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Short-term in vivo evaluation of zinc-containing calcium phosphate using a normalized procedure

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ABSTRACT

The effect of zinc-substituted calcium phosphate (CaP) on bone osteogenesis was evaluated using an in vivo normalized ISO 10993-6 protocol. Zinc-containing hydroxyapatite (ZnHA) powder with 0.3% by wt zinc (experimental group) and stoichiometric hydroxyapatite (control group) were shaped into cylindrical implants (2 × 6 mm) and were sintered at 1000 °C. Thermal treatment transformed the ZnHA cylinder into a biphasic implant that was composed of Zn-substituted HA and Zn-substituted β-tricalcium phosphate (ZnHA/βZnTCP); the hydroxyapatite cylinder was a highly crystalline and poorly soluble HA implant. In vivo tests were performed in New Zealand White rabbits by implanting two cylinders of ZnHA/βZnTCP in the left tibia and two cylinders of HA in the right tibia for 7, 14 and 28 days. Incorporation of 0.3% by wt zinc into CaP increased the rate of Zn release to the biological medium. Microfluorescence analyses (μXRF-SR) using synchrotron radiation suggested that some of the Zn released from the biomaterial was incorporated into new bone near the implanted region. In contrast with previous studies, histomorphometric analysis did not show significant differences between the newly formed bone around ZnHA/βZnTCP and HA due to the dissolution profile of Zn-doped CaP. Despite the great potential of Zn-containing CaP matrices for future use in bone regeneration, additional in vivo studies must be conducted to explain the mobility of zinc at the CaP surface and its interactions with a biological medium.

1. Introduction

There is a critical demand for bone substitute materials in medicine and dentistry. Bone defects that result from congenital, infectious, traumatic or neoplastic processes represent the most important challenges in reconstructive treatment. Autologous bone grafting is considered to be the gold standard in reconstruction, but the supply of autologous bone is limited, and the harvesting of the graft is associated with morbidity [1].

Hydroxyapatite (HA) is widely used as a biomaterial to fill bone defects and to coat the metal parts of prostheses. Synthetic HA is well known as an implant material and has excellent biocompatibility characteristics, including non-toxicity, low biodegradability and bone affinity [2,3]. HA is considered to be osteoconductive due to its ability to strongly bond with natural bone tissue [4,5].

Despite having these optimal properties, synthetic HA differs from biological apatite. Several research studies have attempted to mitigate these differences by doping synthetic HA with small amounts of impurities. These ionic substitutions can alter the properties of HA, including its crystallinity, morphology, lattice parameters, stability, solubility and mechanical character [6–8].

The inorganic component of the bone tissue is a nonstoichiometric carbonated apatite containing substitutions of Na^+, K^+, Mg^{2+}, Sr^{2+}, Cl^-, F^-, HPO_4 and Zn^{2+} [8]. Studies have been carried out to investigate the effects of changes in the composition of HA to better understand and improve the tissue response after HA implantation. The presence of trace elements affects bone formation and resorption through direct or indirect effects on bone cells or bone mineral [9]. The gradual release of divalent cations (Mg^{2+} and Zn^{2+}) incorporated into HA may favor bone repair by improving the cytocompatibility conditions for osteoblast adhesion or may serve as an in vivo model of heavy metal toxicity [7]. These modified forms of HA, known as proactive bioceramics, stimulate the desired responses from the surrounding cells and tissues necessary to promote bonding of orthopedic and dental implants to the bone [6].

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Zinc is an essential trace element for many animals, including humans, and it is known to play a role in growth and development [10]. In vitro studies revealed that granules consisting of 5% mol Zn^{2+} incorporated into hydroxyapatite are cytocompatible [11]. Recent in vitro studies showed that zinc-containing hydroxyapatite decreased inflammation and increased chemotaxis [2] and demonstrated in vitro antimicrobial effects towards *Streptococcus mutans* and *Staphylococcus aureus* [12,13]. In addition, doping HA with 2% mol Zn^{2+} significantly increased osteoblast adhesion compared to undoped HA [6], while doping HA with concentrations of Zn^{2+} between 0.6 and 1.2 wt.% enhanced the proliferation of mouse osteoblast-like cells in composite ceramic [14]. A recent study showed that ZnHA possessed enhanced bioactivity because an increase in the growth of human adipose-derived mesenchymal stem cells, along with the bone cell differentiation markers, was observed [13].

In vivo evaluations of Ca substitution for Zn in CaP have been performed using implant materials with different compositions (βZnTCP, αZnHA, ZnHA/βZnTCP, βZnTCP/HA and ZnHA), variable Zn^{2+} concentrations (from 0.01 to 2.7 wt.%), different preparation routes and animal models (rats, rabbits). Although these works demonstrated that Zn^{2+} had stimulatory effects on bone formation and an inhibitory effect on osteoclastic bone resorption [4,5,15–20], many doubts remain concerning the optimum amount of zinc in implants and the role of zinc in osteogenesis involving Zn-containing CaP biomaterials.

Based on the background discussed above, the purpose of the present work was to compare the in vivo efficacy of a biphasic CaP (ZnHA/βZnTCP) with 0.3 wt.% Zn and sintered HA using the normalized ISO 10993-6 protocols. According to this standard procedure, CaP cylinders that were 2 mm in diameter and 6 mm in height were implanted into rabbit tibias. The efficacy of the cylinders in promoting bone formation was evaluated using histological and histomorphometric techniques. In this work, the ZnHA/βZnTCP cylinders were sintered at 1000 °C to reduce the influence of porosity and to induce a partial decomposition of ZnHA as well as to form a biphasic system containing a more soluble βZnTCP phase in addition to ZnHA.

