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Brief communication

Cobalt, chromium and nickel affect hydroxyapatite crystal growth in vitro

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ABSTRACT

Metals are widely used in orthopaedics and recent studies have reported that patients with metal implants have a significant increase of metal levels in serum and synovial fluid. Femoral neck fracture occurred in some patients with metal-on-metal implants for unknown reasons. Recently, bone quality has emerged as an important factor of bone strength and few studies have investigated the effects of metal ions on hydroxyapatite properties. In the present study, we investigated the effects of Co^{2+}, Cr^{3+} and Ni^{2+} on hydroxyapatite (HA) growth in vitro, using carboxymethylated poly(2-hydroxyethyl methacrylate) (pHEMA) as a biomaterial for calcification. We have demonstrated that metal ions reduced the quantity of mineral formed at the surface of the polymer and decreased the ratio Ca/P by 1.12-, 1.05- and 1.08-fold for Cr^{2+}, Cr^{3+} and Ni^{2+} respectively. Furthermore, the size of calcospherites was significantly increased in the metal-doped HA compared to the controls, indicating a possible effect of metal ions on the crystal lattice. Indeed, the presence of metal ions increased the crystal size as well as the crystallinity of HA and reduce the lattice parameter *c* of the HA framework. The information obtained from this work suggests that the quality of the mineral around metallic implants could be altered. However, further investigation should be conducted to further elucidate the effects of metal incorporation on bone mineral and the functional consequences.

1. Introduction

Metals are essential for the survival of animals, plants and microorganisms [1]. Because of their different state of oxidation, some of them are a useful component of enzymes and metalloproteins [2]. In healthy volunteers, metal levels are very low and are considered as trace elements. However, metal levels are considerably raised in patients with metal prostheses independently of the bearing couple [3], and the consequence of such concentrations on the body is relatively unknown. Recent studies have reported that patients undergoing metal-on-metal (MoM) resurfacing have elevated serum levels of Co (1.0 $\mu g\,l^{-1}$) and Cr ions (0.8 $\mu g\,l^{-1}$) [4,5], as well as increased levels in synovial fluids (0.3 $mg\,l^{-1}$ of Co and 1 $mg\,l^{-1}$ of Cr) [6,7]. Some of these patients also developed neck narrowing and, in a recent cohort of 377 patients, Little et al. [8] reported that 4% of the patients had undergone femoral neck fracture. Although there is a growing body of evidence that the bone mineral density (BMD) is decreased around the implant in the first postoperative months, this could be attributed to the operation itself, as BMD seemed to increase after 3 months post-surgery [9–11]. Recent reports suggest that, besides bone density, other factors, often referred as "bone quality", influence bone strength. Nowadays, it is widely accepted that bone strength depends on both bone quantity and quality, a term encompassing structural and material properties. Inside the cells, metals undergo oxido-reduction reactions which lead to the generation of free radicals (reactive oxygen species and reactive nitrogen species) (see the review in Ref. [12]). Fleury et al. [13] reported that metal ions induce osteoblast death mainly by increasing the levels of oxidated and nitrated proteins. These free radicals can also attack the DNA double strand and induce damage to purine and pyrimidine bases, as well as to the deoxyribose backbone. Free radicals can also induce crosslinks in DNA and they catalyse the oxidation of phospholipids (a process known as lipid peroxidation) [14–16]. Furthermore, Nichols and Puleo [17] showed that in bone marrow cultures exposed continuously to cobalt and chromium ions there was a significant decrease in the total area resorption. On the other hand, Rousselle et al. [18] documented that exposure of mature rabbit osteoclasts to cobalt ions increased the number of osteoclasts. Metal overload can damage not only the cellular compartment of the bone matrix but also the organic phase [19,20]. Recent studies have reported that strontium, aluminium and iron can be incorporated in the mineral during the mineralization process and could affect its crystal properties [19,21–23]. However, it is still unknown whether cobalt, chromium and nickel ions can affect the mineral properties during physiological crystal growth.

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The aim of the present study was to investigate the role of cobalt, chromium and nickel ions on the in vitro growth of hydroxyapatite crystals, which represents the mineral component of bones. We employed a polymer poly(2-hydroxyethyl methymethacrylate), modified by carboxymethylation (pHEMA-CM), which can mimic the calcification of woven bone in the absence of cells [19,24,25]. Polymer disks were incubated in a synthetic body fluid containing metals at a concentration found in the vicinity of the implant [6]. The hydroxyapatite growth in the presence of metal ions was assessed by chemical analysis, scanning electron microscopy and X-ray diffraction. We found that cobalt, chromium and nickel ions can be incorporated into the mineral during crystal growth and that they dramatically affected the crystal size and crystallinity of hydroxyapatite.

2. Material and methods

Commercial 2-hydroxyethyl methacrylate (HEMA) was purchased from Sigma–Aldrich Chemical (Illkirsh, France). Commercial HEMA contains residual methacrylic acid and crosslinkers due to the fabrication process. The polymerization inhibitor 4Ùmethoxyphenol (added by the manufacturer before shipping, at a concentration of 350 ppm) also needs to be removed. HEMA was purified and distilled under reduced pressure (5×10^{-2} mBar, 65 °C). Others chemicals were used as received.

