

Synthesis and cytotoxicity evaluation of granular magnesium substituted β -tricalcium phosphate

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ABSTRACT

Objective: The aim of this study was to produce dense granules of tricalcium phosphate (β -TCP) and magnesium (Mg) substituted β -TCP, also known as β -TCMP (Mg/Ca=0.15 mol), in order to evaluate the impact of Mg incorporation on the physicochemical parameters and *in vitro* biocompatibility of this novel material. Material and Methods: The materials were characterized using X-ray diffraction (XRD), infrared spectroscopy (FTIR), electron microscopy and inductively coupled plasma (ICP). Biocompatibility was assayed according to ISO 10993-12:2007 and 7405:2008, by two different tests of cell survival and integrity (XTT and CVDE). Results: The XRD profile presented the main peaks of β -TCP (JCPDS 090169) and β -TCMP (JCPDS 130404). The characteristic absorption bands of TCP were also identified by FTIR. The ICP results of β -TCMP granules extract showed a precipitation of calcium and release of Mg into the culture medium. Regarding the cytotoxicity assays, β -TCMP dense granules did not significantly affect the mitochondrial activity and relative cell density in relation to β -TCP dense granules, despite the release of Mg from granules into the cell culture medium. Conclusion: β -TCMP granules were successfully produced and were able to release Mg into media without cytotoxicity, indicating the suitability of this promising material for further biological studies on its adequacy for bone therapy.

Key words: Magnesium. Bone substitutes. Biocompatibility.

INTRODUCTION

Research on bone tissue regeneration is constantly expanding due to the improved quality of life and the resulting increase in the world population's life expectancy¹⁰. The regeneration of lost or injured human osseous components is limited; when bone is subjected to extensive damage, a graft is required. Hydroxyapatite (HA) and tricalcium phosphate (TCP) are the most common synthetic biomaterials used as bone grafts, especially in the field of oral and craniofacial surgery, due to their similarity to the mineral phase of the bone. There are two major distinct phases of tricalcium phosphate crystals: α -TCP and β -TCP.

In spite of their similar chemical composition, their different crystallographic features result in different resorption patterns: α -TCP is obtained by heating above 1170°C and is more soluble than β -TCP. In addition, β -TCP is more stable at room temperature than α -TCP, it presents higher solubility than HA and, consequently, can be degraded faster in the body, allowing a desirable gradual replacement by the newly formed bone^{2,8,13}.

Due to the β -TCP instability in water, the most common method to obtain tricalcium phosphate is the wet precipitation of a calcium-deficient apatite (CDA) followed by heat treatment in the range of 700-1170°C^{9,16,24}. Studies concerning β -TCP efficiency as a bone graft were already

conducted. *In vivo* studies with β -TCP implanted in the rat femoral condyle were conducted by Kondo, et al.¹⁴ (2005), concluding that β -TCP has a good biocompatibility, since both bioresorption and bone formation started at an early phase after implantation. Shiratori, et al.²⁶ (2005) also considered β -TCP an osteoconductive biomaterial, based on the histological and molecular findings of bone tissue withdrawn from bone defects in rat femurs previously implanted with β -TCP granules.

Synthetic bone grafts are basically made of calcium phosphate ceramics and, nowadays, research is being directed to calcium phosphates partially substituted by several ions in order to improve the material's properties⁷. Among all substituting cations, magnesium (Mg) is of special importance. Briefly, it is the fourth most abundant cation in the body, the second most abundant in intracellular medium and the most abundant (around 6 mol%) in cartilage and bone tissue during the initial stages of osteogenesis. Mg is an essential cofactor in many biochemical reactions involving ATP as second substrate. In addition, Mg is mitogenic for osteoblasts and its depletion causes inhibition of cell growth *in vitro* due to the resulting reduction in the synthesis of DNA, RNA and proteins. Dietary Mg deficiency is associated with risk of osteoporosis and it also plays an important role in many enzymatic reactions. Additionally, Mg incorporation into TCP crystal lattice reduces the crystallinity of the material and therefore accelerates the degradation process, which may favor bone formation by the osteoblasts^{17,23,29}.

To date, there are very few biological studies about Mg-substituted β -TCP, also known as β -TCMP. Sader, LeGeros and Soares²⁴ (2009) cultivated human osteoblast cells onto sintered tablets of β -TCP and β -TCMP (Mg/Ca=0.2 mol) for 4 h, 24 h and 7 days. The results showed greater cell proliferation on β -TCMP when compared to β -TCP. However, it was recently assumed that part of the Mg could be adsorbed onto the material's surface, since the maximum substitution of Mg for calcium (Ca) in the structure of TCP is 0.15 mol¹. Therefore, the aim of this study was to produce dense granular β -TCP and β -TCMP (Mg/Ca=0.15 mol) in order to investigate β -TCMP biocompatibility by means of an *in vitro* cytotoxicity assay that evaluates two different parameters of cell survival and integrity.

