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Osteogenic activity and antibacterial effect of zinc ion implanted titanium

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ABSTRACT

Titanium (Ti) and its alloys are widely used as orthopedic and dental implants. In this work, zinc (Zn) was implanted into oxalic acid etched titanium using plasma immersion ion implantation technology. Scanning electron microscopy and X-ray photoelectron spectroscopy were used to investigate the surface morphology and composition of Zn-implanted titanium. The results indicate that the depth profile of zinc in Zn-implanted titanium resembles a Gaussian distribution, and zinc exists in the form of ZnO at the surface whereas in the form of metallic Zn in the interior. The Zn-implanted titanium can significantly stimulate proliferation of osteoblastic MC3T3-E1 cells as well as initial adhesion, spreading activity, ALP activity, collagen secretion and extracellular matrix mineralization of the rat mesenchymal stem cells. The Zn-implanted titanium presents partly antibacterial effect on both *Escherichia coli* and *Staphylococcus aureus*. The ability of the Zn-implanted titanium to stimulate cell adhesion, proliferation and differentiation as well as the antibacterial effect on *E. coli* can be improved by increasing implantation time even to 2 h in this work, indicating that the content of zinc implanted in titanium can easily be controlled within the safe concentration using plasma immersion ion implantation technology. The Zn-implanted titanium with excellent osteogenic activity and partly antibacterial effect can serve as useful candidates for orthopedic and dental implants.

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1. Introduction

Zinc (Zn) has been recognized as an essential trace element for the function or structure of more than 300 proteins [1,2], and is involved in a great number of cellular processes, like DNA synthesis, enzyme activity and cell division [3,4]. It is believed that zinc can stimulate bone formation [5,6], increase osteogenetic function in osteoblasts through exciting cell proliferation, alkaline phosphatase activity, collagen synthesis and protein synthesis [7–10]. It is also well established that zinc can enhance osteoblast marker gene expressions [11].

In addition, zinc possesses excellent antibacterial ability [12,13]. Zinc ions are demonstrated to inhibit multiple activities of bacteria, such as transmembrane proton translocation, glycolysis and acid tolerance [14]. Cummins et al. [15,16] found that many of its antibacterial actions may be caused by the reaction between zinc ions and sulfhydryl groups. Applerot et al. [17] reported that ZnO exhibits antibacterial effect on both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) due to a significant enhancement of the oxidative stress.

Zinc has thus attracted the interest of implanted materials, and has been incorporated into various kinds of biomaterials [18–20]. Our previous study demonstrated that zinc-incorporated TiO_2 coatings on titanium can inhibit the growth of both *E. coli* and *S. aureus* as well as excellent biocompatibility [18]. Huo et al. [21] reported that the incorporation of zinc exhibits excellent osteogenic activity and antibacterial effect. Yamaguchi et al. [22] investigated the influence of zinc sulfate on gene expression of osteoblastic cells. However, stimulatory effect on bone formation is only one side of the coin, high concentration of zinc can cause cytotoxic reactions [1,23]. Therefore, it is significantly important to control the concentration of zinc strictly [24].

Plasma immersion ion implantation (PIII) technology offers unique advantages for treating various biomaterial surfaces in recent years. PIII technique enables to embed various element into the near-surface region of various substrate [25], and has been demonstrated to enhance the corrosion resistance, wear resistance, hardness, bioactivity and antibacterial effect of biomaterials [26–30]. In addition, one of the most valuable advantages is that PIII can strictly control the concentration and depth distribution of

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implanted ions in substrate by adjusting the implantation parameters [31].

In this work, zinc ions were implanted into titanium for various times using PIII technology to prepare the Zn-implanted titanium samples with various concentration of zinc. The behaviors of osteoblastic cells and rat mesenchymal stem cells (rMSCs) on the various surfaces were investigated. The antibacterial effect of the samples against both *E. coli* and *S. aureus* was also examined.

2. Materials and experimental details

2.1. Specimen preparation and characterization

2.1.1. Preparation of Zn-implanted titanium

Commercially pure titanium plates (Cp Ti, Grade 2) with size of 10 mm × 10 m × 1 mm were used. The plates were then mirror polished and ultrasonically cleaned with acetone or ultrasonically cleaned in ethanol and deionized water several times, followed by acid etching in 5 wt% oxalic acid at 100 °C for 2 h and ultrasonically cleaned with deionized water, ethanol and ultra pure water successively. Before Zn ion implantation, the titanium samples were cleaned by radio frequency (RF) argon ions sputtering for 15 min at a bias of −550 V. Zn ions were then implanted into titanium plates at 15 kV of implantation voltage for 0.5 h, 1.0 h, 1.5 h and 2.0 h (represented as Zn0.5, Zn1.0, Zn1.5, Zn2.0, respectively). During implantation, the sample stage was cooled by circulating water in order to keep the sample temperature at room temperature. Detailed implantation parameters are shown in Table S1.

2.1.2. Surface chemistry and structure characterization

The surface views of titanium surfaces before and after Zn ion implantation were observed by scanning electron microscopy (SEM) (S-3400, Japan). The phase compositions were analyzed by X-ray diffraction (XRD; D/MAX-2550, Rigaku, Japan). The elemental depth profiles and chemical states were determined by X-ray photoelectron spectroscopy (XPS) (Physical electronics PHI 5802).

