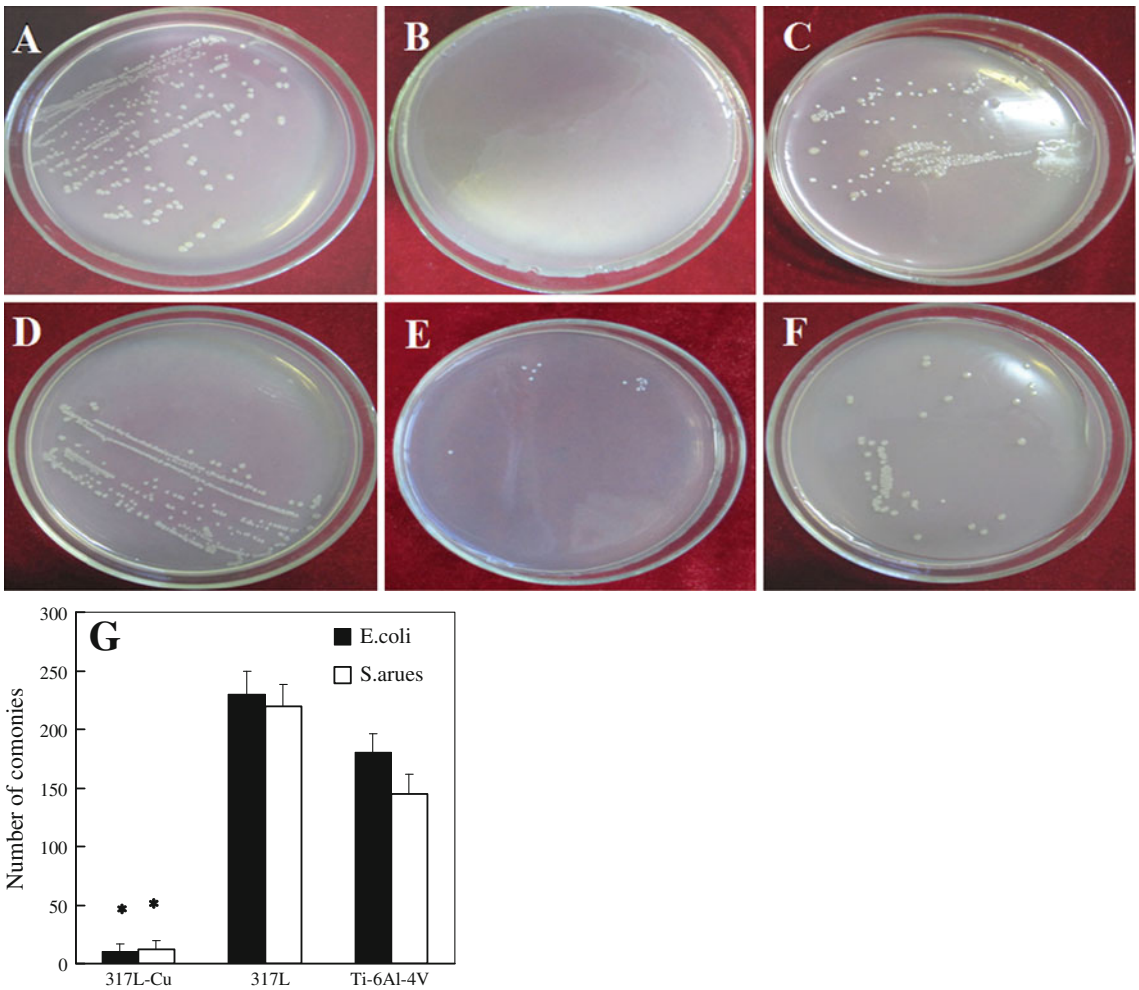


**Fig. 5** Colonization of *E. coli* and *S. aureus* from swabs taken in rabbits at 5 days post surgery from screw tracts. **a** 317L cultured with *E. coli*; **b** 317L–Cu cultured with *E. coli*; **c** Ti–6Al–4V cultured with *E. coli*; **d** 317L cultured with *S. aureus*; **e** 317L–Cu cultured with *S. aureus*; **f** Ti–6Al–4V cultured with *S. aureus*. **g** Histogram illustrates the number of colonies around each screw tracts (*shows signification at $P < 0.05$)

mitosis, during which the chromatin is condensed [26, 27]. Copper also plays a role for the ATP7A Copper-transporting ATPase in macrophage bactericidal activity [28]. Thus, it is critical for organisms to maintain homeostatic concentrations of copper. Here, we demonstrated that 317L–Cu is an excellent biomaterial and is not toxic, and the evidence from our in vivo studies indicates that it may stimulate osteoblast-like cell growth.

