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Incorporation of Strontium up to 5 Mol. (%) to Hydroxyapatite did not Affect its Cytocompatibility

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The aim of this work was to produce hydroxyapatite (HA) granules containing 0, 0.5, 1 and 5 mol. (%) of strontium (Sr), evaluate the physico-chemical properties and also the cytotoxicity by three different parameters of cell viability (ISO 10993-5, 10993-12). The physico-chemical characterization was carried out by using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and X-ray fluorescence (XRF). The XRD profile presented the main peaks of HA (JCPDS 860740) and the absorption bands of HA were identified by FTIR. The XRF results showed that the strontium concentration was close to the theoretical value. Regarding the cytotoxicity assays, the incorporation of strontium up to 5 mol. (%) to the HA did not affected dehydrogenase activity (XTT, 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide), membrane integrity (neutral red uptake) or DNA contend (incorporation of crystal violet), in relation to HA alone. In conclusion, hydroxyapatite containing from 0.5 to 5 mol. (%) of Sr was successfully produced and presented no cytotoxicity.

Keywords: *strontium, hydroxyapatite, cytotoxicity*

1. Introduction

Bone loss due to chronic diseases or trauma can result in large defects, which challenge clinicians, and most of the time bone grafts are needed. Research is being conducted in order to improve bone formation through the implantation of biomaterials with a superior bioactivity. Calcium phosphate ceramics, such as hydroxypapatite (HA), are widely used as bone grafts because of their similarity to the mineral phase of the bone. Moreover, they seem to induce the formation of a biological apatite layer, and are thus considered bioactive materials[1,2]. Biological apatite is a calcium-deficient apatite with several substitutions in its crystal lattice, such as F^-, Cl^-, Na^+, K^+, Fe^{2+}, Zn^{2+}, Sr^{2+}, Mg^{2+}, and CO_3^{-2} [3,4,5]. Granular material is often used for bone filling in order to avoid phagocytosis, since it has been shown that particle size influences the induction of bone resorption[6].

Strontium (Sr) is a bone-seeking trace element and 98% of its total content is in the skeleton[7]. It was reported that low dosages can improve bone formation while high dosages induce defective bone mineralization[8,9]. Recent in vitro studies showed that strontium ranelate, a novel agent used in osteoporosis (characterized by low bone mass and susceptibility to fractures) treatment, inhibits bone resorption via the osteoclasts and promotes osteoblast replication and bone formation[10]. In vivo tests with strontium-substituted hydroxyapatite (Sr/Ca = 0.10) cement were performed using a rabbit hip replacement model and an apatite layer was found six months later between the cement and the cancellous bone[11]. As reported, strontium-containing hydroxyapatite (Sr-HA) appears to be an interesting bone substitute material. Because of the difference in the atomic radius between strontium and calcium, the crystal lattice becomes distorted and therefore the Sr-HA stability decreases, facilitating its replacement by new bone[12].

In vitro studies of Sr-HA containing 0, 1, 5, 10 and 100 mol. (%) Sr presented good biocompatibility, but the cytotoxicity increased as the strontium concentration increased[13]. Moreover, osteoblast-like MG63 cells cultured on Sr-HA nanocrystals containing 0, 1, 3, 7 atom. (%) exhibited good proliferation and increased values of the differentiation parameters[14]. Differences in the responses of biomaterials have been reported depending on the cytotoxicity test used. Therefore, it is important to evaluate this response by using a combination of more than one parameter related to cell functions in order to increase the reliability of results[15].

Previous works indicated that even Sr-HA with 100 mol. (%) of strontium has a good biocompatibility, but which also showed that cytotoxicity increased proportionally to the strontium content. Due to the ambiguous results in the literature, the aim of this study was to produce dense HA granules containing 0, 0.5, 1 and 5 mol. (%) of strontium (which is closer to the biological content in HA), evaluate the physico-chemical properties and also the cytotoxicity of produced biomaterials by three different parameters of cell viability, according to ISO 10993-5[16] and 10993-12[17] standards.

