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Biomaterials 23 (2002) 2035–2042

Biomaterials

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α-Tricalcium phosphate cement: "in vitro" cytotoxicity

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Accepted 25 September 2001

Abstract

Calcium phosphate-based bioceramics have revolutionized orthopedic and dental repair of damaged parts of the bone system. Among these materials, calcium phosphate-based cements, with hydraulic setting, stand out due to their biocompatibility and in situ hardening, which allow easy manipulation and adaptation to the shape and dimensions of bone defects. An investigation was made of the in vitro cytotoxic effect of calcium phosphate cement based on α-tricalcium phosphate, immersed for different lengths of time in simulated body fluid (SBF), based on the ISO-10993 "Biological Evaluation of Medical Devices" standard. The culture medium was Chinese hamster ovary (CHO) cells in contact with diluted cement extracts. The results revealed that the calcium phosphate cement used was cytotoxic and that the material's cytotoxicity decreased the longer the cement was immersed in SBF.

Keywords: Citotoxicity; Calcium phosphate cement; Bioceramic; Biomaterial

1. Introduction

The term "calcium phosphate cement" was introduced by Gruninger et al. [1]. According to them, this type of cement can be prepared by mixing a calcium phosphate salt with water or with an aqueous solution to form a paste that reacts at room or body temperature, giving rise to a precipitate containing one or more calcium phosphates, which sets by the intercrossing of the crystals of this precipitate. This cement consists of two components, one basic and one acid, which react when mixed with water, producing one or more products with an intermediary acidity. The cement is applicable in the grafting and replacement of damaged parts of the bone system, offering a series of advantages such as:

- The graft does not require shaping;
- Minimum filler cavity;
- The cement is prepared during surgery;
- Excellent contact between bone and graft;
- The material is biocompatible and bioactive.

In addition to their easier manipulation and greater adaptation, calcium phosphate cements can serve as drug delivery systems for a variety of remedies such as antibiotics, anti-tumor and anti-inflammatory drugs [2], etc. that can easily be added to them. Despite the wide range of possible applications of calcium phosphate cements, literature contains few reports on in vitro biocompatibility tests with these materials, showing only in vivo tests. The correlation between the products deriving from the setting reaction at different times and cytotoxicity is still unknown. In vitro tests may not represent the real situation of an implant, for the greater the system's complexity, the greater its variability. However, they can provide fast results regarding the material's interactions in biological mediums, thus helping to minimize testing on animals. In vitro tests have been used to evaluate the biocompatibility of materials for over two decades and they are widely used today owing to the easy availability of cell strains on the market. Moreover, there is a wide range of repeatable

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0142-9612/02/$ - see front matter
PII: S0142-9612(01)00333-7

and reproducible methods, which are regulated by national and international standards for commercial use and for the scientific development of new materials and products. The purpose of this study was to determine the cytotoxic level of α-tricalcium phosphate cement based on the method of colony suppression, using Chinese hamster ovary (CHO) cell culture.

2. Materials and methods

2.1. Cement preparation

α-Tricalcium phosphate (α-TCP) was prepared as described in literature [3], through the reaction of $CaCO_3 + \gamma\text{-}Ca_2P_2O_7 = \alpha\text{-}Ca_3(PO_4)_2$. Reagents in equimolar amounts were dry-mixed in a zirconia ball mill for 2 h, calcined at 1300°C for 15 h and quenched on a stainless steel plate [4]. The product of this reaction was milled in a polyethylene jar with zirconia balls in an alcoholic medium (anhydrous ethanol) for 5 h. The particle size was analyzed by sedimentation (Sedigraph-Micromeritics 5100). A solution of 2.5 wt% of Na_2HPO_4 was used to accelerate the setting reaction of the α-TCP cement, with a liquid/powder ratio of 0.32 (ml/g). Three specimens with 21 mm diameter and 10 mm height were prepared using silicon molds. The specimens were kept at 100% relative humidity for 24 h (0D composition), after which they were placed in polystyrene flasks containing simulated body fluid (SBF), with ion concentrations (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 148.8, HPO_4^- 1.0, HCO_3^{2-} 4.2, SO_4^{2-} 0.5 mM/l) nearly equivalent to those of human blood plasma, for 24 h and 7 days (compositions 1D and 7D, respectively) at 37 ± 1°C (liquid/solid ratio = 50 ml/g). After this immersion in SBF, the specimens were placed in anhydrous ethanol to stop the setting reaction, similar to that used by Ginebra et al. [5]. The specimens were allowed to dry at room temperature for 3 days, after which they were crushed in a porcelain mortar and sieved through a #100 mesh ASTM sieve (150 μm), producing calcium phosphate cement powder (CPCp).