In our animal experiments in vivo, 317L–Cu bone screws showed a significantly reduced infection and inflammation; in fact, in animals with contaminated 317L–Cu screws, all bacterial cultures from the screw, screw track and soft-tissues were negative 14 days post surgery. Further, histological analysis confirmed that inflammation from tissues surrounding the 317L–Cu screws was minimal, whereas screw tract inflammation and infection of 317L stainless steel was severe, showing many inflammatory cells gathered by acute infection sites. However, there was no obvious difference in the quality of new bone formation surrounding the 317L–Cu screw tracts but for a short period of implantation (14 days), $Cu^{2+}$ cannot likely stimulate bone growth. This had also been shown by Shirai et al., who found no obvious new bone formation 14 days following implantation of Ti–Cu pins in a rabbit model [1].

In biomaterials science, we often evaluate biocompatibility by analyzing osteoconduction, the growth of bony tissue into the structure of an implant or graft, which is required for osteointegration [29], the structural and functional connection between living bone and the surface of an implant. The extent of osteoconduction depends upon biological factors and the biocompatibility of the implant. In our study, the titanium alloy had good osteoid formation, and therefore was considered to have good osteoconduction. Nevertheless, some authors have reported that bone conduction is impossible on several materials such as copper and silver [16]. Our study found 317L–Cu had

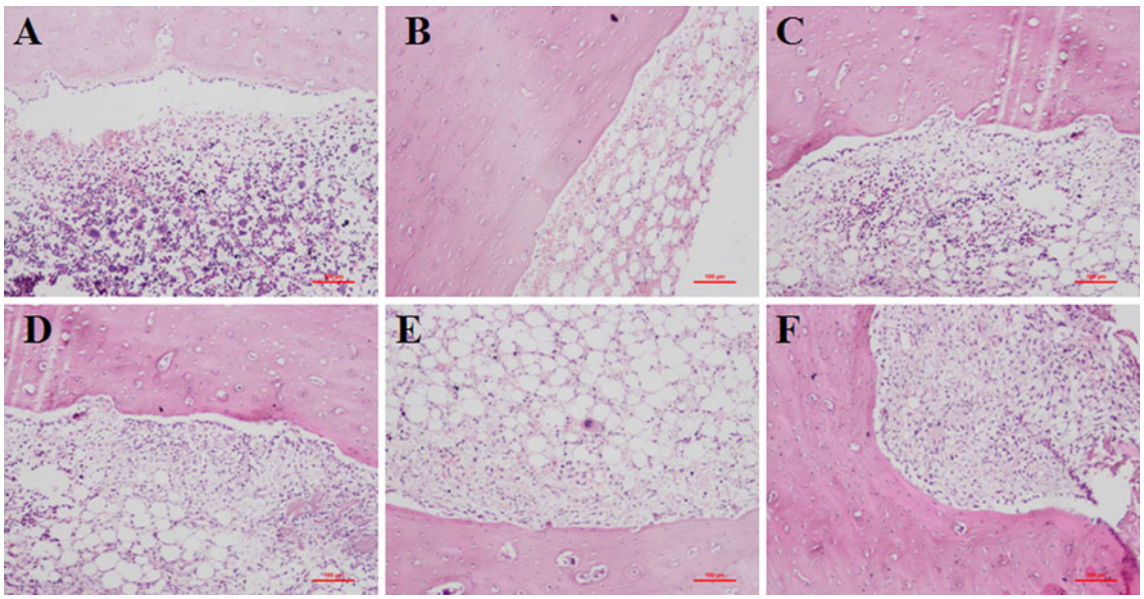


**Fig. 6** Light microscope images from hematoxylin–eosin stained bone and intramedullary tissues. **a** 317L screws with bacteria for 5 days; **b** 317L–Cu screws with bacteria for 5 days; **c** Ti–6Al–4V screws with bacteria for 5 days; **d** 317L screws with bacteria for 14 days; **e** 317L–Cu screws with bacteria for 14 days; **f** Ti–6Al–4V screws with bacteria for 14 days

