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Protocols

Immobilizing bacitracin on titanium for prophylaxis of infections and for improving osteoinductivity: An *in vivo* study

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ABSTRACT

Bacitracin immobilized on the titanium (Ti) surface significantly improves anti-bacterial activity and biocompatibility *in vitro*. In the current study, we investigated the biologic performance (bactericidal effect and bone-implant integration) of bacitracin-modified Ti *in vivo*. A rat osteomyelitis model with femoral medullary cavity placement of Ti rods was employed to analyze the prophylactic effect of bacitracin-modified Ti (Ti-BC). Thirty-six female Sprague Dawley (SD) rats were used to establish the Ti implant-associated infection. The Ti and Ti-BC rods were incubated with and without *Staphylococcus aureus* to mimic the contaminated Ti rod and were implanted into the medullary cavity of the left femur, and sterile Ti rods were used as the blank control. After 3 weeks, the bone pathology was evaluated using X-ray and micro-computed tomography (micro-CT) analysis. For the investigation of the Ti-BC implant osseointegration *in vivo*, fifteen SD rats were divided into three groups (N = 5), namely Ti, Ti-dopamine immobilized (Ti-DOPA), and Ti-BC. Ti rods were implanted into the left femoral cavity and micro-CT and histological evaluation was conducted after 12 weeks. The *in vivo* study indicated that Ti-immobilized bacitracin owned the prophylaxis potential for the infection associated with the Ti implants and allowed for the osseointegration. Thus, the multiple biofunctionalized Ti implants could be realized via immobilization of bacitracin, making them promising candidates for preventing the Ti implant-associated infections while retaining the osseointegration effects.

1. Introduction

Titanium (Ti) and its alloys are widely applied clinically and used as orthopedic and dental implants because of their excellent mechanical properties and good biocompatibility. However, infection and poor osseointegration have been identified as the two major factors contributing to the early orthopedic implant failure [1]. Ti implants require rapid bone-implant osseointegration when implanted *in vivo* [2,3]. To achieve this goal, the Ti implant should be fabricated to prevent infection around the implant, which may result in a severe inflammatory reaction and active osteoclasts, leading to periprosthetic osteolysis and loosening of the implant. In addition, the Ti implant should promote bone-implant osseointegration. However, Ti itself is a bio-inert material and cannot accelerate the osteogenic differentiation of bone marrow mesenchymal stem cells [4]. Surface biofunctionalization of Ti is a main approach to improve its bioactivity [5,6]. Functional groups and nano structures can be easily introduced to the Ti surface to provide an immobilization site for bioactive drugs. NaOH-treated Ti surfaces have a high number of hydroxyl groups and dopamine-treated Ti provides a bioactive layer for drug immobilization [7]. The NH_2 group can be introduced to the Ti surface via (3-aminopropyl) triethoxysilane (APTES) [8–10]. These functional groups can act as binding sites to immobilize bioactive molecules on the Ti surface. The design of a functional Ti surface must take into account antibacterial properties and osteogenicity [11,12]. However, both

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functions are mutually exclusive because the antibacterial Ti surface immobilizes antibacterial agents (antibiotics, Ag), which are cytotoxic to the osteoblasts. To promote the osteogenic differentiation of bone marrow stem cells on the Ti surface, bioactive molecules such as bone morphogenetic protein-2 (BMP-2), vascular endothelial growth factor (VEGF), collagen, and hyaluronic acid are being investigated [13–17].

Many approaches have been applied to biofunctionalize Ti to improve the antibacterial activity and promote superior osteoinductivity. Immobilization of secondary chemical or bioactive compounds in the matrix of the biomaterial has been widely investigated to mitigate the toxicity of the antibacterial agents (Ag, antibiotics). Strontium and zinc have been widely applied as secondary chemicals to optimize the Ag-functionalized Ti for anti-bacterial activity and improve its biological activity [12,18,19]. Sr and Ag released from Ti nanotubes showed no cytotoxic effects, and the Sr could improve the damaged cortical bone and increase trabecular bone micro architecture. Ag also shows excellent antibacterial properties [11]. Dual systems including antibiotics and osteoinductive protein immobilized on Ti substrates could enhance the osteointegration and implant longevity [20]. Osteoblast adhesion and antibacterial properties were enhanced with the use of Ti nanotubes loaded with antibacterial agents and a surface modified with two poly (lactic-co-glycolic acid) (PLGA) and chitosan biopolymer coatings [21]. However, those strategies had some limitations such as the burst or short time release of antibacterial agents and the biological safety concerns [22].

