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Effect of strontium ranelate on bone mineral: Analysis of nanoscale compositional changes

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ABSTRACT

Strontium ranelate has been used to prevent bone loss and stimulate bone regeneration. Although strontium may integrate into the bone crystal lattice, the chemical and structural modifications of the bone when strontium interacts with the mineral phase are not completely understood. The objective of this study was to evaluate apatite from the mandibles of rats treated with strontium ranelate in the drinking water and compare its characteristics with those from untreated rats and synthetic apatites with and without strontium. Electron energy loss near edge structures from phosphorus, carbon, calcium and strontium were obtained by electron energy loss spectroscopy in a transmission electron microscope. The strontium signal was detected in the biological and synthetic samples containing strontium. The relative quantification of carbon by analyzing the C_K edge at an energy loss of $\Delta E = 284$ eV showed an increase in the number of carbonate groups in the bone mineral of treated rats. A synthetic strontium-containing sample used as control did not exhibit a carbon signal. This study showed physicochemical modifications in the bone mineral at the nanoscale caused by the systemic administration of strontium ranelate.

1. Introduction

The anti-osteoporotic drug strontium ranelate can stimulate bone regeneration (Dahl et al., 2001; Marie et al., 2001; Marie, 2006; Boivin et al., 2012) by increasing the number of pre-osteoblast cells and inhibiting bone resorption by osteoclast cells (Marie, 2006; Bonnelye et al., 2008). Mineral density and the mechanical properties of osteoporotic bone tissue could also be positively affected by strontium ranelate treatment, decreasing the risk of bone fractures (Ammann et al., 2004, 2007; Reginster et al., 2008). Although several positive effects of strontium have been shown *in vitro* (Barbara et al., 2004; Bonnelye et al., 2008) and *in vivo* (Boivin et al., 1996; Dahl et al., 2001; Ammann et al., 2004, 2007; Reginster et al., 2008; Meunier et al., 2009; Roschger et al., 2010), the mechanism involved in bone formation in the presence of strontium and the protection against resorption remains poorly understood.

The amount of strontium incorporated into bone mineral is dose dependent over a certain range, up to a plateau level (Dahl et al., 2001). It is heterogeneously distributed in compact bone (low levels of substitution) and cancellous bone (high level of substitution) with a higher uptake in new bone tissue than old one (Boivin et al., 1996, 2010; Farlay et al., 2005; Roschger et al., 2010). These differences are thought to be related to the faster remodeling rate in cancellous bone, allowing a higher incorporation of strontium into the tissue (Dahl et al., 2001).

Two mechanisms of strontium incorporation in bone mineral are currently accepted (Boivin et al., 1996; Dahl et al., 2001; Marie et al., 2001; Boivin and Meunier, 2003; Bazin et al., 2011): (I) a fast mechanism of surface exchange between blood and bone mineral and (II) a slower mechanism of heteroionic substitution, which results in the incorporation of strontium into the crystal lattice replacing calcium. Regarding the chemical aspect of the first mechanism, strontium would be surrounded by oxygen atoms if adsorbed at the surface of apatite crystallites (Bazin et al., 2011). Strontium could also be engaged in a hydrated poorly crystalline apatite phase on the surfaces of the crystals linked to oxygen and phosphate atoms (Cazalbou et al., 2005; Bazin et al., 2011). No structural changes in the lattice parameters would be expected for this mechanism.

The second mechanism is based in the resorption/apposition of bone and depends on osteoclast and osteoblast cells activity. In this case, strontium would substitute for calcium in apatite crystals at

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either crystallographic calcium site I (Ca linked to OH^-) and/or II (Ca linked to PO_4^{3-}) during bone remodeling (Terra et al., 2009; Bazin et al., 2011). Physicochemical changes in the bone mineral would be expected because strontium atoms are larger than calcium atoms.

In synthetic hydroxyapatites, the substitution of calcium by strontium in the crystals caused several changes in the mineral properties, including (1) the expansion of the lattice parameters (Li et al., 2007; Terra et al., 2009), (2) an increase in the local disorder of the lattice (Terra et al., 2009), (3) a decrease in the crystallinity and crystal size (Verberckmoes et al., 2004; Li et al., 2007), (4) a decrease in the number of (OH^-) sites (Verberckmoes et al., 2004; Terra et al., 2009), (5) an increase in the CO_3^{2-} content (Li et al., 2007) and (6) an increase in the solubility (Christoffersen et al., 1997).

In biological apatite, the substitution of calcium by strontium has been described in several studies (Boivin et al., 1996; Boivin and Meunier, 2003; Farlay et al., 2005; Li et al., 2010a). In a recent work on patients treated with strontium ranelate for 5 years, an increase was observed in the lattice parameters of apatite crystals in the regions of bone remodeling (Li et al., 2010a). Based on the variation of the crystallographic parameter along the *c*-axis, it was inferred that up to 5% of the calcium atoms from the crystal lattice had been substituted by strontium atoms. However, few works have explored the effect of strontium in bone mineral at the level of the apatite crystallites (Li et al., 2007, 2010c).

The objective of this study was to evaluate biological bone apatite isolated from rats treated with strontium ranelate, comparing its characteristics at the nanoscale (*e.g.* strontium content, morphology and shape of nanocrystals, Ca/P ratio, presence of carbonate, *etc.*) with those of apatite from untreated rats and of synthetic apatites with and without strontium. Details on the bone mineral properties after strontium incorporation will help to understand the mechanism of biomineralization, bone mineral quality and the potential of strontium salts for clinical applications.

