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Alendronate and Pamidronate calcium phosphate bone cements: Setting properties and in vitro response of osteoblast and osteoclast cells

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ABSTRACT

We have investigated the effect of Alendronate and Pamidronate, two bisphosphonates widely employed for the treatment of pathologies related to bone loss, on the setting properties and in vitro bioactivity of a calcium phosphate bone cement. The cement composition includes α-tricalcium phosphate (α-TCP) (90 wt%), nanocrystalline hydroxyapatite (5 wt%) and $CaHPO_4 \cdot 2H_2O$ (5 wt%). Disodium Alendronate and disodium Pamidronate were added to the liquid phase (bidistilled water) at two different concentrations: 0.4 and 1 mM (AL0.4, AL1.0, PAM0.4, PAM1.0). Both the initial and the final setting times of the bisphosphonate-containing cements increase with respect to the control cement. X-ray diffraction analysis, mechanical tests, and SEM investigations were carried out on the cements after different times of soaking in physiological solution. The rate of transformation of α-TCP into calcium deficient hydroxyapatite, as well as the microstructure of the cements, is not affected by the presence of Alendronate and Pamidronate. At variance, the bisphosphonates provoke a modest worsening of the mechanical properties. MG63 osteoblasts grown on the cements show a normal morphology and biological tests demonstrate very good rate of proliferation and viability in every experimental time. In particular, both Alendronate and Pamidronate promote osteoblast proliferation and differentiation, whereas they inhibit osteoclastogenesis and osteoclast function.

1. Introduction

Biocompatibility, bioactivity, and easiness to adapt to the shape of bone cavities and defects are some of the reasons of the increasing interest towards calcium phosphate bone cements (CPCs) for application in both medical and dental surgery. A further advantage of CPCs with respect to poly-methylmethacrylate (PMMA), the most widely employed material for implant fixation, is the absence of heat development during hardening. Mixing of a calcium phosphate powder with an aqueous solution leads to the hardening of the cement paste through a low temperature dissolution/precipitation reaction [1,2]. The lack of an exothermic reaction is desirable not only to avoid inflammatory tissue response, but also for potential employment of the cements for the delivery of sensitive molecules such as drugs or proteins [3,4]. Bisphosphonates (BPs) are a chemical class of compounds in widespread use since the 1970s for the management of disorders of bone metabolism, associated to bone loss. These compounds bind strongly to hydroxyapatite crystals, they suppress osteoclast-mediated bone resorption, they are retained for a long time in the skeleton, and they are excreted unmetabolized in urine [5]. In bisphosphonates, the oxygen atom that binds the two phosphate groups of pyrophosphate (P–O–P) is substituted by a carbon atom (P–C–P). Individual bisphosphonates are characterized by the two covalently bonded sidechains attached to the central carbon atom, termed R1 and R2. The presence of these groups allows the introduction of numerous substitutions that led to a large number of compounds with different properties. Binding to bone is enhanced when R1 is a hydroxyl group, whereas the R2 side group has some effect on binding but predominantly determines the antiresorptive potency of the bisphosphonates. In particular, the R2 sidechain of nitrogen-containing BPs (N–BPs) can also influence overall bone affinity as a result of the ability of the nitrogen moiety to interact with the crystal surface of bone mineral [5–8]. The utility of bisphosphonates in managing specific disorders of bone resorption is widely recognized. On the other hand, osteonecrosis of the jaws is a recently recognized condition reported in patients treated with bisphosphonates, in particular potent aminobisphosphonates [9,10]. Local administration of these drugs might be helpful against the potential negative effects of over suppression of bone metabolism caused by prolonged use [11–13]. We have investigated the possibility to introduce Alendronate and Pamidronate, two potent aminobisphosphonates, into the composition of a calcium phosphate cement. Herein we report the results of the structural and mechanical characterization carried out on the composite cements

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in order to investigate the modifications of the setting properties as a function of bisphosphonate content, together with the results of in vitro cultures of osteoblasts and osteoclasts.

2. Materials and methods

2.1. Materials

α-TCP (α-$Ca_3(PO_4)_2$) was obtained by solid state reaction of a mixture of $CaCO_3$ and $CaHPO_4 \cdot 2H_2O$ in the molar ratio of 1:2 at 1300 °C for 5 h [14], crushed in a ball mill and sieved through a 400-mesh sieve (20 μm). Nanocrystalline hydroxyapatite (HA) was prepared by dropwise addition of a $(NH_4)_2HPO_4$ solution to a $Ca(NO_3)_2 \cdot 4H_2O$ solution at 90 °C under N_2 atmosphere [15]. The cement powder was prepared by accurately mixing 90% α-TCP with 5% HA and 5 wt% of DCPD ($CaHPO_4 \cdot 2H_2O$, Merck). For the cement paste preparation a liquid/powder ratio of 0.3 ml/g was used. The biphosphonate disodium Alendronate and disodium Pamidronate (Sigma Aldrich) were added in the liquid phase (bidistilled water) at two different concentrations: 0.4 and 1 mM (AL0.4, AL1.0, PAM0.4, PAM1.0). The control samples (CCs) were prepared using bidistilled water as the liquid phase. The two phases were mixed in a mortar for 60 s and the paste was introduced in cylindrical steel moulds for the compaction that was then performed by using a 4465 INSTRON dynamometer set at 300 N for 1 min. The samples were let inside the moulds for 1 h and then extracted and immersed in physiological solution (NaCl 0.9%) at 37 °C for different periods of time up to 21 days.