2. Materials and Methods

2.1. Synthesis of HA and processing the dense granules

Strontium-substituted hydroxyapatites were synthesized by a wet chemical route. A KH_2PO_4 solution (MERCK, 99.5 wt. (%) pure) was prepared and dropped into the $Ca(NO_3)_2$ (VETEC, 99 wt. (%) pure) and $Sr(NO_3)_2$ (MERCK, 99 wt. (%) pure) solution over a period of about 2-3 hours, with constant heating at 40 °C and stirring. The

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pH of the solution was adjusted to 11 using an aqueous solution of ammonium 50% (v/v), with a final concentration of 12.5%. The reaction product was kept in suspension by constant stirring and heating to 40 °C for 2 hours. The precipitate was centrifuged and washed several times with ultra-pure water until pH 7 was reached. Finally, the precipitate was dried for 24 hours at 37 °C. The dried powder was crushed and sieved using a 125 μm sieve to obtain the fine fractions. All materials were uniaxially pressed into cylindrical tablets under a compressive strength of 216 MPa, followed by sintering at 1150 °C for 4 hours in air (2.8 °C/min heating rate). The tablets were crushed and sieved to select dense granules in the range of 300-600 μm.

The Ca + Sr/P molar ratio was equal to 1.5 according to the apatite chemical formula of $Ca_{9-x}Sr_x(PO_4)_6(OH)_2$ in order to produce non-stoichiometric apatites, which are usually more soluble than the stoichiometric ones. Table 1 gives the concentrations used for each apatite composition.

2.2. Physico-chemical characterization

The calcium phosphate phase of the granular material was determined using an X-ray diffractometer (SHIMADZU XRD 6000) with monochromatized Cu kα radiation and an operational tube with a voltage and current of 40 kV and 30 mA, respectively. The angle of diffraction was from 10 to 60° and the granular material was crushed. The resulting trace was analyzed and compared with the standard library of known diffraction patterns. The granules (mixed with KBr) were also examined by FTIR in order to identify the vibrational modes of the molecules (Perkin Elmer, Spectrum 100) in the range of 500-4000 cm^{-1}. At least 16 scans were conducted and the average of the results was reported. The chemical composition of the samples was determined by X-ray fluorescence (XRF) on RIGAKU RIX 3100 equipment with a rhodium tube (4 kW). Therefore, the strontium content and also the Ca+Sr/P ratio could be verified.

2.3. Cytotoxicity test

Samples were extracted in a culture medium (100 $mg.mL^{-1}$, apatite granules/DMEM free of bovine fetal serum) at 37 °C for 24 hours and the extracts were collected for the cytotoxicity assay according to ISO 10993-5[16] and 10993-12[17]. As control were used 1% phenol solution (positive) and titanium powder (100 $mg.mL^{-1}$, as a negative control). MC3T3 osteoblasts (CRL 2594 – ATCC) were seeded into a 96-well cell culture plate (1×10^4 . $well^{-1}$) and cultured in DMEM containing $NaHCO_3$ (1.2 $g.L^{-1}$), ampicillin (0.025 $g.L^{-1}$), and streptomycin (0.1 $g.L^{-1}$) supplemented with 10% bovine fetal serum for 24 hours at 37 °C under 5% CO_2/95% air conditions. Afterwards, the medium was replaced by each extract and supplemented with 10% bovine fetal serum (BFS) and maintained at 37 °C under 5% CO_2/95% air conditions.

After 24 hours of cell exposure to each extract media, cytotoxicity was evaluated using a commercial kit (Cytotox, Xenometrix, Germany) which allows the use of three different parameters of cell survival and integrity on the same sample: 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), neutral red (NR) and crystal violet dye elution (CVDE). The XTT cell proliferation assay is based on the ability of mitochondrial dehydrogenase enzymes to convert the yellow water-soluble tetrazolium salt XTT into orange colored soluble compounds of formazan, measured by their absorbance at 480 nm[18]. Neutral red is a survival/viability test based on the ability of living cells to incorporate the neutral red dye in their lysosomes, where it accumulates on membrane-intact cells[19]; the quantity of dye incorporated can be measured at 540 nm. The CVDE assay evaluates cell density by staining DNA; the absorbance at 540 nm is proportional to the amount of cells in each well[20]. The absorbance data were obtained with a microplate UV/Vis spectrophotometer (PowerWave MS2, BioTek Instruments, USA).