2.2. Scanning electron microscopy (SEM)

Some pieces of calcium phosphate cement were crushed, covered with gold and examined under scanning electron microscopy (SEM—Carl-Zeiss I model DSM 940A). This was carried out to give complementary information about the transformations occurring during the hardening of the cement.

2.3. X-ray diffraction (XRD) analysis

The α-TCP powder and the cements were analyzed by qualitative and quantitative X-ray diffraction, using a model D 5000 X-ray diffractometer (Siemens, Karlshüre) with Cu K_α radiation, Ni filter, 40 kV and 40 mA. The angular interval 2θ analyzed was from 20° to 40°, with 2°/min for the qualitative analysis. The quantitative analysis method used was the internal pattern, with point-to-point scanning at 0.017° intervals and an integration time of 2 s for the quantitative analysis. Diffraction lines (0 2 1 0) for β-TCP, (2 1 1) for HA and (1 7 0) for α-TCP, to make the phase % calculations, was used. The standard deviation was taken as the square root of the number of phase counts [6].

2.4. pH measurements

The pH of the CPCp and α-TCP powder was measured in distilled water using an Analion PM 608 pH meter with a liquid/powder ratio of 50 ml/g, at room temperature (about 27°C), after 1 h of immersion.

2.5. Cytotoxicity procedure

Testing for cytotoxicity was performed by adding dilutions of CPCp extract to a CHO cell culture on a Petri plate ($15 \times 60\ mm^2$). The positive and negative controls used were, respectively, a 0.02 vol% phenol solution and alumina (Al_2O_3) with medium particle size of 5 μm [7,8].

2.5.1. Preparation of CPCp extracts

Six grams of CPCp and 6 g of Al_2O_3, both sterilized by gamma radiation (25 kGy) were poured into 100 ml glass flasks. Sixty milliliter of the culture medium MEM-BFS (minimum eagle medium contents, 10 vol% of bovine fetal serum and 1 vol% of penicillin and streptomycin solution) was added and incubated for 48 h at 37°C. The supernatant was then filtered through a Millipore® membrane with 0.22 μm pores and serial dilutions were made of the CPCp, the Al_2O_3 and the 0.02 vol% phenol solution (100, 50, 25, 12.5 and 6.25 vol%) extracts. Alumina and phenol solutions were used, respectively, as negative and positive controls.

2.5.2. Preparation of the primary culture

The CHO cells were cultivated in plastic bottles in an MEM-BFS medium, placed in an incubator at 37°C with a humid atmosphere of 5 vol% CO_2, until a cell layer was obtained. The culture medium was then removed from the incubator and the cells washed with a calcium and magnesium free saline phosphate buffer (PBS-CMF—phosphate buffer saline-calcium magnesium free). A 0.2 wt% tripsine solution was added to detach the cells from the bottle. The cells were washed twice with PBS-CMF, re-suspended in MEM-BFS, the suspension adjusted to 100 cells/ml.

2.5.3. Cytotoxicity testing

From the above suspension, 2 ml was distributed on each culture plate ($15 \times 60\,mm^2$) and incubated for 5 h for cell adhesion. After this period, the culture medium was removed and 5 ml of the pure extract (100 vol%) and 5 ml of each serial dilution (50, 25, 12.5 and 6.25 vol%) were added to the same plates. Fresh medium (MEM-PBS, 5 ml) was then placed on the plate of the CHO cell control. Each concentration of the tested extracts was made in triplicate. The plates were incubated in a humid incubator with 5 vol% CO_2 at 37°C for 7 days, after which the medium was removed and the colonies fixed with a solution of 10 vol% formaldehyde diluted in a 0.9 wt% saline solution and stained with Giemsa. The visible colonies on each plate were counted and compared with the number of colonies of the CHO cell control plate. The material's cytotoxic potential was expressed by an index of cytotoxicity (IC_{50} (%)), which represents the concentration of the extract that suppresses the formation of cell colonies by 50% in comparison to the control [7].