The final dimensions of the samples were 6 mm in diameter and 12 mm high for structural and mechanical characterization, 6 mm in diameter and 2 mm high for cell culture.

2.2. Structural and mechanical characterization

Initial and final setting times were determined by the Gillmore method according to the ASTM: C 266-89 standard. A needle of certain diameter and weight is rested, at control times, onto a flat surface of a volume of cement. When no visual mark is let down onto the surface by the needle, the cement is considered to be set. The light and thick needle of 113.4 g of weight and 2.13 mm of diameter was used to measure the initial setting time and the heavy and thin needle of 453.6 g of weight and 1.06 mm of diameter for the final setting time. Each experiment was performed in triplicate using 1 g of cement compacted in a 15 mm diameter mould and the measurements performed every minute.

Mechanical characterization was carried out after immersion in physiological solution for different periods: the cements were demoulded and immediately submitted to compression tests by using the INSTRON testing machine, equipped with a 1 kN load cell. At least six specimens for each composition were tested at a crosshead speed of 1 mm/min.

For X-ray diffraction investigation, the samples were demoulded and immediately immersed in liquid nitrogen for 15 min, in order to stop the setting reaction, dried at 37 °C for one night and then ground in a mortar. The samples were packed into recessed silicon slides. X-ray diffraction analyses were carried out by means of a Philips X'Pert powder diffractometer equipped with a graphite monochromator in the diffracted beam. Cu Kα radiation was used (40 mA, 40 kV). The spectra had been obtained in the 3°–50° 2θ range using a 0.03° step and a 3°/min speed.

The relative amount of α-TCP conversion into calcium deficient hydroxyapatite (CDHA) was determined through the measurement of the integrated intensities of the reflections at 25.9° and 24.3° of 2θ, corresponding to the 002 reflection of hydroxyapatite and to the 131 reflection of α-TCP, respectively. The percentage of CDHA in the samples was determined from the value of $I_{002}/(I_{002} + I_{131})$ ratio, using a calibration curve obtained from weighted mechanical mixtures of hydroxyapatite and α-TCP.

Morphological investigation of the fractured surfaces of the cement samples was performed using a Philips XL-20 scanning electron microscope. The samples were sputter-coated with gold prior to examination.

2.3. Cell experiments

2.3.1. Osteoblast culture

MG63 human osteoblast-like cells were cultured in DMEM medium (Sigma, UK) supplemented with 10% FBS, and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin). Cells were detached from culture flasks by trypsinization, washed, and cell number and viability were checked with trypan blue dye exclusion test.

MG63 osteoblast-like cells were plated at a density of 2×10^4 cells/ml in 96-well plates containing sterile samples of the control cement (CC) as reference, and samples of cement prepared with different concentration of Alendronate (AL0.4, AL1.0) or Pamidronate (PAM0.4, PAM1.0). The same concentration of cells was seeded in empty wells for control (CTR). Plates were cultured in standard conditions, at 37 °C with 95% humidity and 5% CO_2. At 24 h medium was changed with DMEM addition with β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml) to activate osteoblasts. For the production of osteocalcin the culture medium was enriched with $1,25(OH)_2D_3$ 48 h before end of experimental time.

2.3.2. Osteoblast proliferation

Cell proliferation reagent WST1 test was done to assess cell proliferation and viability (3 and 7 days): 100 μl of WST1 solution and 900 μl of medium (final dilution: 1:10) were added to the cell monolayer, and the multi-well plates were incubated at 37 °C for a further 4 h. Supernatants were quantified spectrophotometrically at 450 nm with a reference wavelength of 625 nm. Results of WST1 are reported as optical density (OD).

2.3.3. Osteoblast activity and differentiation

Finally, at the end of experimental times the supernatant was collected from all wells and centrifuged to remove particulates, if any. Aliquots were dispensed in Eppendorf tubes for storage at −70 °C and assayed for Type I Collagen (CICP, Prolagen-C enzyme Immunoassay Kit, Metra Biosystem, CA, USA), Osteocalcin (OC, enzyme Immunoassay Kit, Bender MedSystems, Vienna, A), Osteoprotegerin (OPG, Immunoassay Kit, Bender MedSystems, Vienna, A), TNF-related activation-induced cytokine receptor (TRANCE, Immunoassay Kit, Bender MedSystems, Vienna, A); alkaline phosphatase activity (ALP, kinetic assay, Biosystems S.A., Barcellona, E), was tested on supernatants immediately after collection. All the measured concentration and activity were normalized by cell number to take into account the differences in cell growth.

2.3.4. Isolation and characterization of mononuclear cells for osteoclast culture

Peripheral human blood was obtained from healthy adult volunteers. To prevent clotting blood was collected in heparinated tubes. Density gradient centrifugation was used to separate the mononuclear cells from the other elements of blood. Briefly, a volume of peripheral blood was diluted 1:1 with pre-warmed PBS and carefully layered on an equal volume of Histopaque1077 in a 50 ml tube. The tube was centrifuged with 400 g at 20 °C for 30 min. After centrifugation, the mononuclear cells accumulated at the interface between PBS and Histopaque were collected and transferred to another tube. 10 ml of PBS were added and tube was centrifuged with 250 g at 20 °C for 10 min. Pellet was resuspended in 1 ml of culture medium (DMEM + 10% FBS). Trypan-blue method was used to as-