All of the tests were performed in quintuplicate. Mean values and standard deviations were submitted to one-way analysis of variance (ANOVA) and Tukey's post-test considering significant differences if $p < 0.05$.

3. Results and Discussion

The chemical compositions of the powders obtained from the X-ray fluorescence are shown in Table 2. Regarding the X-ray fluorescence results, the strontium concentration incorporated in the HA was very close to the theoretical value (Table 1). Moreover, the (Ca+Sr)/P in all of the substituted HA was close to 1.5, and these results are comparable to the Ca/P ratio in pure HA, 1.53, corresponding to a calcium-deficient apatite.

Figure 1 shows the XRD patterns of hydroxyapatite with the different Sr/Ca molar ratios after sintering. All of the patterns show the mean peaks of hydroxyapatite at 25.8, 31.8, 32.3 and 32.9° (JCPDS 860740), compatible with a Sr-HA solid solution, since the crystalline structure of HA was maintained and no other phase containing strontium was formed after the calcium partial substitution by strontium[21], according to XRD data showed in Figure 1. The XRD of 0, 0.5, and 1 mol. (%) Sr-HA granules exhibited a peak at 31.2°, which is typical of β-TCP and a decreased in its intensity was noted with the augmentation of strontium content. The presence of β-TCP is, probably, a consequence of the transformation of HA to TCP that occurs in calcium-deficient apatites. A previous report had already described the presence of TCP in Sr-HA, and its formation was attributed to the non-stoichiometry of the apatite structure causing structural and compositional changes in elevate temperatures[22]. Structural changes to TCP upon calcining calcium deficient HA at temperatures between 500 and 1100 °C (up to 1450 °C) were also reported earlier[23]. Furthermore, other undesirable phases can be found as reported by Dagang et al.[24] during the synthesis of Sr-HA. One of the most critical parameters in determining properties and thermal stabilities of HA powder is the Ca/P ratio[25]. Accordingly, the β-TCP peak was not observed in 5% Sr-HA samples probably due to its Ca + Sr/P molar ratio, which was higher when compared to the

Table 1. Molar concentrations used for the powder synthesis of $Ca_{9-x}Sr_x(PO_4)_6(OH)_2$, where x = 0.0, 0.5, 1.0 and 5.0.

Sr (x)	KH_2PO_4	$Ca(NO)_2$	$Sr(NO_3)_2$
0.0	0.80	0.91	0.000
0.5	0.80	0.90	0.009
1.0	0.80	0.90	0.018
5.0	0.80	0.86	0.090

Table 2. XRF analysis[1] of granular material showing (Ca + Sr)/P and Sr/Ca ratios.

Samples	(Ca + Sr)/P	Sr/Ca (%)
HA	1.53	0
0.5% Sr-HA	1.48	0.55
1.0% Sr-HA	1.50	1.0
5.0% Sr-HA	1.55	5.0

[1]The data represent one analysis of the sample.

other apatites synthesized herein (Table 2). In addition, the partial replacement of Ca^{2+} by Sr^{2+} increases the solubility of HA. This phenomenon was studied by Christoffersen et al.[26], who reported that the solubility of apatite increases with an increasing strontium content.

