The anti-bacterial activity of titanium-copper sintered alloy against *Porphyromonas gingivalis* in vitro

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This study investigates the anti-bacterial property of Ti-Cu sintered alloys against *Porphyromonas gingivalis*. The anti-anaerobic property of Ti-Cu sintered alloys against *P. gingivalis* was investigated by antibacterial activity test, DNA measurement, DAPI staining and morphology observation. The antibacterial rates of the Ti-5Cu against *P. gingivalis* after 18 and 24 h incubation were 36.04 and 54.39%, and those of Ti-10Cu were 68.69 and 75.39%, which were lower than their anti-aerobic abilities. The concentration of *P. gingivalis* DNA gradually decreased with the increasing Cu content, which was nearly 50% after 24 h incubation on Ti-10Cu. SEM results showed that the shape of *P. gingivalis* changed and the bacteria broke apart with the addition of Cu and the extension of the culture time. Ti-Cu sintered alloys could not only kill anaerobic bacteria but also reduce the activity of the survived bacteria. The anti-anaerobic mechanism was thought to be in associated with the Cu ion released from Ti-Cu alloy.

Keywords: Antibacterial activity, *Porphyromonas gingivalis*, Ti-Cu sintered alloy

INTRODUCTION

Periodontitis is one of the major reasons for teeth extraction. The incidence of periodontitis unresponsive to treatment depends on pretreatment progression rate, extent and severity of disease, tooth type, smoking, high levels of putative periodontal pathogens, a deficient immune response, and the type of therapy provided¹. *Porphyromonas gingivalis*, a gram-negative black-pigmented anaerobe, has been characterized as a periodontal pathogen and associated with various types of periodontal diseases² and peri-implantitis³. Periodontal disease was considered as a more important risk factor than high blood pressure, and second only to having long-term diabetes⁴. Control of bacterial infection and periodontal therapy would reduce atherosclerotic changes and improve endothelial function. The interrelationships between periodontal infection and systemic conditions such as cardiovascular disease (CVD), adverse pregnancy outcomes, head-and-neck cancer, diabet, pneumonia and end-stage renal disease have become increasingly appreciated in recent years^{5,6}.

The organism possesses a number of pathogenic properties including the abilities to adhere to and colonize on the oral surfaces and to invade periodontal tissues. Oral epithelia is the primary site for *P. gingivalis* infection, and this bacteria can enter the circulation following tooth brushing and other dental procedures⁷. It can invade human epithelial cells, likely to avoid

the immune defense and find its privileged niche⁸. A variety of cellular and extracellular components including fimbriae, proteases, hemagglutinins and lipopolysaccharide participate in the adherence of *P. gingivalis*^{9,10} and a number of salivary components are capable of interacting with *P. gingivalis*¹¹. Salivary proline-rich proteins (PRPs)/proline-rich glycoproteins (PRGs) and statherin were found to act as salivary receptors for the organism as well as several other plaque-forming bacteria¹². It should be noted that the oral surfaces including teeth, gingival margin, mucosal membrane, and the surface layer of plaque-forming bacteria are all coated with salivary fluid. *P. gingivalis* also occurs when these salivary components are deposited onto apatitic mineral such as hydroxyapatite¹³, metal and porcelain¹⁴ via protein-protein interactions through definitive domains of salivary proteins and fimbriae, respectively. Control of bacterial infection and periodontal therapy would reduce atherosclerotic changes and improve endothelial function.

At the present stage, many kinds of metal materials have been used in prosthodontics, orthodontics and orthopedic surgery operation, such as removable partial denture, porcelain, metal crown, implant, mini-implant, bracket, arch wire, screw and plate. Most of them involve inevitably contact with the mouth saliva, and *P. gingivalis* can deposit onto them through salivary components. From the point of view of biological materials, can we develop an anti-anaerobic bacteria implant? Some experimental approaches include the development of anti-bioadhesion coatings, coating surfaces with antimicrobial agents (Vancomycin,

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F, Cu, Ag, Zn) or antimicrobial releasing coatings (calcium phosphate, polylactic acid, chitosan)^{15,16}. But if these membranes and antibacterial surface coatings are destroyed for different reasons, the antibacterial property will disappear. Therefore, it is very necessary to develop a metal material that has antibacterial activity in the whole alloy rather than on the surface.