A previous *in vitro* study demonstrated that Ti modified by bacitracin showed potential antibacterial activity, promoting osteogenic differentiation of bone marrow mesenchymal stem cells and reduction of macrophage inflammation [23]. It is essential to evaluate the *in vivo* performance of bacitracin functionalized on Ti, since the complex biological environment may influence its bioactivity. Hence, the current study was designed to further investigate the effects of Ti implants in an experimental infection model (rat femur implant-related infection model) using a standard *Staphylococcus aureus* (ATCC 25923) strain inoculation.

2. Materials

2.1. Materials

The materials used included Ti6AL4V rods (measuring 1.5 mm in diameter and 20 mm in length), sodium hydrate (Aladdin Industrial Corporation, Shanghai, China), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Tokyo, Japan), *N*-hydroxysulfosuccinimide (NHS, Shanghai, China), dopamine (Sigma-Aldrich), bacitracin (Sigma-Aldrich), *Staphylococcus aureus* (*S. aureus*, ATCC 25923), alizarin red S (Sigma-Aldrich), and calcein (Sigma-Aldrich).

3. Methods

3.1. Preparation of the Ti rod

The Ti implant samples were machined into the rods of 1.5 mm diameter and 20 mm height. The surface stains were removed by ultrasonic cleaning. The bacitracin-modified Ti was prepared according to a previous study [23]. Briefly, the cleaned Ti rod samples were soaked in 5 M NaOH solution at 80 °C for 24 h followed by deionized water at 80 °C for 1 week to remove the residual NaOH. Then, the rod was immersed into 2 mL of dopamine solution (2 mg/mL) with 10 mM Tris buffer (pH = 8.5) in a six well plate. After 12 h, the Ti rod samples were sonicated and rinsed twice with water to remove the residual dopamine on the surface. Then, bacitracin was dissolved in 5 μM ethanoic acid solution. The concentration of bacitracin (1 mg/mL) was selected to ensure that the amino group on the Ti reacted completely with EDC and NHS. The reaction was carried out at room temperature for 8 h. Then, the Ti samples were sonicated and rinsed with water to remove any ungrafted bacitracin on the surface. Each Ti rod sample was sterilized with ethylene oxide.

3.2. In vivo *establishment of anti-infective animal model*

All *in vivo* animal experimental procedures were approved and performed according to the guidelines of the Animal Ethics Committee of Shanghai Ninth People's Hospital, China. Thirty-six female Sprague-Dawley, specified pathogen free (SPF), 12-week-old rats were used. The rats were divided into three groups. The Ti and Ti-bacitracin (BC) rod samples were contaminated with *Staphylococcus aureus* bacteria (concentration: 10^8 CFU/mL, *S. aureus*, ATCC 25923) and were respectively denoted as Ti + *S. aureus* and Ti-BC + *S. aureus* with the addition of Ti+ phosphate buffer saline (PBS) as the negative control. Then, the Ti rods in the three groups (N = 12) were implanted into the left femurs of the rats via the femoral condyles. Briefly, the knee of the rat was opened to expose the condyle, and the bone cavity was expanded with electroporation until it was large enough to hold the Ti rod. After implantation of the Ti rods, the surgical site was closed layer by layer after the opening hole in the femur condyle was sealed with bone wax. The rats were kept in separate cages and were allowed to eat and drink *ad libitum*. The animals were sacrificed after 3 weeks to characterize the Ti implant-associated infection. No antibiotics were administered post surgery.

3.3. Bone pathology and radiographic evaluation

After 1 day and 3 weeks, X-rays were used to evaluate the cortical bone destruction of femurs and knee joints in all the groups. The radiographic scores were evaluated as previously described (N = 5) [24]. Five rats from each group were sacrificed at 3 weeks post-surgery and the femurs were removed for evaluating the gross bone pathology. The remaining rats in each group were sacrificed, and the femurs were scanned with micro-computed tomography (micro-CT) at a resolution of 20 μm. The shafts of the femurs were chosen as the "region of interest" (ROI) to analyze bone histomorphometry. High-resolution images in three-dimensions were obtained from overall, longitudinal, and transverse sections. Three main parameters including the mean trabecular thickness (Tb.Th), bone volume fraction (BV/TV), and the mean trabecular separation (Tb.Sp) were analyzed.

3.4. Microbiological analysis in vivo

After 3 weeks, the rats were sacrificed and femurs were harvested in sterilization (N = 5). Gross bone pathology scores were taken according to the criteria that described preciously using a grading system with the femurs cut sagittally, briefly, 0 represents no local abscess, sequestrum formation, reactive bone formation, or red skin colouration; 1 represents red skin coloration, but no local abscess, sequestrum formation, 2 indicates that reactive new bone formation; 3 indicates that local abscess formation, periosteal reaction, purulent secretions, or sinus formation; 4 indicates that severe bone resorption, abscess formation [24,25].

3.5. Quantitative determination of S. aureus colony forming units (CFU)

The number of CFUs of bacteria dwelling in the Ti rod and around the bone tissue was determined by the spread plate method. The