MATERIAL AND METHODS

β -TCP and β -TCMP granules preparation

Mg-substituted calcium deficient apatite (Mg-CDA) containing 0.15 mol of Mg was synthesized by the wet precipitation method as previously described by Sader, LeGeros and Soares²⁴ (2009). Briefly, a solution containing 1.3 M $\text{Ca}(\text{OH})_2$ (96%

pure, Merck, Darmstadt, Germany) and 0.17 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck) was simultaneously introduced with a 0.17 M H_3PO_4 (85% pure, Merck) solution into water over 3 h, with constant heating at 39°C and pH equal to 9. The obtained powder and the commercial product tricalcium phosphate (β -TCP, dried extra pure; Merck) were then uniaxially pressed into cylindrical tablets under a compressive pressure of 216 MPa, followed by sintering at 1000°C for 4 h in open air. The tablets were crushed and sieved to select the dense granules in the range of 250-500 μm .

Physicochemical characterization

The phases presented in the granular materials were analyzed using an X-ray diffractometer (XRD; Shimadzu XRD 6000, Japan) with monochromatized Cu ka radiation and operational tube with 40 kV and 30 mA. The resulting trace was analyzed and compared with the standard library of known diffraction patterns (JCPDS). The samples mixed with KBr (99.9% pure, Sigma Aldrich, St. Louis, MO, USA) in the proportion of 1:20 were also examined by Fourier-transformed infrared spectroscopy (FTIR; PerkinElmer, Spectrum 100, Norfolk, VA, USA) in order to identify the vibration modes of the molecules in the range of 500-4000 cm^{-1} . For XRD and FTIR analysis, the granules were crushed prior to use.

Calcium, phosphorus (P) and Mg contents in both phosphates were determined by inductively coupled plasma optical emission spectroscopy (ICP; PerkinElmer Optima 3000, USA). The analysis was performed in triplicate, and the mean values and standard deviations were subjected to one-way ANOVA and Tukey's post-test test ($p < 0.05$). Scanning electron microscopy allowed the morphological characterization of the granules (SEM; JEOL JSM 6460-LV, Japan).

Cytotoxicity assay

Samples were extracted from culture medium (100 mg of TCP granules/mL of DMEM) at 37°C for 24 h and the extracts were collected for cytotoxicity assay according to ISO 10993-12:2007¹¹ and 7405:2008¹². ICP analysis was performed in triplicate to determine the Ca and Mg content dissolved in the culture medium for 24 h (biomaterial extract). A 1% phenol solution was used as positive control for cytotoxicity, while untreated cells were used as a negative control (cultivated with DMEM only). Subsequently, MC3T3 pre-osteoblasts (CRL 2594 - ATCC) were seeded in 96-well cell culture plates (1×10^4 /well) and subcultured in DMEM containing ampicillin (0.025 g/L), streptomycin (0.1 g/L) and supplemented with 10% fetal bovine serum for 24 h at 37°C and 5% CO_2 /95% air.

After 24 h of cell exposure to each extract

medium, cell viability was evaluated with a commercial kit (In Cytotox, Xenometrix, Germany) by two different tests of cell survival and integrity on the same sample: 2,3-bis[2-methoxy-4 nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) 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 nm²⁵. The CVDE assay evaluates cell density by staining DNA and the absorbance at 540 nm is proportional to the amount of cells in each well¹⁵. The absorbance data were obtained with a microplate UV/Vis spectrophotometer (PowerWave MS2, BioTek Instruments, Winooski, VT, USA). All the tests were performed in quintuplicate.

Normal distribution was confirmed by a D'Agostino-Pearson omnibus test. Mean values and standard deviations were submitted to one-way ANOVA and Tukey's *post-hoc* test considering statistical significance at $p < 0.05$.

RESULTS

As shown in Figure 1, the diffraction pattern of granules obtained from the commercial TCP showed the mean peaks of β -TCP or whitlockite (JCPDS 090169); on the other hand, the diffractogram of granules produced with Mg-CDA presented the mean peaks of β -TCMP or magnesia whitlockite (JCPDS 130404). Regarding the morphology of the granules, a lower surface roughness was verified on β -TCMP when compared to β -TCP granules on SEM images (Figure 2).