In our previous studies, the Cu element was added to titanium by a powder metallurgy in order to obtain an alloy with antibacterial properties. The alloy showed excellent antibacterial properties against *Staphylococcus aureus* and *Escherichia coli*¹⁷ and exhibited very good cell biocompatibility^{18,19}. But the role of Ti-Cu sintered alloy against the oral anaerobic bacteria is still unknown. Here, we propose that Ti-Cu sintered alloy shows promising antibacterial activity against *P. gingivalis*. The result will raise the application possibility of the Ti-Cu sintered alloy as an anti-bacterial metal in dentistry, and the application has not been considered until now.

MATERIALS AND METHODS

Materials

High purity titanium powder (99.99% w/w) and 5, 10% w/w, respectively, high purity copper powder (99.99% w/w) were ball milled for 6 h and were then hot pressure sintered under vacuum condition at 30 MPa pressure and 1,050°C to prepare Ti-Cu sintered alloys (named Ti-5Cu and Ti-10Cu alloy, respectively) with 40 mm in diameter. Ti-Cu samples for further experiments with a diameter of 15 mm and a thickness of 1 mm were sliced from the sintered Ti-Cu alloys. Commercial available pure titanium (cp-Ti) samples with the same dimension were also used for comparison. All samples were ground with SiC paper up to 1000 grits, polished with 1 µm polishing liquid and ultrasonically cleaned in alcohol and dried in warm air⁹.

Anaerobic bacteria culture

P. gingivalis ATCC 33277, provided by the Central Laboratory, School of Stomatology, China Medical University was cultured in BHI-S blood agar plates. The bacterial strains were cultured in an anaerobic condition at 37°C for 72 h, identified by Gram staining as the pure culture, and then cultured for another 48 h. The bacteria suspension with a concentration of 1×10^9 CFU/mL was prepared with BHI fluid nutrient at pH 7.2.

BHI-S blood agar diffusion assay

The hot BHI-S blood agar solution was spread evenly with a conrage stick on 90 mm petri dishes and was allowed to cool down to room temperature under a sterile condition. A suspension of *P. gingivalis* (50 µL, 1.5×10^9 CFU/mL) was sprayed over the dish and incubated in an anaerobic condition for 4 days. The sterile Ti-Cu samples, cp-Ti samples and antibiotic tablets (Erythromycin, 0.1 g, Jinhong Pharmaceutical; Metronidazole, 0.1 g, Symviantiane Pharmaceutical) were separately placed in petri dishes according to the National Standard of China (GB/T 2738-2005). The petri dishes were

incubated in an anaerobic condition for 3 days. The antibacterial activity was accessed by the width of an inhibition zone around the sample. The width (W) of the inhibition zone was calculated by the following formula:

$$W = (D_{\text{zone}} - D_{\text{sample}}) / 2$$

D_{zone} is the diameter of the antibacterial zone observed around the sample, and D_{sample} is the diameter of the sample. The larger width value displays better antibacterial property.

Plate-count method

Put Ti-Cu and cp-Ti samples and same size coverslips into petri dishes. Add 200 µL bacterial suspension (5.0×10^8 CFU/mL) to each sample. Then, samples were covered and incubated in an anaerobic condition for 6, 12, 18 and 24 h, respectively. The inoculated sample was washed in a sterilized Petri dish by 4.8 mL BHI. After it was diluted 5×10^3 times, 100 µL bacterial suspension was smoothly spread over BHI-S blood agar plates and cultured anaerobically for 7 days. The bacteria colonies were counted by an automatic Colony Counter (V3, Shineso Science & Technology, China), and the antibacterial rate (R) was calculated:

$$R (\%) = (N_{\text{control}} - N_{\text{sample}}) / N_{\text{control}} \times 100\%$$

N_{control} is the average number of the bacterial colonies on coverslips, and N_{sample} is the average number of bacterial colonies on the Ti-Cu and cp-Ti samples, respectively.