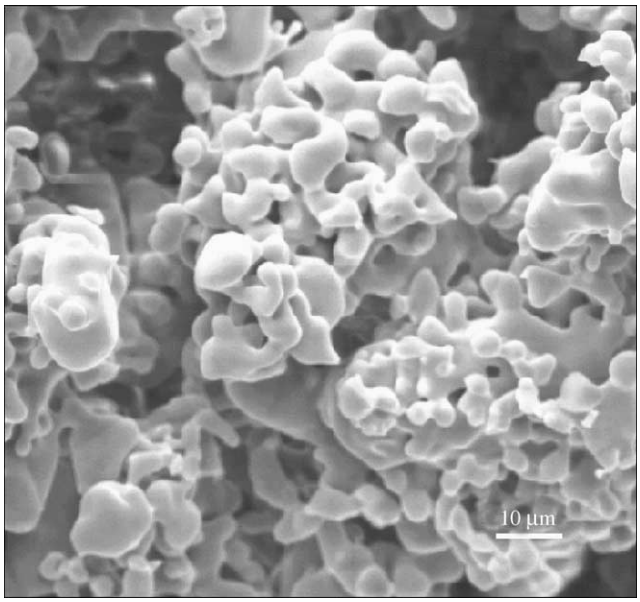

Fig. 1. Fracture SEM micrograph of the α-tricalcium phosphate obtained.

3. Results and discussion

3.1. Particle size measurements

The particle size distribution by sedimentation of the material after grinding in an alcoholic medium varied from 25.0 to 2.0 μm, with an average size of 8.0 μm, which is similar to that reported in literature, i.e., a distribution of 20 to 2 μm. The amount of liquid, containing 2.5 wt% Na_2HPO_4 (0.32 ml/g), used is the same as that reported in literature [9,10] to produce a paste. The particle size distribution of the powder, in all kinds of cements, is a fundamental factor of liquid/powder ratio required to produce a paste with the proper consistency.

3.2. Scanning electron microscopy

The material's microstructure showed interconnected grains (see Fig. 1), revealing that the reacted material entered the initial stages of sinterization through the formation of necks and joining of adjacent particles. However, this joining was too weak to hinder the grinding process of the material. The porosity can also be explained by the evolution of CO_2 during the reaction to obtain α-TCP.

Fig. 2 presents the micrographs of the fracture surfaces of the cements reacted under different conditions. Contrary to our expectations, due to the 57% calcium deficient hydroxyapatite (CDHA, due to the transformation reaction $\alpha\text{-}Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5OH$) [23]), measured by quantitative X-ray analysis, no CDHA structures were observed in the material kept at 100% humidity for 24 h (Fig. 2a). The appearance of needles or petal-like plates is characteristic of α-TCP-based calcium phosphate cements [1–3,5]. The absence of needles or petal-like plates in the microstructure shown in Fig. 2(a) was ascribed to the lack of time for CDHA growth to occur besides insufficient time and conditions for the material to react completely. Figs. 2(b) and (c) present the cement

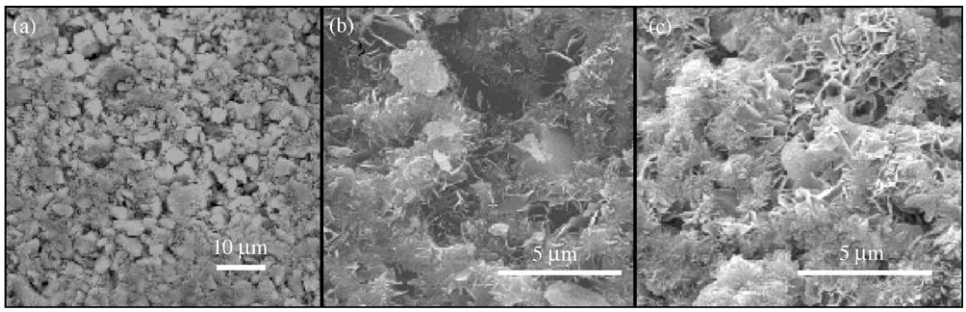

Fig. 2. Fracture SEM micrograph of the compositions: (a) 0D, (b) 1D and (c) 7D.