2.1. Assessment of osteoclast formation and activation

The linear polymer was prepared by bulk polymerization. Briefly, the polymerization mixture was composed of HEMA (10 ml) and 0.2 g of benzoyl peroxide was used as the initiator. The mixture was accelerated by *N*,*N*-dimethyl-*para*-toluidine. The ratio of benzoyl peroxide to *N*,*N*-dimethyl-*para*-toluidine was 100:1 (mol:mol). Monomers were polymerized at 4 °C for 2 h in polypropylene wells (Delta Microscopies, Labege, France). In that way, calibrated disks of pHEMA (150 ± 5 mg) were regularly obtained. Carboxymethylation of the disks was done as reported previously [24].

2.2. Incubation of pellets in synthetic body fluid

A standard synthetic body fluid (SBF) mimicking lymph fluid was prepared according to Yamada et al. [26]. Its composition was as follows: Na, 142.19 mmol l^{-1}; Ca, 2.49 mmol l^{-1}; Mg, 1.5 mmol l^{-1}; HCO_3, 4.2 mmol l^{-1}; Cl, 141.54 mmol l^{-1}; HPO_4, 0.9 mmol l^{-1}; SO_4, 0.5 mmol l^{-1}; and K, 4.85 mmol l^{-1}. Disks of carboxymethylated pHEMA (pHEMA-CM) were sterilized by ultraviolet radiation (360 nm for 3 h) and were distributed in sterile-capped Falcon tubes containing 50 ml of SBF during 21 days at 37 °C to allow formation of calcospherites at the surface. The SBF was replaced every other day. To investigate the effects of metal ions on the mineralization process, the SBF was enriched with 100 μmol l^{-1} of Co^{2+}, Cr^{3+} or Ni^{2+}. At the end of the incubation period, the disks were rinsed in deionized water three times for 10 min to remove any noncrystallized ions. Thirty-six disks were incubated and randomly allocated into four groups. Among the nine disks per group, three underwent chemical analysis, three were prepared for scanning electron microscopy and three were used for X-ray diffraction.

2.3. Chemical analysis

Transfer of the disks into 1 ml of 0.2 M HCl for 24 h led to the complete dissolution of calcospherites. The fluid was then collected and used to determine the amount of free ions on an automated Hitachi 917 spectrophotometer (Roche, France) with standardized clinical reagents for calcium (Calcium Infinity™ Arsenazo III) and phosphate (the reduced phosphomolybdate method) obtained from the manufacturer. Measurement was performed on the fluid obtained from three disks incubated in the same conditions, and the mean ± SD of the triplicate was considered.

2.4. Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX)

Disks to be examined by SEM were processed as previously described [27]. SEM was performed on a JEOL 6301F (Paris, France) field emission microscope equipped with an energy-dispersive X-ray microanalysis machine (EDX, Link ISIS, Oxford Instruments, Oxford, UK). EDX was done by point analysis at the surface of calcospherites to determine their composition. In each group, the size of 30 calcospherites was measured using ImageJ freeware (NIH, Bethesda, MD) on SEM photographs.

2.5. X-ray diffraction (XRD)

Phase analysis of the synthesized HA or metal-doped HA was conducted primarily using XRD employing a PANalytical X'PERT MRD (PANalytical Ltd, Almelo, The Netherlands) using a Cu *K* radiation (λ = 1.5458 Å) and equipped with an X'Cellerator detector, which allows ultrafast data collection with no compromise with the diffractogram resolution. The diffractometer was operated at 40 kV and 45 mA at a 2θ range of 10°–37° with a step size of 0.0125° and an integration time of 75 s per step. Crystallographic identification of the metal-substituted HA was accomplished by comparing the experimental XRD patterns to standards complied by the Joint Committee on Powder Diffraction and Standards (ICDD PDF-2 Release 2004). The size of individual HA crystallites were calculated from XRD data using the Scherrer equation as previously reported [28]. Briefly, the peak of 25°–27° 2θ (002) was fit to define its full width at half maximum intensity ($B_{1/2}$):

$$t = \frac{k\lambda}{B_{\frac{1}{2}} \cos\theta}$$

where t is the crystal size, as calculated for the (002) reflection, λ is the wavelength of the Cu K_α radiation and k is the broadening constant varying with crystal habit and chosen as 0.9 for the elongated apatite crystallites, as previously reported [28].

The crystallinity noted by X_c corresponds to the fraction of crystalline apatite phase in the investigated volume of powdered sample. An empirical relation between X_c and the $B_{1/2}$ was deduced according to the equation as below:

$$K_A = B_{\frac{1}{2}} \times \sqrt[3]{X_c}$$

where X_c is the crystallinity degree, $B_{1/2}$ is the full width of the peak at half intensity of the (002) reflection in 2θ, and K_A is a constant set at 0.24, as previously established [28].

Interplanar distances (d values) obtained by X-ray diffraction (peak 25°–27° 2θ = planes 002, peak 33–34° 2θ = planes 300) allowed the calculation of parameters a and c from the crystal lattice. In a hexagonal system such as hydroxyapatite [21],

$$a = \frac{6}{\sqrt{3 \times d_{(300)}}}$$

$$c = 2 \times d_{(002)}$$

The error of the measurements being greater on plane (300) than on plane (002), the results obtained with parameter c are given more weight.