DNA of *P. gingivalis*

Two hundred µL bacteria suspension (1×10^7 CFU/mL) was inoculated onto the Ti-Cu samples and cp-Ti samples in 24 well plates and then cultured in an anaerobic condition for 12 and 24 h, respectively. Then, the supernatant liquids were harvested, and total DNA was extracted using a Bacterial DNA Extraction kit (DN11, Biomed Biological Technology, Beijing, China). Real-time PCR was performed on 7500 Real time PCR system (Applied biosystems, New York, USA) using a SYBR® Select Master Mix Kit (Applied biosystems) according to the manufacturer's instructions. PCR for gene was carried out in 20 µL reaction mixture containing 1 µL DNA template. The PCR conditions were 50°C for 2 min; 95°C for 2 min followed by 40 cycles of 95°C for 15 s; 60°C for 30 s and 72°C for 1 min. The reactions were performed in triplicate for each sample. The gene-specific primers were designed with the Primer 5 software (Premier Biosoft International, Palo Alto, CA, USA), as presented: Forward (5'-3'): CATAGATATCACGAGGAAGTCCGATT; Reverse (5'-3'): AAAGT TTAGCAACTACCGATGTGG.

Total bacterial DAPI staining

Four hundred µL bacteria suspension (1×10^7 CFU/mL) was inoculated onto the cp-Ti and Ti-Cu samples and cultured in an anaerobic condition at 37°C for 12 and 24 h, respectively. Then, the samples were rinsed 3 times with PBS. For staining, the samples were covered with 400 µL Triton X-100 (Jiang biological technology, China) and 0.4 µL DAPI (Merck, Darmstadt, Germany) in a dark

chamber. After 15 min the DAPI solution was removed. Then, the specimens were dried at room temperature and were analyzed using a light microscope (Eclipse 80i, Nikon, Tokyo, Japan) with a heat-barrier, filtered, epifluorescent light source (Model C-SHG1, Nikon) at the wave length of 435 nm.

Morphology of *P. gingivalis*

Two hundred μL bacteria suspension (1×10^7 CFU/mL) was inoculated onto the cp-Ti and Ti-Cu samples and cultured in an anaerobic condition at 37 °C for 12 and 24 h, respectively. Then, the samples with bacteria were fixed in 2.5% glutaraldehyde solution for 2 h at room temperature and rinsed 3 times with PBS, followed by dehydration in a gradient ethanol/distilled water mixture (50, 60, 70, 80, 90, and 100%) for 10 min each. The surface of the samples was sputter-coated with gold, and the morphology of *P. gingivalis* was observed on a scanning electronic microscope (S-4700, Hitachi, Tokyo, Japan).

RESULTS

BHI-S blood agar diffusion assay

The antibacterial activity was tested against *P. gingivalis* in BHI-S blood agar plates by the agar diffusion method. Figure 1 shows the inhibition zones around different samples against *P. gingivalis*. The significant inhibition zone existed around the Metronidazole, as shown by a black circle in Fig. 1c. On the contrary, no inhibition zone was observed around the cp-Ti and Ti-Cu samples and Erythromycin, as shown in Figs. 1b, d, e and f. Table 1 summarizes the inhibition zone width around different samples. These results clearly indicate that no antibacterial activity of the Ti-Cu alloy and cp-Ti was detected by this method.

Plate-count method

Figure 2 shows typical *P. gingivalis* bacterial colonies incubated on the surface of different samples for 6, 12, 18 and 24 h, respectively. A large amount of *P. gingivalis* were observed on the control sample and the cp-Ti sample (a and b), corresponding to the fact that the control sample and the cp-Ti alloy do not have antibacterial properties. On the contrary, less bacterial colonies were found on the Ti-5Cu and Ti-10Cu samples, especially after incubation for 18 and 24 h (c-3, c-4, d-3 and d-4), strongly demonstrating that the Ti-5Cu and Ti-10Cu alloys had antibacterial property.