The FTIR spectra of HA and Sr-doped HA are shown in Figure 2 and different modes of molecular vibration characteristics of the apatites can be observed. The bands at 1091 (v3), 1049 (v3), 967 (v3), 598 (v4) and 568 cm^{-1} (v4) are typical of phosphate (PO_4^{-3}) molecular vibration and were present in all of the spectra from the synthetic apatites. The sharp band at 3572 and 632 cm^{-1} corresponds to the OH^- stretching mode, characteristic of HA. A well-defined band of carbonate, at 1385 cm^{-1}, was detected when the strontium concentration was equal or superior to 1% Sr. The band present in this region suggests that phosphate ions are partially substituted by carbonate. This carbonate substitution may be due to the reactive absorption of atmospheric carbon dioxide by the alkaline solution during the slurry preparation and reaction. A similar carbonate absorption band in the region of 1367-1527 cm^{-1} was previously reported on Sr-HA[27], which increased of intensity with strontium content. Another study showed that carbonate incorporation decreases the crystallinity of the apatites and enhances solubility[28].

Cytotoxicity assays provide a measure of cell death caused by contact with materials or their extracts, and only one test is not capable of detecting the cytotoxicity of biomaterials[29,30]. Hence, membrane integrity (NR), metabolic activity (XTT) and cell density (CVDE) were evaluated. Figure 3 shows the in vitro cytotoxicity measured by XTT, NR, and CVDE after 24 hours in culture with the apatite (partially substituted with Sr and pure HA) extracts.

The XTT analysis showed no significant difference among the Sr-HA or HA extracts or cells exposed only to DMEM plus BFS. Metabolic activity was around 1.5 fold greater in the Sr-HA groups, irrespective to Sr concentration, in relation to cells in DMEM alone ($p < 0.05$). As expected, the positive control (1% phenol) significantly decreased cell viability.

A similar trend was presented by Fu et al.[13] when the strontium concentration was up to 5%, using the same assay. Capuccini et al.[31] showed that the presence of strontium significantly improved osteoblast adhesion in early culture phases. Moreover, cell adhesion increased with increasing strontium content (1, 5, and 10 Sr at.%). The cytotoxicity of Sr-HA containing 1, 5, 10, and 100 mol. (%) was also evaluated by Yuan-Fei et al.[32] via methyltetrazolium (MTT) assay and showed that all of the bioceramics studied had good biocompatibility. Furthermore, osteoblast-like MG63 cells cultured on the Sr-HA nanocrystals containing 0, 1, 3, and 7 atom. (%) displayed good proliferation and increased values of the differentiation parameters[14]. Osteoprecursor cell behavior was examined through MTT reduction at 3, 7, and 14 days of incubation by culturing them onto 10 mol % Sr-HA surface. The authors concluded that Sr-HA induced better cell attachment and proliferation when compared to HA[33].

Regarding the NR assays it is possible to observe that no effect on the membrane integrity occurred by the exposition to extracts, except in the phenol group (almost 100% reduction), as already reported[4,34] (Figure 3b).

The crystal violet intercalate to DNA and correlates to the cell number. Despite the slightly more CV intensity for 5% Sr-HA group ($p < 0.05$, 30%), no other significant difference was observed. In resume, this data indicates no effect of Sr-HA extracts on cell viability. Also, phenol reduced 70% the number of adhered cells ($p < 0.05$).

It is well known that different HA can be synthesized by substituting calcium, phosphate or hydroxyl groups resulting in biomaterials with different biological and chemical properties. Lima et al.[4] assessed the number of viable Balb/c 3T3 fibroblasts (through an hemocytometer) after exposure to several metal modified apatites extracts for 24 hours as follows: $Ca_{9.5}M_{0.5}(PO_4)_6(OH)_2$ (M=Fe, Zn, Cu, Co, Sr, Pb) and $Ca_{10}(VO_4)_6(OH)_2$. Sr-HA did not present any toxicity while phenol killed 82% of the cells. It was observed an increase of 70% and 20% in the number of cells exposed to the extracts of Fe-HA and Sr-HA, respectively. This study showed that cells response is dependent on the metal that substitutes calcium or phosphate ions in the crystal lattice of HA, and that 0.5% Sr-HA did not affect cell viability.