The FTIR spectra in Figure 3 showed phosphate

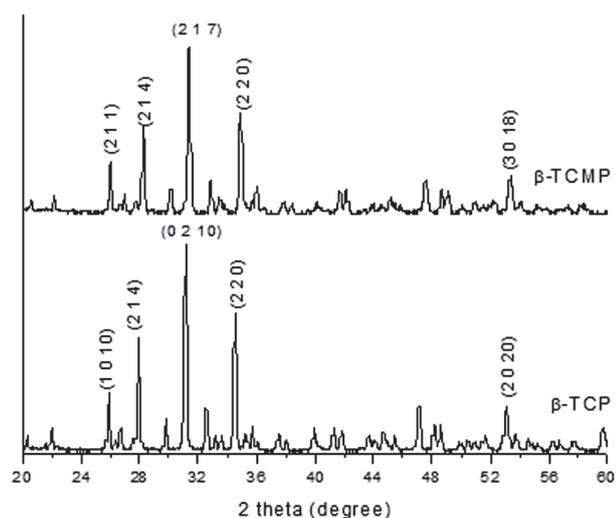


Figure 1- X-ray diffraction (XRD) patterns of tricalcium phosphate (β -TCP) and magnesium-substituted β -TCP (β -TCMP) heat-treated at 1000°C in air for 4 h

bands at 1126 and 1025 (ν_3) cm^{-1} , as well as at 604 and 554 (ν_4) cm^{-1} in both materials, which are characteristic of tricalcium phosphates. Based on ICP results, the (Ca+Mg)/P molar ratio of β -TCMP was calculated to be 1.45, containing 0.14 mol of Mg, which is close to the theoretical values [(Ca+Mg)/P=1.5 and 0.15 mol of Mg]. Also, the Ca/P ratio of β -TCP was 1.38.

In relation to DMEM alone, the ICP analysis of the β -TCMP granule extract medium (DMEM) showed that there was a significant decrease in the Ca concentration in opposition to an increase ($p < 0.05$) in Mg (Figure 4).

The results of *in vitro* cytotoxicity tests for β -TCP and β -TCMP granules from XTT and CVDE viability assays are in Figure 5. Mitochondrial activity was similar in the cells exposed to β -TCP and β -TCMP extracts and in the untreated cells (control group) but, as expected, 1% phenol abolished all dehydrogenase activity in the treated cells (positive

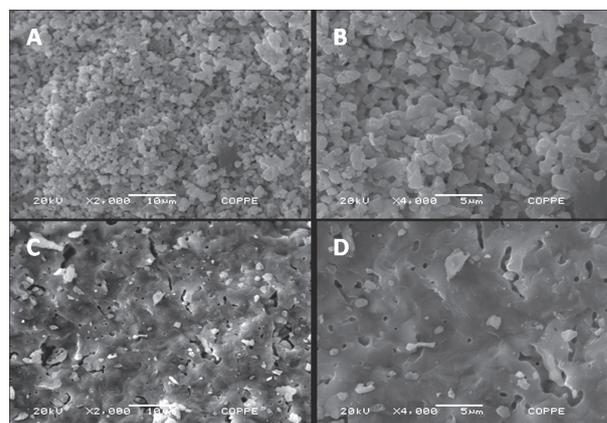


Figure 2- Scanning electron microscopy (SEM) images of tricalcium phosphate granules (250-500 μm): β -TCP (A,B) and magnesium-substituted β -TCP (β -TCMP) (C,D)

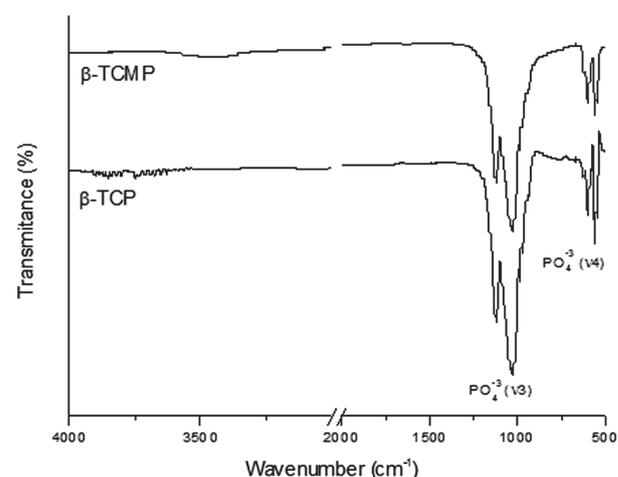


Figure 3- FTIR spectra of tricalcium phosphate (β -TCP) and magnesium-substituted β -TCP heat-treated at 1000°C in air for 4 h