Figure 3 shows the time-dependent antibacterial rates of the Ti-Cu sample and cp-Ti sample against *P. gingivalis*. The antibacterial rate of the cp-Ti stabilized at a very low level (about 5%) during the whole duration process, confirming that the cp-Ti did not have antibacterial ability. The antibacterial rates of the Ti-5Cu and Ti-10Cu samples increased significantly with the culture time. For example, the antibacterial rates of the Ti-10Cu sample against *P. gingivalis* after 18 and 24 h incubation were 68.69 and 75.39% while the antibacterial rates of the Ti-5Cu sample were 36.04

Fig. 1 Inhibition zones around different samples against *P. gingivalis*.

The antibacterial activity was tested against *P. gingivalis* in BHI-S blood agar plates by the agar diffusion method; a) blank control. b) no inhibition zone was observed around Erythromycin. c) significant inhibition zone existed around the Metronidazole, as shown by a black circle. d) no inhibition zone was observed around cp-Ti. e) no inhibition zone was observed around Ti-5Cu. f) no inhibition zone was observed around Ti-10Cu.

Table 1 Width of inhibition zones for different samples against *P. gingivalis* (mm)

Samples	Width of inhibition zones (mm)
Erythromycin	0
Metronidazole	11.14±0.22
cp-Ti	0
Ti-5Cu	0
Ti-10Cu	0

Width of inhibition zones (mm) for Erythromycin (negative control), Metronidazole (positive control) and cp-Ti, Ti-5Cu and Ti-10Cu on BHI-S blood agar diffusion were measured (mean±standard deviation, $n=3$), as described in “Materials and Methods” section.

Fig. 2 Typical colonization by *P. gingivalis* on different samples. a-1)–a-4), The amount of *P. gingivalis* were observed on the blank control after 6, 12, 18 and 24 h incubation. b-1)–b-4), The amount of *P. gingivalis* were observed on cp-Ti sample after 6, 12, 18 and 24 h incubation. c-1)–c-4), The amount of *P. gingivalis* were observed on Ti-5Cu sample after 6, 12, 18 and 24 h incubation. d-1)–d-4), The amount of *P. gingivalis* were observed on Ti-10Cu sample after 6, 12, 18 and 24 h incubation. Less bacterial colonies were found on the Ti-5Cu and the Ti-10Cu samples from 6 to 24 h incubation than those on blank control and cp-Ti samples. The least bacterial colonies were found on the Ti-10Cu samples after 24 h incubation.

and 54.39%, respectively. These results indicate that the antibacterial rate of the Ti-Cu alloys is time-dependent and Cu content-dependent. High Cu content results in a high antibacterial rate in the whole investigated condition.

DNA of *P. gingivalis*

Figure 4 shows the DNA values of *P. gingivalis* bacteria, which was inoculated onto the cp-Ti and Ti-Cu samples for 12 and 24 h. Increased DNA expression was observed on the cp-Ti, Ti-5Cu and Ti-10Cu samples with the culture time, but the increase in the DNA expression on the Ti-Cu samples was not significant as that on the cp-Ti sample. At each time point, significant down-regulation of DNA values was observed for the *P. gingivalis* cultured on the Ti-5Cu ($p < 0.05$) and the

Ti-10Cu sample ($p < 0.05$) in comparison with that on the cp-Ti sample, especially on the Ti-10Cu sample at 24 h, from 103.9 $\mu\text{g/mL}$ on the cp-Ti sample to 51.9 $\mu\text{g/mL}$ on the Ti-10Cu sample. These results demonstrate the sterilization function of the Ti-Cu alloy. According to the DNA data, the antibacterial rates of the Ti-5Cu sample and Ti-10Cu sample at 24 h were about 40 and 50%, respectively, which were less than the values shown in Fig. 3 at 24 h. In addition, the DNA data also indicated that the Cu content in the Ti-Cu alloy influenced the antibacterial property in a dose-dependent way, which is in agreement with the results shown in Fig. 3.

Bacterial DAPI staining

After 12 and 24 h incubation, the pronounced effects of the cp-Ti and Ti-Cu samples on the amount of adherent

Fig. 3 Change of the antibacterial rates of the cp-Ti and Ti-Cu samples against *P. gingivalis* with culture time.

