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In vitro and *in vivo* studies of anti-bacterial copper-bearing titanium alloy for dental application

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ABSTRACT

Objective. A novel copper-bearing titanium alloy (Ti–Cu) was fabricated for dental application that is expected to efficiently restrain the growth of bacteria and discourage biofilm formation. The aim of this study was to investigate both the antibacterial activity and biofilm inhibition of Ti–Cu alloy *in vitro*, and the antibacterial effect of Ti–Cu implant in early stage of peri-implantitis *in vivo*.

Methods. Staphylococcus aureus and *Escherichia coli* were selected to evaluate the antibacterial activity of Ti–Cu alloy and Ti served as control. The antibacterial rate, attached bacteria and developed biofilms were studied from quantitative antibacterial test, biofilm observation and bacterial morphological examination. Electrochemical tests were used to investigate the corrosion property of Ti–Cu alloy. Furthermore, both Ti and Ti–Cu dental implants were manufactured and then implanted in the mandibular premolar sites of beagle dogs for 3 months with ligature-infected treatment. Implant-tissue samples were prepared for radiographic analysis, Micro-CT evaluation and histological examination.

Results. Ti–Cu alloy was found to efficiently kill the attached bacteria by ways of damaging cell membranes and cell walls and strongly inhibit the biofilm formation. However, Ti–Cu alloy had excellent corrosion resistance similar with Ti. Further, Ti–Cu dental implants showed superior capacities of inhibiting the bone resorption caused by bacterial infection and enhancing bone formation.

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Significance. Ti–Cu alloy strongly inhibited biofilm formation *in vitro* and prevented bacterial infection associated with dental implant *in vivo*, making it great potential for application in dental implants with excellent antibacterial viability and positive effect against bone resorption induced by peri-implantitis.

1. Introduction

Titanium (Ti) and its alloys are extensively applied in clinic as preferred metallic medical materials due to their excellent combination of mechanical properties, corrosion resistance and biocompatibility [1]. However, titanium and its alloys are inert materials in biological environment, *i.e.*, they have less bioactive functions, such as antimicrobial activity, promotion of osteogenesis, *etc.* [2]. It has been known that the adherence and colonization of bacteria on the surface of implants can lead to formation of biofilm, a firm layer composed of bacteria with the secreted glycoproteins and polysaccharides, which can cause infection and inflammation like peri-implantitis probably resulting in the failure of implantation [3]. Peri-implantitis inflammatory reactions have been detected in about 10% to 45% of dental implants within 10 years after implantation [4]. Therefore, peri-implantitis remains a persistent clinical problem without an easy treatment. It is an instinctive reaction for the bacteria to adhere on the surface and then form a biofilm for self-protection, which is a cycling dynamic process [5]. An important characteristic of bacterial biofilms is their innate resistances to both immune system and antibiotics [6,7]. Resistance to antibiotics of the bacteria in a biofilm may improve 10–1000 times compared with that of planktonic bacteria [8]. It has been estimated that over 60% of bacterial infections currently treated in hospitals are caused by the bacterial biofilm [5]. Therefore, by inhibiting the formation of biofilm on the surface of implant, it should be an effective way to control the implant-associated infection including peri-implantitis.

Copper (Cu) has long been known as a necessary trace element in the human body, and shows excellent biological properties, such as anti-inflammatory, anti-microbial and anti-proliferative properties [9]. Previous researches have confirmed the favorable antibacterial ability of copper-containing coatings. Li et al. prepared a Cu-containing bioactive glass nano-coating with uniform nanostructure on natural eggshell membrane (Cu-BG/ESM) that improved osteogenesis, angiogenesis and antibacterial activities of the material [10]. A Ti–Cu coated layer on 316L stainless steel (316L-SS) was fabricated to improve the antibacterial activity, corrosion and tribological properties [11]. Longevity and safety, however, are the main concerns for clinical adoption [7]. Bacterial contamination may occur not only peri-operatively but also hematogenously later during the life time of the implant [12,13]. This problem cannot be circumvented by coating with a releasing strategy because the antibacterial agent or antibacterial ions in the coating would run out eventually [14]. Therefore, it is of vital importance to develop the integral antibacterial materials by immobilizing antibacterial agent into materials for long-term effective antibacterial activity. A proper incorporation of Cu into stainless steel through the material melting has been reported to be an effective way to develop the integral antibacterial stainless steels with excellent antibacterial activity [15,16].

Meanwhile, there have some studies in terms of antibacterial Ti–Cu sintered alloys with the microstructure of β-transform phase [17,18]. However, the plasticity of the above Ti–Cu alloys would be not enough to machine dental implant and then difficult to gain application in clinic. Considering this, equiaxed α phase Ti–Cu alloys with optimized microstructure and mechanical property were obtained after optimizing heat treatment. Based upon this work, our previous *in vitro* study [19] proved that Ti–Cu had antibacterial and anti-bioflm ability against oral bacterial strains of *Porphyromonas gingivalis* (*P. gingivalis*) and *Streptococcus mutans* (*S. mutans*). Consequently, the aim of the present work was to further and systematically evaluate the antimicrobial activity of Ti–Cu alloy from both *in vitro* and *in vivo* tests. Various properties were assessed *in vitro* by using *Staphylococcus aureus* and *Escherichia coli*, including bacteria adhesion, cell morphological change, structure and viability of the biofilm on Ti–Cu alloy as well as the antibacterial activity. In addition to this, Ti–Cu implants were fabricated and a modified peri-implantitis dog model was established in order to evaluate the antibacterial activity of Ti–Cu alloy dental implant *in vivo* by means of X-ray, Micro-CT and histological assessment.

2. Material and methods

2.1. Material preparation

A Ti–Cu alloy with 5 wt.% Cu content was prepared in a vacuum consumable furnace from high purity Ti and Cu. The ingot was initially re-melted three times and hot forged to bar with 40 mm in diameter and then heat treated at 850 °C for 2 h followed by cooling in furnace.

For Cu ion release test and *in vivo* bacterial-related tests, including quantitative antibacterial test, SEM observation, biofilm staining assay and TEM observation, Ti–Cu alloy and commercially pure Ti were machined to small samples with diameter of 10 mm and thickness of 2 mm. Samples were further ground with SiC paper up to 2000 grit, washed by acetone, ethanol and sterile deionized water, dried by warm air, and finally disinfected by ultraviolet light prior to experiments.

Samples for electrochemical tests were cut with diameter of 10 mm and thickness of 5 mm and linked up with a copper wire at the back. Every sample was put into a polymer sample holder with only one side of 20 mm in diameter exposed and then mounted in epoxy resin that covered the entire back. Before test, samples need to be polished with SiC paper up to