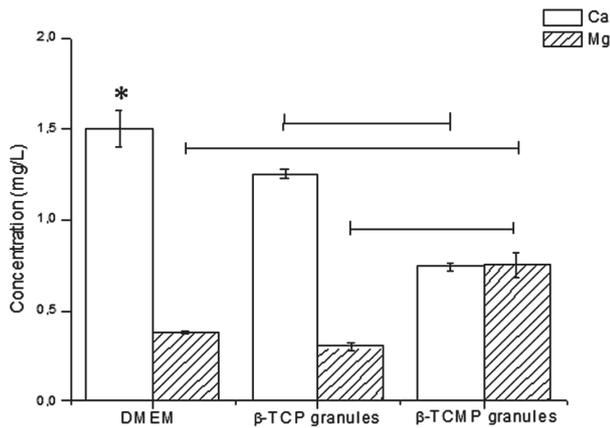


Figure 4- Calcium (Ca) and magnesium (Mg) contents of β -TCP and β -TCMP granule extracts or cell culture medium (DMEM) determined by ICP analysis, expressed as means and corresponding standard deviations. Bars and asterisks indicate significant differences ($p < 0.05$, ANOVA)

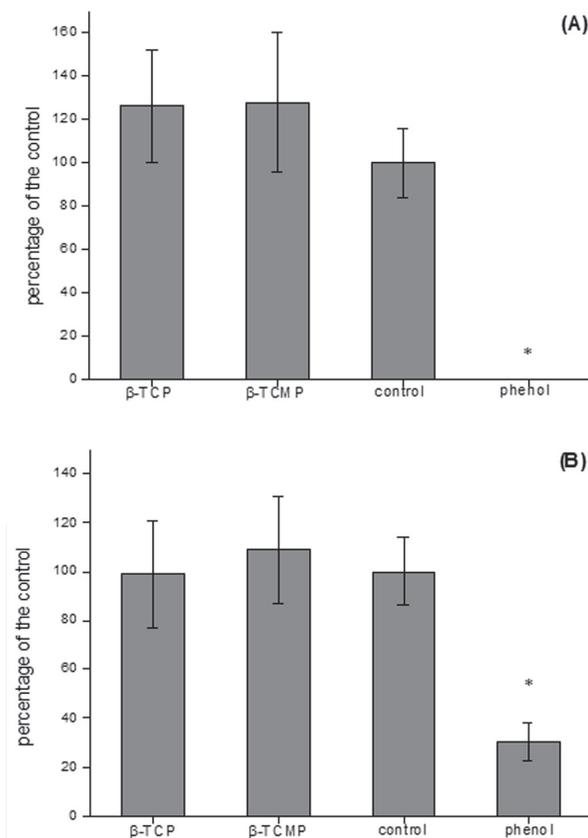


Figure 5- Cytotoxicity assay of tricalcium phosphate (β -TCP) and magnesium-substituted β -TCP on mouse osteoblasts, indicated as means and corresponding standard deviations. (A) 2,3-bis[2-methoxy-4 nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) reduction. (B) Crystal Violet dye elution. The asterisk indicates that the phenol group presented a significantly lower absorbance than all other groups ($p < 0.05$, ANOVA)

control). Similar behavior was observed for the crystal violet test with no statistical difference between the biomaterials, but a strong reduction in the DNA content of cells in the phenol-treated group. Additionally, the results of both tests reveal that the overall viability of the cells exposed to the biomaterial extracts did not decrease, when compared with the untreated cells.

DISCUSSION

The treatment of large bone fractures still remains a challenge for dental and medical professionals because of many factors such as, biomaterials lacking adequate physicochemical or biological characteristics, e.g. bioresorption, even those of natural origin^{5,22}. Recently, research has been performed in the field of engineered polymer scaffolds to be used as structural bone substitutes. The incorporation of calcium phosphate into these scaffolds could improve their efficacy as bone grafts. The slow degradation of calcium phosphate materials compared with a number of polymers should maintain the material's stability during the bone formation phase, and rapid degradation of the material would be no longer an important issue²⁸. In spite of the myriad of materials being developed and tested in the last decades, the ideal biomaterial has not yet been developed, and research must be conducted to investigate physicochemical and biological improvements to the currently available repertoire of biomaterials. In this context, the present work showed the synthesis and characterization of dense granules of β -TCMP, a novel biomaterial aiming to associate the osteoconductivity of β -TCP and the stimulatory effect of Mg on osteoblasts. However, before performing *in vivo* studies on the impact of such material in bone tissue therapy, an *in vitro* evaluation of cytocompatibility should be conducted.