The antibacterial rate of the cp-Ti stabilized at a very low level (about 5%) during the whole duration process. The antibacterial rates of the Ti-5Cu against *P. gingivalis* after 18 and 24 h were 36.04 and 54.39%, and the rates of the Ti-10Cu sample were 68.69 and 75.39%. The antibacterial rate increased significantly with the culture time and the increasing percentage of the Cu element of Ti-Cu alloys.

Fig. 4 The amount of DNA by *P. gingivalis* on different samples during the culture.

The DNA values of *P. gingivalis* bacteria, which were inoculated onto the cp-Ti and Ti-Cu samples for 12 and 24 h. All experiments were performed in triplicate, and representative results were presented as the means±standard deviations. The statistical analyses for quantitative assays were performed using the SPSS 11.0 software. $P < 0.05$ (*) was considered to be statistically significant. The DNA values were increased with the culture time, and significant down-regulation of DNA values was observed on the Ti-5Cu ($p < 0.05$) and Ti-10Cu sample ($p < 0.05$) in comparison with that on the cp-Ti sample.

Fig. 5 DAPI staining of *P. gingivalis* distributed on different samples.

The effects of the cp-Ti and Ti-Cu samples on the amount of adherent bacteria were observed with DAPI staining in comparison with the control samples after 12 and 24 h incubation. a, b) The adherent bacteria on blank control at 12 and 24 h. c, d) The adherent bacteria on cp-Ti samples at 12 and 24 h. e, f) A significant reduction of the adherent bacteria on Ti-5Cu samples at 12 and 24 h. g, h) A significant reduction of the adherent bacteria Ti-10Cu samples at 12 and 24 h.

bacteria were observed with DAPI staining in comparison with the control samples. Figure 5 shows a significant reduction of the adherent bacteria on Ti-5Cu and Ti-10Cu samples after 12 h in comparison with that on the cp-Ti sample. In general, Ti-5Cu alloy was slightly less effective than Ti-10Cu alloy. Also, after 24 h, the effect of the Ti-10Cu alloy increased significantly.

Morphology of *P. gingivalis*

Figure 6 shows the morphology of *P. gingivalis*, which adheres to the surfaces of the cp-Ti and Ti-Cu samples after 12 and 24 h incubation. It was seen that the

Fig. 6 Morphology of *P. gingivalis* on the different samples. The morphology of *P. gingivalis* on the surfaces of the cp-Ti, Ti-5Cu and Ti-10Cu at 12 and 24 h incubation. a-1 (12 h, $\times 5,000$), a-2 (12 h, $\times 10,000$), a-3 (24 h, $\times 5,000$), a-4 (24 h, $\times 10,000$) The bacteria on the cp-Ti alloy grew well during the culture and showed regular spherical or rod shapes with uniform distribution density. b-1 (12 h, $\times 5,000$), b-2 (12 h, $\times 10,000$), b-3 (24 h, $\times 5,000$), b-4 (24 h, $\times 10,000$). Less bacteria were observed on the Ti-5Cu samples. Most bacteria on the Ti-5Cu surface were elongated with clusters or piles. c-1 (12 h, $\times 5,000$), c-2 (12 h, $\times 10,000$), c-3 (24 h, $\times 5,000$), c-4 (24 h, $\times 10,000$) A little bacteria were observed on the Ti-10Cu samples. Many bacteria fragmentations were seen on the Ti-10Cu alloy surface after 24 h incubation (c-4, white arrow).

bacteria on the cp-Ti alloy grew well during the culture and showed regular spherical or rod shapes with uniform distribution density after 12 h incubation, as shown in Figs. 6a-1 and 6a-2. On the Ti-Cu samples, less bacteria were observed in comparison with the cp-Ti sample, as shown in Figs. 6b-1, 6b-2, 6c-1 and 6c-2. Also, most bacteria on the Ti-5Cu and Ti-10Cu alloy surface were elongated with clusters or piles, and many bacteria fragmentations were seen on the Ti-10Cu alloy surface after 24 h incubation (Fig. 6c-4, white arrow). These results also suggest that the many bacteria were killed on the Ti-Cu samples surface. With the extension of the culture time, the amount of *P. gingivalis* increased on the surfaces of both the cp-Ti and Ti-Cu samples, which is in agreement with the results in Fig. 4.