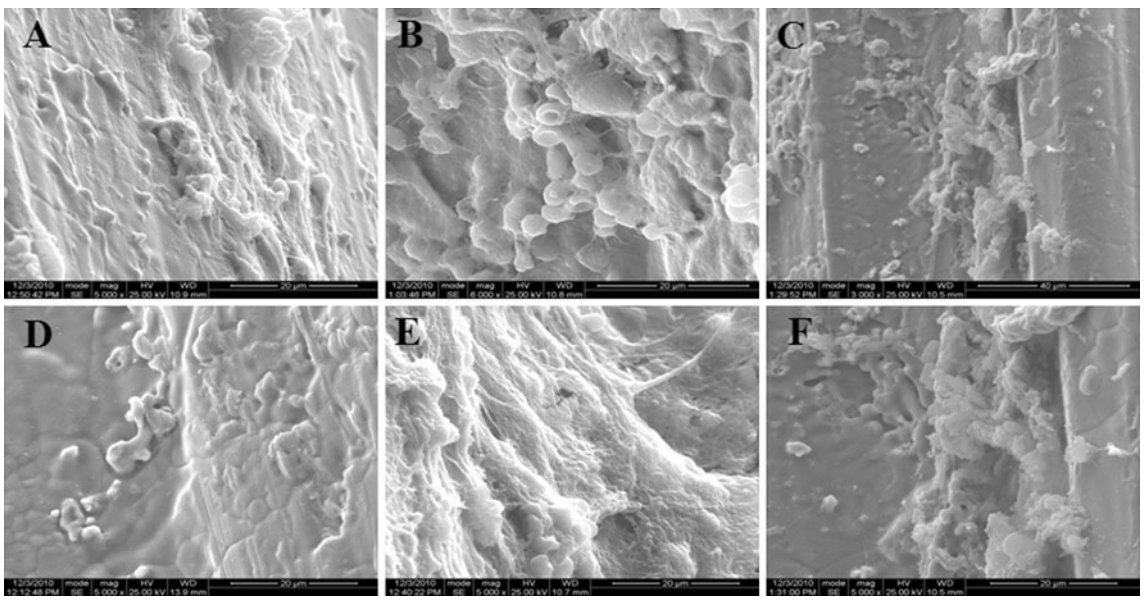


**Fig. 7** SEM images of implanted screws. **a** 317L screws implanted for 5 days; **b** 317L–Cu screws implanted for 5 days; **c** Ti–6Al–4V screws implanted for 5 days; **d** 317L screws implanted for 14 days; **e** 317L–Cu screws implanted for 14 days; **f** Ti–6Al–4V screws implanted for 14 days

osteoconduction for excellent osteoid formation on surface of screws implanted for 5 and 14 days; thus, this alloy was an excellent material not only for its antimicrobial properties but osteoconduction and osteointegration.

In addition, we also examined the expression of IGF-1 by immunohistochemistry. IGF-1 levels and functioning in osteoblast-like cells are mediated and enhanced by $Cu^{2+}$ [23] and $Cu^{2+}$ may participate the release of IGF-1 and could enhance its cellular expression [23, 30]. Hirukawa reported that IGF-1 clearly stimulates the multiplication and differentiation of osteoblast-like cells [23] and recent findings by Roughead and Lukaski showed that low levels of copper induces IGF-1 and promotes bone strength [23]. IGF-1 staining in osteoblast-like cells on surface of 317L–Cu, whereas tissues surrounding 317L and Ti–6Al–4V screws showed only weak IGF-1 activity. We conclude that