2.1.3. Zinc ion release

The Zn-implanted titanium samples were immersed in 10 ml physiological saline solution (0.9% NaCl at a pH of 7) for 21 days at 37 °C without stirring. The amounts of released zinc ions at 7, 14 and 21 days were determined by analyzing the resulting solutions using inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

2.2. In vitro cytocompatibility evaluation

2.2.1. Cell culture

The rat mesenchymal stem cells (rMSCs) (Cells Resource Center, Shanghai Institutes of Biological Science, Shanghai, PRChina) were seeded on oxalic acid etched titanium surfaces before and after Zn ion implantation to evaluate the cytocompatibility. The cells were cultured at 37 °C in a 5% CO_2 incubator in 75 cm^2 flasks (Corning Incorporated, USA) containing 8 ml of α-minimum essential medium (α-MEM) (Minimum Essential Medium alpha-Medium, Gbico, Invitrogen Inc.), 10% fetal bovine serum (FBS) (Hyclone, USA), 1% antimicrobial of penicillin and streptomycin (Antibiotic–Antimycotic, Hyclone, USA). The cells were subcultured every 3 days, washed twice with a phosphate buffer saline (PBS) (pH = 7.4, Hyclone, USA), and incubated in a trypsin/EDTA (0.25% trypsin, 0.02% EDTA) (Gibco, Invitrogen) solution for 3 min at 37 °C to detach the cells. Then centrifuged at 1000 r/min for 5 min and resuspended in the complete medium for reseeding on the various surfaces. The specimens were sterilized in 75% ethanol for 2 h and put into a 24-well plate (Nunc, USA). Afterwards, 1.0 ml of the cell suspension with a cell density of 5.0×10^4 cell/ml was added to each well, the culture plate was transferred to the 37 °C incubator.

2.2.2. Cell morphology

In the fluorescence microscopy (Olympus, Japan) observation, a 1.0 ml cell suspension with a cell density of 5.0×10^4 cell/ml was seeded on the various surfaces. After cultured for 1, 4, and 24 h, the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde (PFA) solution (Sigma, USA) for 10 min at room temperature, followed by three times PBS rinses. The cells were permeabilized with 0.1% (v/v) Triton X-100 (Amresco, USA) for 2 min at room temperature, followed by three times PBS rinses. Add 1 wt% BSA (Sigma, USA) to each sample for 5 min. They were then stained with FITC-Phalloidin (Sigma, USA) at room temperature in the dark for 1 h, followed by three times PBS rinses and further staining with DAPI (Sigma, USA) for 5 min. The cytoskeletal actin and cell nuclei were observed by fluorescence microscopy.

2.2.3. Alkaline phosphatase activity assay

Rat MSCs were seeded on the various surfaces in 24-well plates to evaluate the alkaline phosphatase (ALP) activity, the cell density was 1.0×10^4 cell/ml (7 days) and 0.5×10^4 cell/ml (14 days). After cultured for 7 and 14 days, the cells were rinsed with PBS twice and immersed in lysis buffer at 4 °C for 40 min. After 40 min, the cells were dissociated off the surfaces and incubated with p-nitrophenyl phosphate (pNPP) (Sigma, USA) at 37 °C for 30 min. ALP activity was assayed by measuring the optical density (OD) values of absorbance at 405 nm. A BCA protein assay was used to calculate the total protein content, the result was normalized with a series of BSA (Sigma, USA) standard at 570 nm. At last ALP levels were normalized to the total protein content, and described as μM/μg of total proteins. The measurements were done in triplicate.

2.2.4. Collagen secretion

Collagen secretion on the specimens was quantified by Sirus Red staining. Rat MSCs were seeded on the various surfaces, the cell density was 1.0×10^4 cell/ml (7 days) and 0.5×10^4 cell/ml (14 days). After cultured for 7 and 14 days, the cells were rinsed with PBS three times then fixed in 4% PFA for 20 min, followed by three times PBS rinses, and then stained in a 0.1% Sirius Red solution (Sigma) in saturated picric acid for 18 h. The cells were washed with 0.1 M acetic acid until no more red color appearing. After taking images, in the quantitative analysis, the stain was dissolved in 0.5 ml of the destain solution (0.2 M NaOH:methanol = 1:1). Then measure the OD values of absorbance at 492 nm. The experiment was carried out in triplicate.

2.2.5. Extracellular matrix mineralization

Extracellular matrix (ECM) mineralization was evaluated by Alizarin Red staining. After cultured for 7 and 14 days, the cells were rinsed with PBS three times and then fixed in 75% ethanol for 1 h. The cells were stained with 40 mM Alizarin Red in distilled water for 10 min at room temperature. Then the cells were rinsed with distilled water until color disappeared and images were taken. In the quantitative analysis, the stain was eluted in 10% cetylpyridinum chloride in 10 mM sodium phosphate. Then measure the OD values of absorbance at 600 nm. The measurements were done in triplicate.

2.3. Antibacterial test

The antibacterial effect of titanium surfaces before and after Zn ion implantation against *E. coli* (Gram negative) (ATCC 25922) and *S. aureus* (Gram positive) (ATCC 25923) was evaluated by the bacterial counting method. *E. coli* was cultured in Luria–Bertani (LB) broth or LB agar plates, while *S. aureus* was cultured in tryptic soy broth (TSB)

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