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Synergistic effects of bisphosphonate and calcium phosphate nanoparticles on peri-implant bone responses in osteoporotic rats

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ABSTRACT

The prevalence of osteoporosis will increase within the next decades due to the aging world population, which can affect the bone healing response to dental and orthopedic implants. Consequently, local drug targeting of peri-implant bone has been proposed as a strategy for the enhancement of bone-implant integration in osteoporotic conditions. In the present study, an established *in-vivo* femoral condyle implantation model in osteoporotic and healthy bone is used to analyze the osteogenic capacity of titanium implants coated with bisphosphonate (BP)-loaded calcium phosphate nanoparticles (nCaP) under compromised medical conditions. After 4 weeks of implantation, peri-implant bone volume (%BV; by μCT) and bone area (%BA; by histomorphometry) were significantly increased within a distance of 500 μm from implant surfaces functionalized with BP compared to control implants in osteoporotic and healthy conditions. Interestingly, the deposition of nCaP/BP coatings onto implant surfaces increased both peri-implant bone contact (%BIC) and volume (%BV) compared to the deposition of nCaP or BP coatings individually, in osteoporotic and healthy conditions. The results of real-time PCR revealed similar osteogenic gene expression levels to all implant surfaces at 4-weeks post-implantation. In conclusion, simultaneous targeting of bone formation (by nCaP) and bone resorption (by BP) using nCaP/BP surface coatings represents an effective strategy for synergistically improvement of bone-implant integration, especially in osteoporotic conditions.

1. Introduction

The use of bone implants in dental or orthopedic rehabilitation is generally successful as reflected by 10-year survival rates of 95% in healthy patients [1]. In the clinic, however, the aging patient population challenges the use of implants due to general as well as oral health issues in modern societies. For instance, osteoporosis, a common systemic bone disease, develops with age and is more prevalent in women and men aged above 50 years [2]. The prevalence of osteoporosis has been reported to increase up to 70% in patients at 80 years old. Worldwide, osteoporosis affects approximately 200 million people and the national osteoporosis foundation (NOF) estimates that over 40 million people in the USA already have osteoporosis or are at high risk in 2020 [3,4]. Osteoporosis is characterized by a severe decrease in bone mass and alteration of trabecular bone microstructure due to an imbalance between bone resorption (by osteoclasts) and bone formation (by osteoblasts) [5]. Further, it has been shown that bone healing in osteoporosis is impaired and the biological activity of bone cells is negatively influenced [6]. For bone implant treatment in osteoporotic patients, the osteoporotic condition impedes primary stability, biological fixation and final osseointegration [7]. As such, the application of bone implants in osteoporotic patients remains a clinical challenge in dental and orthopedic surgery.

For successful implant osseointegration, the bone-implant interface has to interact optimally with the bone tissue in the implant vicinity. For medically healthy patients, new bone (i.e. woven bone) is formed directly in contact with the implant surface by osteoblastic cells, where after it transforms into mature bone [8]. This interaction can be improved by implant-related factors, such

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as implant design, surgical technique, and osteophilicity of the implant surface [9]. Because the implant surface directly interacts with bone tissue, a variety of surface modifications have been explored [10]. The currently available surface modifications aim to combine advantages of physical properties (e.g. roughness) with bioactive cues (i.e. bone-bonding) to improve implant integration [11]. Over the past two decades, calcium phosphate (CaP) coatings have demonstrated to favor the healing response to the implant surface and hence to enhance peri-implant bone formation [12,13]. The presence of a CaP coating at the implant surface is anticipated to facilitate colonization by mesenchymal precursor cells and upregulate specific gene expression in the vicinity of the implant [14]. Recently, it was shown that electrostatic spray deposition (ESD) enables the functionalization of implant surfaces with CaP nanoparticles (nCaP) that mimic the mineral component of natural bone [15]. An additional advantage of ESD is that it enables the deposition of nCaP in combination with organic biomolecules such as collagen proteins, growth factors, peptides, or other therapeutic agents [16]. As a consequence, a novel generation of therapeutic implant coatings can be synthesized that instruct bone cells by releasing drug molecules locally around the implant surface, especially for compromised conditions such as osteoporosis [17,18]. In clinical practice, the deposition of nCaP in combination with therapeutic implant coatings might have a dual effect during bone-implant integration, and their concomitant use may offer a simultaneous targeting of peri-implant bone anabolic/catabolic processes. Particularly bisphosphonates (BPs) are appealing for such purpose, as their therapeutic function to inhibit osteoclast proliferation and activity can be exploited for local effects in the vicinity of an implant surface toward improved implant fixation [19]. In view of this, the idea for therapeutic nCaP/BP coatings for bone implants represents an appealing approach to improve bone responses and implant integration in osteoporotic conditions.