DISCUSSION

Many scientists have tried to find simple and effective methods to reduce the bacterial adhesion to the surface of artificial dental restorative materials, which would promote the colonization of host tissue and suppress the colonization of bacterial species. These materials have many different action mechanisms. Materials can interfere with bacterial adhesion by modifying surface energy²⁰⁾ and immobilizing molecules onto the surface (bactericidal)²¹⁾, photocatalytic²²⁾, releasing metal ions²³⁾

or antibiotics²⁴⁾. Because of material corrosion, which is usually caused by saliva electrolyte solution and tooth brushing, the function period of the strong and active bactericidal activity of the above materials was short, sometimes only 1 month *in vitro*²⁵⁾. So it is necessary to develop an antibacterial alloy that exhibits antibacterial activity in the whole alloy rather than on the surface and in turn can resist the colonization of anaerobic and aerobic bacteria for a long time.

As we known, the majority of dental implants today are made from commercially pure titanium (cp-Ti) or titanium alloys and cp-Ti is produced with various degrees of purity. Grade 1 has the highest purity because of its low oxygen and iron content *versus* cp-Ti grade 4, which has the highest maximum oxygen and iron percentage, according to an American standard (ASTMF67)²⁶⁾. For example, Brånemark system® implants (Nobel Biocare, Goteborg, Sweden) are made from grade 1 cp-Ti, while ITI implants (Straumann, Basel, Switzerland) are made from grade 4 cp-Ti. In the previous studies¹⁷⁾, 10 wt% high purity copper (99.99 wt%) was added into high purity titanium powder (99.99 wt%) by a powder metallurgy to produce Ti-Cu sintered alloy. It has been shown that Ti-Cu sintered alloy consisted of intermetallic compound Ti_2Cu and α -Ti matrix (HCP) and low amount of solid solution of Cu in titanium was also detected due to high temperature

diffusion of Cu atom. In this study, we want to know the effect of Ti-Cu sintered alloys on cells and bacteria. The Cu element is an essential trace element, which takes part in the metabolism of cholesterol and glucose and the synthesis and release of life and it is also an element for titanium alloy, which has been used commercially to develop high-strength alloy²⁷. Previous studies show that the micro-hardness and compressive strength of cp-Ti are 160.3 HV and 333.3±69.0 MPa, but the strength of Ti-10Cu are 369.5 HV and 1707.9±45.4 MPa, indicating that the addition of Cu element increases the hardness significantly¹⁷.

And Cu element has been widely used as an antibacterial agent. This antibacterial ability of Ti-Cu sintered alloys has been proved by our previous studies. The Ti-Cu sintered alloys exhibited very strong antibacterial activity against *S. aureus* and *E. coli* even after polishings¹⁷. In this study, the antibacterial activity of the Ti-Cu sintered alloys to *P. gingivalis* was investigated. In the plate-count method, the antibacterial activity of the Ti-Cu samples against *P. gingivalis* was detected clearly, and the antibacterial rate increased gradually with the addition of Cu and the extension of the culture time. But in the agar diffusion assay, a significant inhibition zone existed around the positive samples (metronidazole) with no inhibition zone around the cp-Ti and Ti-Cu samples. Metronidazole, which is mainly active against anaerobes, is the most frequently investigated antibiotics in the treatment of periodontal disease. Poulet has poured metronidazole in Columbia agar plates to find the susceptibility of anaerobic bacteria to metronidazole *in vitro*. As for *P. gingivalis*, the average value was 122 µg/mL, and the MIC90 was 7.8–750 µg/mL *in vitro*²⁸. These results indicate that the Ti-Cu alloy could kill *P. gingivalis* by direct contact but not by the diffusion in blood agar.