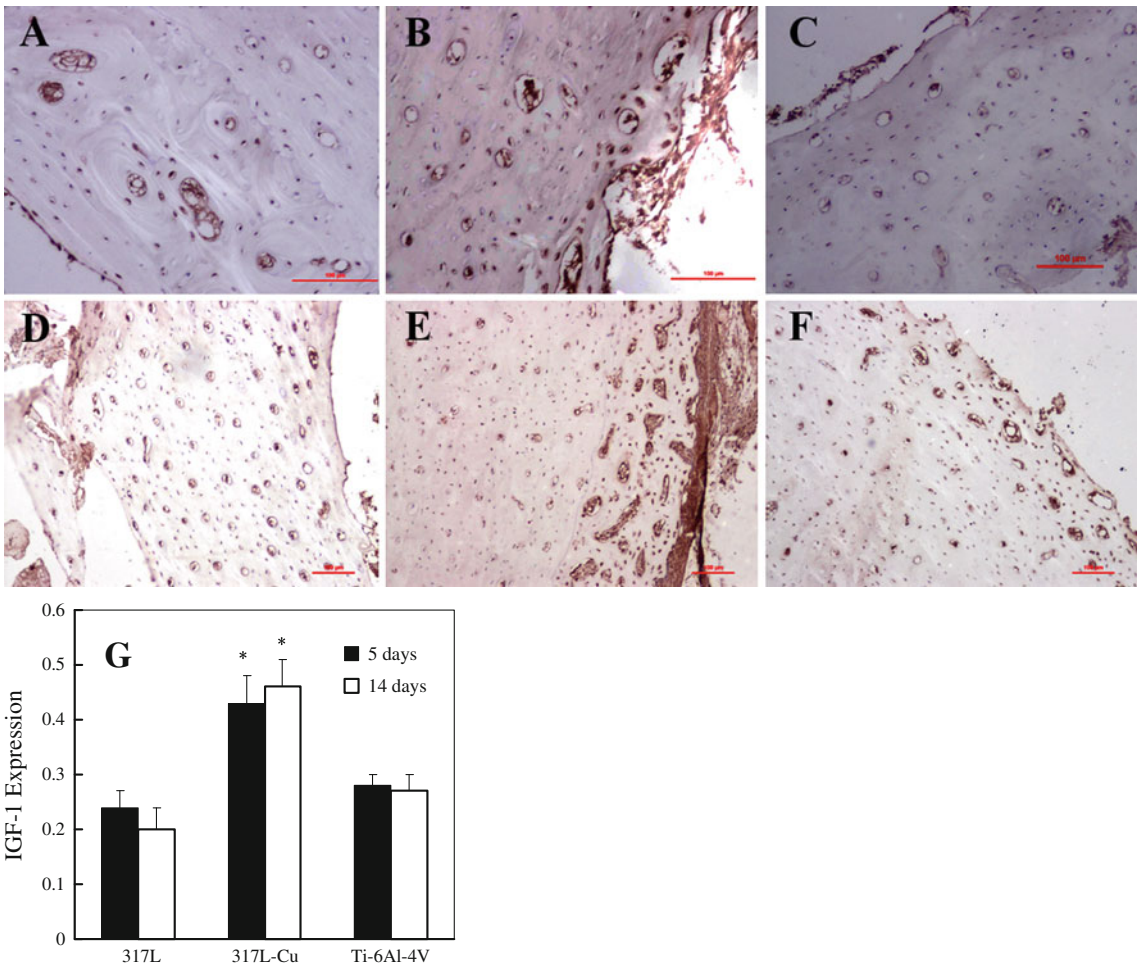


**Fig. 8** IGF-1 expression by immunohistochemistry of bone tissues around implanted screws. **a** 317L implanted for 5 days; **b** 317L–Cu implanted for 5 days; **c** Ti–6Al–4V implanted for 5 days; **d** 317L implanted for 14 days; **e** 317L–Cu implanted for 14 days; **f** Ti–6Al–4V implanted for 14 days. **g** Histogram illustrates IGF-1 levels expressed in tissues around each screw alloy (*shows signification at $P < 0.05$)

$Cu^{2+}$ released into the bone preserved and possibly stimulated IGF-1, which played an important role in preserving osteogenesis in our animal model.

As we have seen, at higher concentrations copper causes serious toxic effects, as is clearly documented in patients with Wilson's disease, in which copper accumulates, causing serious liver disease, neurological symptoms, and damage to organ systems [31]. In healthy individuals, the release of copper from implants can be toxic; $Cu^{2+}$ has been found in toxic levels in hepatocytes from copper-containing implants in liver [32]. In our study, we found no statistically significant differences between preoperative and postoperative blood copper levels in rabbits. The 317L–Cu alloy released very low levels of $Cu^{2+}$ locally and systemically after 14 days.

Our study had certain limitations. We examined only *S. aureus* and *E. coli.* Further study should be done to delineate the activity of the released $Cu^{2+}$ against other bacteria species. Moreover, further studies will be needed to elucidate the antibacterial mechanism of this alloy.

In conclusion, our study clearly demonstrated that the 317L–Cu alloys has significant antimicrobial activity and is biocompatible in vitro and in vivo such that it would be suitable as a biomaterial to reduce surgical implant-related infections.

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