The present study aimed to evaluate the efficacy of an ESD-derived nCaP/BP coating on peri-implant bone response in osteoporotic as well as healthy conditions using an established rat femoral condyle implantation model [20,21]. At 4 weeks post-implantation, histological, histomorphometrical, and micro-computed tomography (μCT) were performed. In addition to conventional histological analysis, we also performed real-time polymerase chain reaction (RT-PCR) after 4 weeks of healing to evaluate osteogenic gene expression in the peri-implant bone in osteoporotic and healthy conditions.

2. Materials and methods

2.1. Preparation of implants

Pin-shaped implants were made of commercially-pure titanium with main diameter of 3.1 mm and length of 7.0 mm. The implant model was featured by a pin part (diameter: 1.5 mm and length: 4 mm) to facilitate a standard method of harvesting bone-implant tissues for histological and genetic analyses. All implants were grit-blasted (roughness, $Ra = 0.5$ μm) and cleaned ultrasonically in nitric acid 10% (15 min), acetone (15 min), and ethanol (15 min) and thereafter air-dried.

2.2. Electrostatic spray deposition (ESD) of coatings

ESD coatings were deposited as previously described [22] using a commercially available electrostatic spray deposition (ESD) device (ES-2000S, Fuence Co., Ltd., Japan). The following standardized conditions were applied: 20% relative humidity; 30oC substrate holder temperature; 40 mm nozzle-to-substrate distance; 4 μl/min liquid flow rate; and 10–12 kV applied voltage. The medium used for deposition of all the coating groups consisted in a solution of 50% ethanol. Thereafter, ESD coating was performed in three separate runs (with in between implant turning of 1200) of 30 min each to obtain complete coating coverage. Titanium implants were provided with 3 types of coating or left non-coated:

A: non-coated implants
B: nCaP
C: nCaP/BP
D: BP

2.2.1. nCaP coating

For deposition of nCaP coatings, nano-sized apatite crystals were synthesized according to a previously described method [23]. Briefly, nCaP crystals were obtained from a suspension of $Ca(CH_3COO)_2$ (0.35 M) by the slow addition (1 drop/second) of an aqueous solution of H_3PO_4 (0.21 M). The pH was kept constant (pH = 10) by the addition of a $(NH_4)OH$ solution. After 24 h, the solid residue was collected by centrifugation, washed four times with ultrapure water and then suspended in 100 ml of ethanol. The amount of nCaP coating was measured using the ortho-cresolphthalein (OCPC) method (Sigma–Aldrich, Munich, Germany) as previously described [23,24]. In brief, 300 μL of work reagent was added to 10 μL aliquots of sample or standard in a 96-wells plate. The plate was incubated for 10 min at room temperature, and then the plate was read at 570 nm. Serial dilutions of $CaCl_2$ (0–100 $\mu g \cdot mL^{-1}$) were used for the standard curve.