From the typical *P. gingivalis* bacterial colonies incubated on the surface of the Ti-Cu sintered alloys and the control sample, it was also found that the diameter of the bacterial colonies in Fig. 2d-4 was ranged widely, meaning that the Ti-10Cu alloy could kill the bacteria but also reduce the activity of the survived bacteria. This conclusion was verified by the subsequent experiments results (real-time PCR, DAPI staining and SEM) at 12 and 24 h. The DNA of *P. gingivalis* gradually increased with the culture time and decreased with the increase in the Cu content, but the increasing DNA concentration on the Ti-Cu sintered alloy samples was less than that on the cp-Ti sample. In addition, the decreasing rates of DNA concentration, which were nearly 50% after 24 h incubation on the Ti-10Cu sample, were much lower than the decreasing rate in the number of bacterial colonies in the plate-count method and 75.5% after 24 h incubation on the Ti-10Cu sample. This difference was considered to be attributed to the reduced-proliferation ability bacteria. The genomic DNA of these reduced-proliferation ability bacteria was detected by the real-time PCR, but these bacteria did not have the ability to amplify and form colonies on BHI-S blood agar plates in the plate-count method. Therefore, fewer colonies were

found in the plate-count method and high DNA expression was detected in the real-time PCR for the same sample. It is suggested that we should use RNA-based methods to detect active bacteria in future experiments, although RNA is rapidly degraded and more unstable than DNA. Not only could DAPI rapidly take up into DNA and specificity for the AT-clusters of double-stranded DNA but could also show highly fluorescent nuclei without cytoplasmic fluorescence²⁹. DAPI staining could be used to quantify the bacteria in the adherent state on Ti-Cu sintered alloys. The reproduction of *P. gingivalis* decreased gradually with the addition of Cu in titanium and the culture time. The greatest anti-bacterial of *P. gingivalis* was shown in the Ti-10 Cu sample at 24 h. SEM results showed that the shape of *P. gingivalis* changed and the fragmentation of *P. gingivalis* increased with the addition of Cu and the extension of the culture time. Both DAPI image and SEM morphology demonstrated the addition of Cu in titanium, reduced the bacteria reproduction on the surface and changed the shape of the adhered bacteria, which suggest that Ti-Cu sintered alloy was not favorable for the growth of *P. gingivalis* bacteria.

As a strong antibacterial agent, Cu, Ag and Zn have been widely used to prepare antibacterial surface coating in many methods, such as ion implantation³⁰ and plasma spraying³¹. It was reported that the antibacterial properties of a Cu ion containing liquid were related to the Cu²⁺ concentration, the initial bacteria concentration and the incubation duration³². In this research, the Cu element was added in a commercial pure titanium by a powder metallurgy in order to obtain an antibacterial titanium alloy. However, it was noticed that the anti-anaerobic bacteria properties of Ti-Cu sintered alloy were lower than the anti-aerobic bacteria properties. This result is associated with the characteristics of *P. gingivalis*. Firstly, *P. gingivalis* grows slowly, going into an exponential phase in 24–48 h, forming gray colonies after 1 week incubation in an anaerobic environment and forming black colonies after 10 days. On the contrary, *E. coli* and *S. aureus* grow fast, forming colonies after only 1 day incubation in an aerobic environment. Secondly, the sensitivity of *P. gingivalis* to antibiotics is less than that of aerobic bacteria. For example, the MIC of fluoroquinolone derivatives against *E. coli* and *S. aureus* was 0.25–0.5 µg/mL and 0.125–8 µg/mL³³; the MIC of gentamicin against *E. coli* was 2 µg/mL³⁴; the MIC of penicillin G against *S. aureus* was 2 µg/mL; the MIC of ampicillin against *S. aureus* was 32 µg/mL and the MIC of cloxacillin, ceftiofur and tetracycline against *S. aureus* was <2 µg/mL³⁵. All these MICs against aerobic bacteria are much lower than the MIC of metronidazole against *P. gingivalis* (122 µg/mL)²⁸. The data might explain the lower antibacterial rate and bacteriostasis of the Ti-Cu sintered alloy against *P. gingivalis*.