immersed for 24 h and 7 days in SBF, respectively; however, the presence of these microstructures, which are characteristic of reacted α-tricalcium phosphate cement, can clearly be seen. The interlocking of these crystals enhances the mechanical strength of the cement [1]. Another important aspect of the material's microstructure and bioactivity refers to the surface of the cements immersed in SBF for 24 h and for 7 days (Fig. 3), which showed globular precipitates in both cases. The morphology shown in Fig. 3 is similar to that obtained during biomimetic procedures for the coating of the $BaTiO_3$ substrate [11] by immersion of the substrate in SBF, after previous treatment in a solution containing G glass or sodium silicate. This finding, allied to the X-ray diffraction of the compositions showing only diffraction lines corresponding to the hydroxyapatite phase, suggest a hydroxyapatite precipitated in a spherical shape. Several authors have considered the materials to be biocompatible judging from the presence or absence of a superficial layer of hydroxyapatite formed after immersion in simulated physiological solutions [12–14].

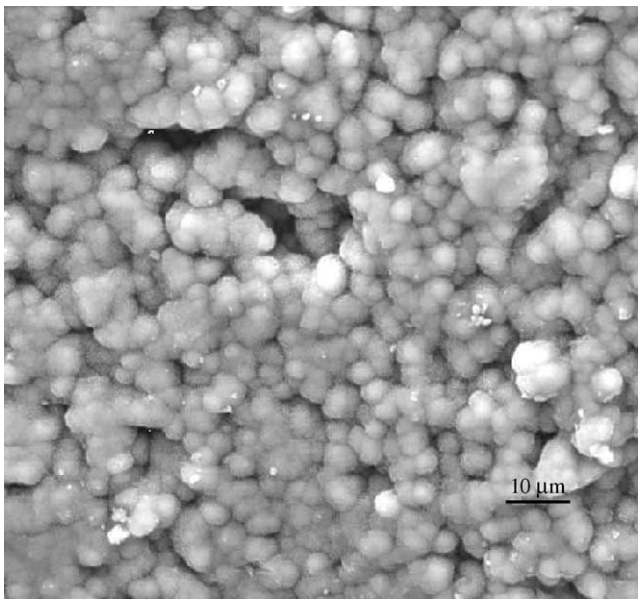

Fig. 3. Surface SEM micrograph of composition 7D.

3.3. X-ray diffraction

Fig. 4 shows the X-ray spectra of the α-TCP used, showing only α- and β-TCP diffraction lines. Unlike previous studies [15–17], no hydroxyapatite X-ray diffraction lines were observed. The presence of β-TCP as an impurity in α-TCP has been reported by several authors [18–21], but to our knowledge, no reports exist regarding the production of pure α-TCP, and the α-TCP obtained usually contains about 15% β-TCP [22]. The material analyzed by quantitative X-ray diffraction showed the presence of 3% β-TCP. The absence of the hydroxyapatite phase may have probably been due to the short grinding time of $CaCO_3 + \gamma\text{-}Ca_2P_2O_7$ used in this study, resulting in coarser β-TCP particles, less susceptible to the transformation reaction of the equilibrium: HA ⇔ β-TCP ⇔ α-TCP, in which the first reversion reaction is slower than the direct reaction [19], and the low content of β-TCP.