In general, the assays mainly used to evaluate cell responses on bioceramics are the XTT (reduced in soluble formazan compounds) and the MTT (reduced in insoluble formazan compounds), which are based on similar principles. Therefore, to the best of our knowledge, the literature is poor regarding NR uptake and CVDE assays to compare with the results of the Sr-modified apatites synthesized herein.

Cytotoxicity is a challenging process to analyze, as there are several ways to activate cellular disruption. In addition, all of these assays have their disadvantages that must be taken into consideration when analyzing the obtained data[29,35]. For instance, metabolic impairment assays such as MTT and XTT can be biased by occasional

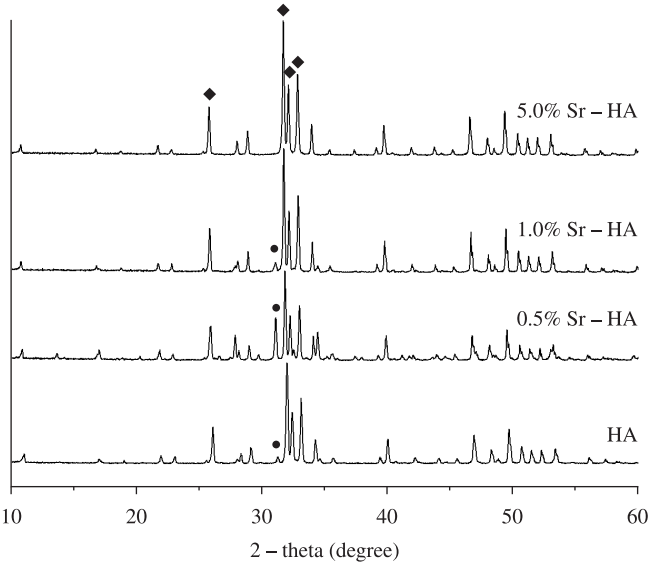

Figure 1. XRD patterns of pure and strontium-doped hydroxyapatites (•-β-TCP and ♦-HA).

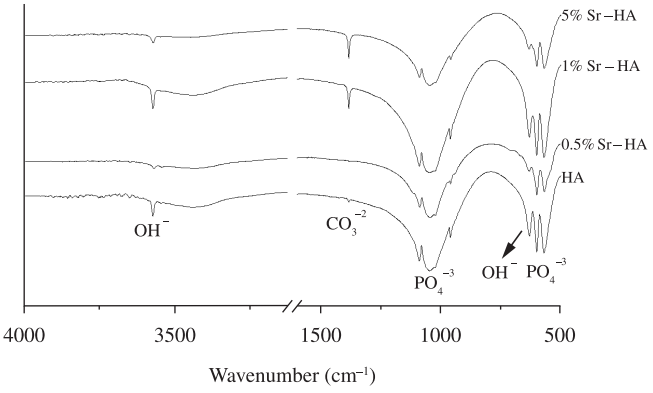

Figure 2. FTIR spectrum of pure HA and strontium-doped hydroxyapatites at different concentrations.