As for the antimicrobial mechanism of Cu, researchers have confirmed that Cu could react with H₂O₂ and lead to highly toxic hydroxyl radical (•OH) and hydroxyl anion (OH⁻) in phagocytic cells, which

could damage lipids, nucleic acids, proteins and oxidant DNA³⁶). Other researchers observed that Cu could displace Fe atoms from dehydratase Fe-S clusters and block branched-chain amino acid synthesis in *E. coli*³⁷. Various scientific studies account for the beneficial use of copper and its alloys, most notably with its multitoxicity, which also renders copper effective against multiresistant germs like the “superbug” MRSA (methicillin resistant *Staphylococcus aureus*)³⁸ or carbapenemase-resistant bacteria³⁹. On the other hand, we know that a high concentration of Cu can cause upset stomach, nausea and diarrhea and can lead to tissue injury and disease⁴⁰. Therefore, the balance of the Cu ion release concentration between the antibacterial activity and the cell compatibility will play a very important role in the development of the antibacterial Ti-Cu alloy. We calculated the Cu ion concentration in the immersion solution and Cu ion release rates in a previous study. After 72 h immersion of the Ti-10Cu alloy, the Cu ion concentration in the immersion solution was 0.050 mg/L, corresponding to a Cu ion release rate of 2.83×10^{-2} µg/cm², which is lower than the Cu ion release rate of several commercial dental alloys (0.58–1.9 µg/cm² after 168 h immersion)^{17,41}. In another research, it was reported that the antimicrobial activity of the Cu alloys (60–99% w/w copper) against *Salmonella enterica* increased with the increase of the copper content⁴⁰, which is in agreement with the antibacterial rate and bacteriostasis of the Ti-Cu sintered alloy against *P. gingivalis* in this research.

Because of the bio-inert of cp-Ti and Ti-Cu sintered alloy, it is necessary to use surface treatments to promote cell adhesion and proliferation and enhance bone/implant bonding strength, which including sandblasted and large-grits acid etching (SLA), plasma spraying physical vapor deposition (PVD), sandblasting (SB), micro-arc oxidation (MAO), anodic oxidation, acid etching and alkaline heat treatment. Our experiments proved that SLA and SB treatments produced a rough but discontinued TiO₂ layer on the Ti-10Cu sintered alloy and reduced the corrosion resistance. On the other hand, alkaline heat treatment formed a smooth and microporous TiO/titanate on Ti-10Cu sintered alloy and slightly improved the corrosion resistance. And all of these treatment did not reduce the antibacterial activity of the Ti-10Cu sintered alloy to *S. aureus*⁴².

And we analyzed the biocorrosion of Ti-10Cu by immersing it in Hank's solution, Tyrode's solution, Saliva (pH 3.5), Saliva (pH 6.8) and Saliva (pH 6.8)+0.2 F, which were chosen to simulate human body fluids with different pH values, blood system, oral condition and oral condition with F⁻ ion. We found that the corrosion rate of Ti-10Cu alloy was in a order of Hank's, Tyrode's, Saliva (pH 6.8), Saliva (pH 3.5) and Saliva (pH 6.8)+0.2 F from slow to fast. The highest concentration of Cu ion and Ti were found in Saliva (pH 6.8)+0.2 F group for 30 days, which were 5.8 ± 4.9 and 51.5 ± 0.0 mg/L, respectively. The concentration of Cu ion is lower than the LD50 for different cell lines^{43–46}, indicating that Ti-10Cu alloy in the five simulated environments will not lead to cells toxicity⁴⁷. And we have proved the good biocompatibility

of Ti-10Cu and cp-Ti implants by implanting them in rabbit femurs *in vivo*⁴⁸.

In conclusion, our results demonstrated that the Ti-Cu sintered alloy exhibited strong antibacterial activity to aerobic bacteria but also antibacterial ability to *P. gingivalis*, thus showing the potential application as dental implant materials. Next, we would continue our investigation for the control of Cu ion release in oral condition with F⁻ ion, to adjust mechanical properties in order to adapt to implant, to enhance corrosion resistance and to assess bone integration ability *in vivo* in the following research.

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