Mg incorporation into TCP granules reflected in the shift of the XRD peaks due to partial Mg-for-Ca substitution, causing a contraction in the unit cell dimension and stabilizing the structure. This effect is attributed to the smaller ionic radius of Mg (0.65 Å) in relation to Ca (0.99 Å) since substitutions with smaller ions give rise to lattice strain, favoring the contraction and at the same time stabilizing the structure²⁷. This was proven when SEM images were observed: due to the higher volumetric contraction of β -TCMP, a different level of densification was observed, consequently leading to a variation on surface microporosity between β -TCMP and β -TCP granules, as already reported²⁴. The FTIR spectra exhibit the characteristic bands of tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$], attesting the accuracy of the biomaterial synthesis^{9,16,24}.

ICP analysis showed that there was release

of Mg and precipitation of Ca into the β -TCMP granule extract, when compared to the composition of DMEM. The material's solubility is a vital characteristic since *in vivo* resorption is controlled by two factors: its own dissolution into the medium and cell-mediated degradation. Li, et al.¹⁸ (2008) immersed β -TCMP powders doped with 0, 2.3, 4.9, 7.3 and 10.1% of Mg in a buffer solution of 0.08 M $\text{CH}_3\text{COOH}-\text{CH}_3\text{COONa}$ (pH 5.5) at 25°C for up to 27 months. It was observed that the reduced solubility of the material with increasing Mg content could be attributed to the greater structural stability: the β -TCP structure can accommodate ions with an ionic radius in the range of 0.06-0.08 nm (the ionic radius of Mg is 0.072 nm).

Biocompatibility is defined as the ability of a biomaterial to perform in the context of a suitable host response in a particular application. Hence, metallic ions released in excess by a biomaterial can be toxic if they induce undesirable changes at higher concentrations^{6,7}. Lima, et al.²⁰ (2011) assessed the number of viable Balb/c 3T3 fibroblasts after exposure to several metal-modified apatite extracts for 24 h and concluded that cells respond to the metal that substitutes Ca or phosphate ions in the crystal lattice of HA. For that reason, soluble biomaterials such as metallic ion-substituted calcium phosphates must be evaluated in terms of their cytocompatibility before clinical use. The general screening assays for biomaterial testing are divided into four phases: general toxicity (*in vitro* cytotoxicity tests), local tissue irritation (animal implantation), pre-clinical (proof of concept in animal model) and clinical evaluation (human trials). *In vitro* cytotoxicity assays provide a measure of cell death caused by biomaterials or their extracts and it is important to recognize the limitations of each test to formulate an appropriate judgment²¹. For instance, metabolic impairment tests (XTT) are more complex and can be biased by bacterial contamination and are prone to overestimating cell viability⁴. On the other hand, the crystal violet assay can stain dead cells that are still attached to the plate³. Nevertheless, both are important tools to predict cell growth and survival.

The XTT result shows that β -TCMP granules are cytocompatible. Concerning the CVDE assay result, considerable cell density was observed in the cells cultivated with the phenol solution. Still, those cells were in a death process since no metabolic activity was detected by the XTT assay, and yet CV also dyed the dead cells that were still attached to the plate. This reinforces the necessity to analyze more than one aspect of cell survival and integrity before attesting biomaterial cytocompatibility.

Landi, et al.¹⁷ (2008) implanted HA granules containing 5.7% of Mg in rabbit femoral bone defects. The osteoconductivity and resorption of

the material was higher compared with commercial HA. Another study investigated the activity of osteoclasts on 6.8 mol% Mg-containing tricalcium phosphate discs in different Mg-containing culture media. Even 100 ppm of Mg ions in the culture medium did not increase osteoclast apoptosis and Mg ions in the TCP structure had no effect on osteoclast apoptosis¹⁹. Moreover, an assay using osteoblast culture indicated that the presence of Mg in TCP structure stabilized the cell-material interface and consequently enhanced cell attachment and growth³⁰.

To the best of our knowledge, there are insufficient data in the literature regarding β -TCMP granules cytotoxicity assays that allow comparison of the results. However, Sader, LeGeros and Soares²⁴ (2009) showed that dense β -TCMP (Mg/Ca=0.2 mol) tablets could sustain the adhesion and proliferation of human osteoblast cells *in vitro*, suggesting that this is a promising biomaterial for bone grafting. Conversely, the reported beneficial effect could be due, at least in part, to the Mg adsorbed by the granule surface¹.

CONCLUSION

Granular β -TCMP (Mg/Ca=0.15 mol) was successfully produced and the resulting biomaterial was shown to be biocompatible, while being capable of releasing Mg in the cell culture medium. These results indicate the suitability of this promising material for further biological *in vivo* studies on its adequacy as an efficient bone substitute material.

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