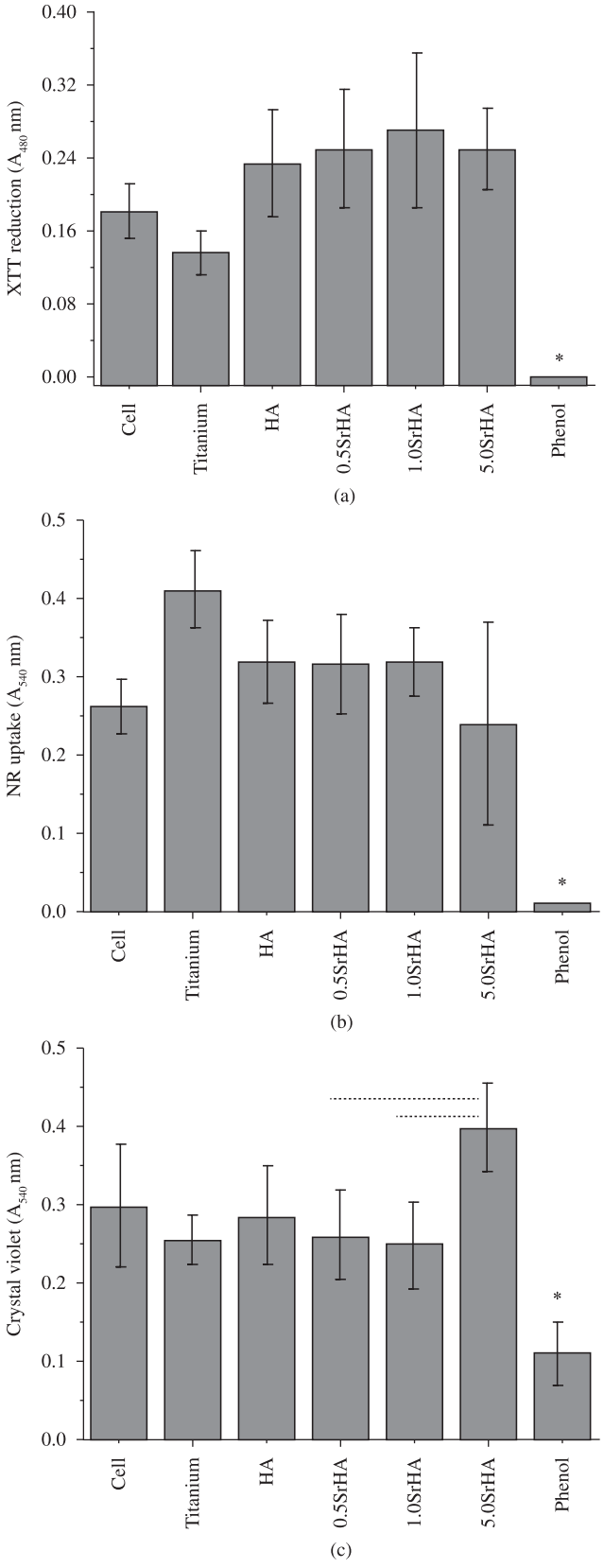

Figure 3. Cytotoxicity assay for pure HA and strontium-doped hydroxyapatites at different concentrations on mouse osteoblasts: a) XTT reduction; b) neutral red uptake; and c) crystal violet dye elution. The bars in the figure represent the standard deviation of means. Phenol group was statistically different from all samples tested in the three assays (*, $p < 0.05$). (- - -) indicate a statistical difference between the samples ($p < 0.05$).

bacterial contamination, which can overestimate cell viability[36]; in contrast, some reducing agents and respiratory chain inhibitors of mitochondrial MTT reduction could underestimate cell viablity[29]. An increase in NR uptake by lysosomal swelling agents such as weakly basic substances[37] could lead to an underestimation of cytotoxicity and CV dye can also stain dead cells due to its non-specificity in binding to DNA[29]. Concerning the CV test result, a considerable cell number was observed in the phenol group. However, those cells are in a death process since no metabolic activity was detected and CV also dyes dead cells that are still attached to the plate. This reinforces the necessity to evaluate more than one aspect of cell survival and integrity before attesting a biomaterial cytocompatibility. Likewise, biomaterials may affect diverse aspects points of cell viability.

Taken together, our results showed that incorporation of strontium in the hydroxyapatite, despite the potential increase in the Sr-HA solubility, did not affect the viability of murine osteoblasts, since no significant effect was observed in the metabolic activity, cell membrane integrity and DNA contend.

4. Conclusion

Granular hydroxyapatite containing from 0 to 5 mol. (%) of Sr was successfully produced and presented no cytotoxicity. The strontium incorporated in the HA crystal resulted in a solid solution with a minor phase (β-TCP) identified by XRD, except for Sr = 5 mol. (%). Further studies are required to evaluate the potential benefits of Sr-HA as a bone substitute biomaterial and its effects in osteoporosis treatment.

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