2.2.2. nCaP/BP coating

For nCaP/BP coatings, alendronate sodium trihydrate ≥97% powder (A4978; Sigma–Aldrich, Munich, Germany) was added to a suspension containing nCaP crystals at a concentration of 3 mg/ml and weight ratio of 1:10 for 24 h. The attached percentage of alendronate onto nCaP crystals was determined by measuring the drug concentration in the supernatant solution using ultraviolet-visible spectroscopy based on the detection of the primary amino group with ninhydrin as previously described [23,25].To evaluate the total amount of nCaP/BP coating, a standard curve was made by dissolving known amounts of nCaP/BP synthesized according to the method described above. Based on OCPC method, the amount of nCaP/BP coating onto an implant surface was measured 8 ± 0.9 μg/cm^2 and weight ratio of 1:10 (i.e. 8 μg/cm^2 nCaP and 0.8 μg/cm^2 bisphosphonate).

2.2.3. BP coating

For BP coatings, a solution of alendronate sodium powder was dissolved in milli-Q and adjusted with ethanol to a final concentration of 0.3 mg/ml. The quantity of the BP drug onto an implant surface was estimated 0.8 μg/cm^2.

2.2.4. Implant sterilization

All implants were sterilized using ethylene oxide (EO; Synergy Health plc, Venlo, The Netherlands).

2.3. Animal model and surgical procedures

The study was approved by the Animal Ethical Committee of the Radboud University Medical Centre (DEC-2011-258). All *in-vivo* experiments obeyed the guidelines (national and international) for animal care and the Dutch law concerning animal welfare and conformed to the ARRIVE guidelines.

For animal experiments, 60 skeletally-mature male Wistar rats (12-weeks old, weight ~350 g) underwent orchidectomy (ORX) surgery to induce osteoporosis through a loss of gonadal function (hypo-gonadism) as well as sham operations. Osteoporotic conditions were allowed to establish for 6 weeks before implants were installed bilaterally in the femoral condyles as previously described [20,21].

2.3.1. Assessment of osteoporosis condition

In brief, after 6 weeks, ORX rats as well as the sham-operated rats ($n = 3$ of each animals group) were scanned by small-animal *in-vivo* μCT imaging system (Inveon; Siemens Medical Solutions, Knoxville, TN). Then, CT images were acquired with the manufacturer recommended parameters: voltage 80 kVp, anode current 500 μA, angular sampling 1° per projection for a full 360° scan, effective pixel size 30 μm, and exposure time of 1 s. Thereafter, the scanned μCT data for right and left femoral condyles were imported into Inveon Research Workplace 3.0 program (Siemens Medical Solutions USA Inc, Knoxville, USA). Trabecular bone was selected by drawing volume of interest (VOI) in metaphyseal region. The following trabecular bone morphological parameters were automatically computed: (1) bone volume fraction (%BV), (2) trabecular thickness (Tb.Th mm), (3) trabecular number (Tb.N mm^{-1}), and (4) trabecular separation (Tb.Sp mm).

2.3.2. Surgical procedure for installation of implants

After confirmation of the osteoporotic conditions, the pin-shaped implants were installed in the femoral condyles under inhalation anesthesia (2% Isoflurane® by volume). Briefly, at the intercondylar notch, a cylindrical hole (diameter: 1.5 mm and depth: 7 mm) was initially prepared parallel to the long axis of the femur, using dental burs and surgical motor (Elcomed 100, W&H Dentalwerk Burmoos, Austria) with low rotational drill speed (800 rpm) and continuous external cooling with saline. Then, only the top part of the prepared hole (3 mm in depth) was increased in diameter to 3.2 mm thereafter, implants were placed (press-fit) bilaterally into the predrilled holes, resulting in two implants per rat randomly.

2.4. Analytic methods

A power-analysis was performed to calculate the study sample number using the following formula: $n = 1 + 2C(s/d)^2$. We assumed a standard deviation (s) of 12.5 and an effect size (d) of 15. C-value was fixed at 7.85 (resulting from $1 - \beta = 0.8$ and $\alpha = 0.05$). There were a total of four experimental (coated and non-coated implants) groups with at least 30 test animals per condition (osteoporotic and healthy). Each