2. Materials and methods

2.1. Synthesis of HA and ZnHA powders

Colloidal hydroxyapatite (HA) was precipitated by the drop-wise addition of an $(NH_4)_2HPO_4$ solution (99% pure, Merck®, Darmstadt, Germany) to an aqueous solution of $Ca(NO_3)_2$ (99% pure, Merck) at 90 °C. The original solutions were adjusted to pH 9.0 by adding NH_4OH. The precipitate was separated by filtration, repeatedly washed with boiling deionized water at pH 7.0 and dried at 100 °C for 24 h. The dried powder was manually ground, and particle aggregates measuring less than 210 μm were separated with a sieve.

The synthesis of HA doped with Zn (nominal concentration of 1% mol) followed the same procedure as described above, except that solutions of both $Zn(NO_3)_2$ and $Ca(NO_3)_2$ were used.

2.2. Cylinder (implant) preparation for the ISO 10993-6 protocol

The ZnHA and HA powders were compacted into cylinders under 200 N in an isostatic press for 2 min. For each cylinder, 0.70 g of ZnHA or HA powder was used, resulting in 2 mm × 6 mm cylinders that were then sintered at 1000 °C for 2 h.

2.3. Characterization of the powders and cylinders

The crystalline mineral phases present in the samples, their crystallinity and the proportion of the hydroxyapatite and β-tricalcium phosphate phases were examined by X-ray diffraction (XRD). The XRD patterns were obtained with a HZG4 diffractometer operating at 30 kV and 15 mA, with CuKα radiation (λ = 1.542 Å). The data were collected in the 2θ range of 10°–100° with a step of 0.02° point per second. The contents of the hydroxyapatite and β-tricalcium phosphate phases in the cylinders were evaluated by the relative intensities of specific peaks of βTCP and HA XRD patterns in the sample as described by Balmain et al. [21]. Mixtures of powders containing known proportions of HA and βTCP sintered at 1000 °C were used to construct a standard curve, $X = (I^{\beta TCP} / (I^{\beta TCP} + I^{HA})$ versus the βTCP content (wt.%), where $I^{\beta TCP}$ and I^{HA} are the integrated intensities of the (0210) βTCP and (300) HA peaks, respectively. The relative intensity of the (003) and (0210) peaks in the XRD patterns of the standard HA and βTCP structures (60% and 100%) were also considered for the determination of X. The β-tricalcium phosphate content of the cylinder was estimated by comparing the X value of the ZnHA sample after calcination at 1000 °C with the X values from the standard curve.

X-ray fluorescence (XRF) and atomic absorption spectroscopy (AAS) were performed to determine the HA stoichiometry, particularly the Zn/Ca molar ratio and calcium substitutions. The XRF spectrometer (XRF — Phillips PW 2400) operated at 40 kV and 50 mA with a Ge (111) crystal, a collimator of 550 μm and a flow detector for the Kα lines of phosphorus, calcium and zinc. The incorporation of Zn^{2+} into HA was estimated with an AA-6800 Spectrophotometer (Shimadzu) operating with an air–acetylene flame atomizer. The zinc absorbance was measured at 213.9 nm. Vibrational modes of phosphate and hydroxyl groups in ZnHA samples were analyzed by Fourier transform infrared spectroscopy. The spectra were obtained in a FTIR-IR—Prestige 21 (Schimadzu) operating in transmission mode from 400 to 4000 cm^{-1}. The dissolution of the ZnHA cylinders was evaluated by determining calcium and zinc released by inductively coupled plasma-optical emission spectrometry (ICP-OES) in 0.088 M MES (4-morpholineethanesulfonic acid hydrate) with pH 5.9 and 0.088 M Hepes (hydroxyethylpiperazine-N-2-ethane sulfonic acid) with pH 7.4 after 1 and 7 days at 37 °C. The morphology of the implants was examined by scanning electron microscopy (SEM — JEOL JSM 5310).

2.4. Animals

Fifteen skeletally mature male and female New Zealand White rabbits weighing between 2500 and 3000 g were used. The animal experiments and breeding were performed under conditions that were approved by the Institutional Review Board (CEP/UFF) no. 195, in compliance with the NIH Guide for Care and Use of Laboratory Animals and with Brazilian legislation on animal experimentation.

2.5. Surgical procedures

All animals were pre-anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg) and anesthetized with spinal anesthesia (lidocaine, 0.3 mg/kg and morphine, 0.1 mg/kg). Following anesthesia and trichotomy, a 4-cm incision was made in the epithelial lining of the animal leg. After exposure of the tibia bone surface and under constant saline irrigation, two holes separated by a 1-cm margin were drilled; each hole measured 2 mm in diameter and penetrated both tibia cortices in a direction perpendicular to the bone axis. Two ZnHA cylinders and two HA cylinders (control) were implanted in the left and right sets of tibia holes, respectively, of each rabbit. The tissue flap was then placed in its original position, and the incision was closed with interrupted #5-0 nylon sutures (Fig. 1). After surgery, the rabbits were allowed to move freely in their cages. The rabbits were injected intramuscularly with one dose of Pentabiótico® (1 mL/kg; Penicillin, antibiotic) and Maxicam® (1 mL/kg; Meloxicam, an anti-inflammatory). A group of five animals was euthanized at the end of each experimental period (7, 14 and 28 days after surgery).

2.6. Histological and histomorphometric analyses

Two fragments, each containing one of the two cylinders from each tibia, were collected. One fragment was fixed in 10% buffered formalin