2. Materials and methods

2.1. Biological samples

Biological apatites were obtained from the mandibles of male Wistar rats, 6 weeks old, treated with strontium ranelate (HA_B-Sr) and untreated rats (HA_B, control group). The mandible bone was chosen due to its high levels of strontium incorporation (Oliveira et al., 2012). Animals were treated with strontium ranelate (PROTOS® 2 g, Servier Laboratories) at a dose of 1800 mg/kg/day in the drinking water for 90 days. The animals were killed, and the mandible bones were harvested and cleaned manually. Bone samples were further ground and rinsed with distilled water. The mineral phase was extracted by removal of the organic matrix with a 5% sodium hypochlorite solution for approximately 10 min in continuous motion in a vortex mixer. After 2 min of centrifugation at 14,000 rpm, the mineral pellets were washed three times with Milli-Q water and twice with 100% ethanol, and they were dried overnight at 40 °C. The *in vivo* procedures were performed according to the guidelines of the ethical committee on the use and care of animals at Federal University of Rio de Janeiro.

2.2. Synthetic samples

Synthetic hydroxyapatite (HA_S) and strontium-containing synthetic hydroxyapatite (HA_S-Sr) samples were obtained in aqueous solution. HA_S and HA_S-Sr were precipitated by dropwise addition of $Ca(NO_3)_2$ or $Ca(NO_3)_2 + Sr(NO_3)_2$ solutions to a $(NH_4)_2HPO_4$ solution. The pH was adjusted to 10 by NH_4OH addition and the synthesis reactions were conducted at 90 °C. The suspension was kept in digestion for 3 h. The precipitate was then separated by filtration and repeatedly washed with boiling deionized water, and it was dried at 100 °C for 24 h. The dried powder was manually ground, and particles <200 μm were separated by sieving. Only HA_S was sintered at 1100 °C in order to produce a stoichiometric hydroxyapatite sample. The theoretical chemical composition from HA_S and HA_S-Sr were $Ca_{10}(PO_4)_6(OH)_2$ and $Ca_{9.5}(Sr_{0.5})(PO_4)_6(OH)_2$, respectively. Experimental calcium, strontium and phosphorus concentrations were determined by X-ray fluorescence using a PW 2400 Sequential (Philips) apparatus. The Ca/P and Sr/Ca ratios in HA_S and HA_S-Sr were used as references to calibrate the energy dispersive X-ray (EDX) spectra from the biological samples.

To investigate the carbon signal from the samples by electron energy loss spectroscopy (EELS) in the transmission electron microscope (TEM), a B-type carbonated (HA_S-Carbo) apatite was included in this work. This sample contains CO_3 groups in the PO_4 position, $Ca_{10-x}(PO_4)_{6-x}(CO_3)_x(OH)_{2-x-2y}(CO_3)_y$ (with $0 \le x \le 1.1$ and $0 \le y \le 0.2$). The HA_S-Carbo sample was produced by the same procedure by addition of $Ca(NO_3)_2$ solution to $(NH_4)_2HPO_4 + (NH_4)_2CO_3$ solutions at pH 12. The precipitate was obtained at 90 °C and dried at 100 °C for 24 h. The CO_3 content from HA_S-Carbo (1.3 wt.%) was determined by X-ray fluorescence using a PW 2400 Sequential (Philips) apparatus.

In addition to HA_S, HA_S-Sr and HA_S-Carbo, the commercial drug strontium ranelate (PROTOS® 2 g, Servier Laboratories) was analyzed by EELS to evaluate the experimental conditions for the detection of the M and L ionization edges from strontium.

2.3. Analytical methods and experimental conditions

Powders obtained from the synthetic and biological samples were deposited onto lacey formvar/carbon TEM grids (Ted Pella, Inc.). The method consisted of spreading the sample powders between two light microscope glass slides and putting the TEM grids previously exposed to a plasma cleaner in contact with the powder.

The samples were analyzed with a Cs-corrected field emission gun (FEG) Jeol 2100F (TEM/STEM), operated at 200 kV. The microscope was equipped with an EDX detector (NORAN System) and a Gatan imaging filter (GIF TRIDIEM) for elemental analysis and EELS, respectively.

Electron diffraction patterns were obtained in the select area electron diffraction (SAED) mode from regions with similar surface areas and using the same camera length. The high resolution transmission electron microscopy (HRTEM) images were digitized with a 2048 × 2048 CCD camera (Gatan). Fast Fourier transform (FFT) was applied to the HRTEM images using the Digital Micrograph software (Gatan, Inc.) for a better interpretation of the lattice images.

X-ray microanalyses were obtained in the Jeol 2100F using scanning transmission electron microscopy (STEM) mode, from regions of 50 nm × 50 nm. In STEM mode, the beam never stops in the same place more than 0.05 s (high scanning speed of the focused beam was used), allowing analysis of small areas (approximately 50 nm × 50 nm) with minimal damaging. Ca/P and Sr/Ca relative quantifications were performed using the Ca_K, P_K and Sr_K peaks by considering the thin-film approximation. The HA_S and HA_S-Sr samples were used to calibrate the Ca/P and Sr/Ca ratios, respectively.

The energy-loss signal obtained by EELS provides elemental identification related to the ionization of the core–shell electrons and information about the electronic structure of the specimen atoms that reveals details about their bonding state and the nearest-neighbor atomic structure (Colliex et al., 1991; Garvie et al., 1994; Williams and Carter, 2009). EELS spectra were obtained in STEM mode (for energy losses between 150 and 600 eV) and in TEM mode (for higher energy losses >1800 eV) with a 3 mm spectrometer entrance aperture. The energy resolution was approximately