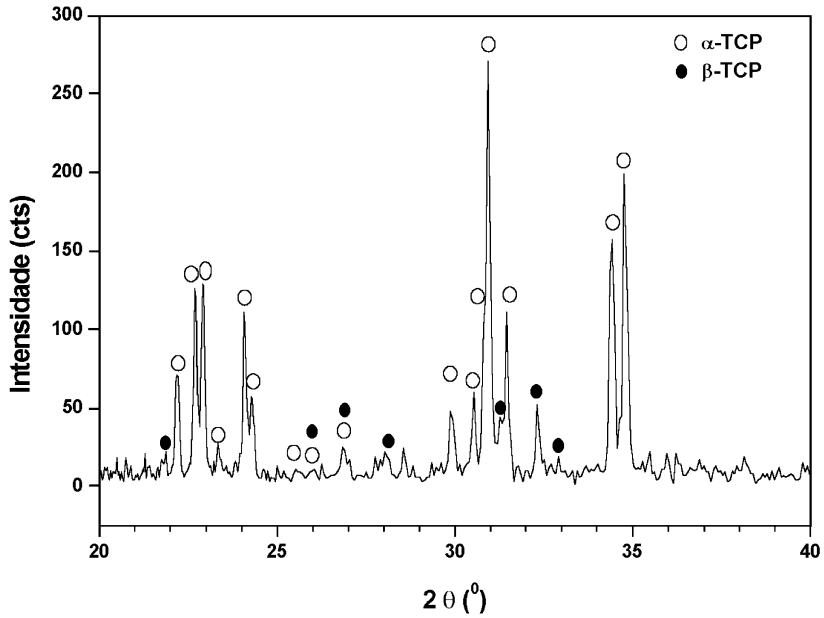

Fig. 4. Qualitative X-ray analysis of the α-tricalicum phosphate obtained.

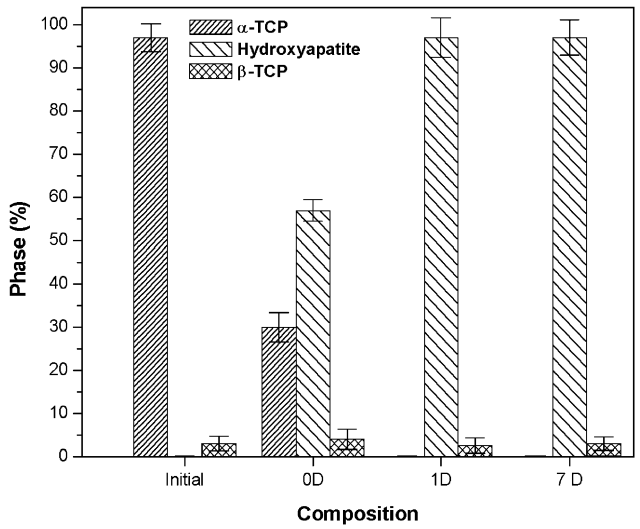

Fig. 5. Quantitative X-ray analysis.

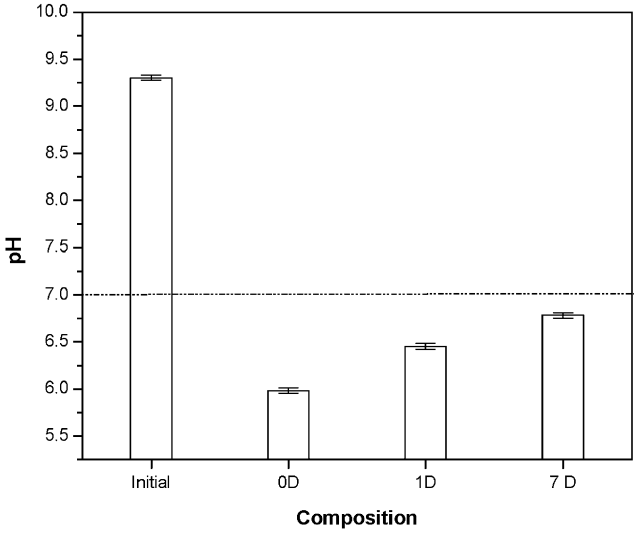

Fig. 6. pH of calcium phosphate cement in distilled water.

Only the α-TCP and β-TCP phases were observed in the starting material, as shown by the qualitative X-ray diffractogram (Fig. 4) and the quantitative X-ray analysis (Fig. 5). However, the presence of peaks corresponding to the α-TCP, β-TCP and CDHA phases was observed in the non-immersed material and kept at 100% relative humidity for 24 h (0D). After the material had been immersed in SBF for 24 h and for 7 days (compositions 1D and 7D), the α-TCP diffraction peaks disappeared and, apart from the β-TCP peaks, only CDHA diffraction peaks were found.

Fig. 5 shows the result of the quantitative X-ray diffraction analysis for the three phases detected in the qualitative analysis. The amount of CDHA was calculated by the subtraction of the amounts of α- and β-TCP, since the low crystalinity of the CDHA crystals, which affect the width and intensity of the diffraction peaks, make a quantitative analysis difficult. The amount of β-TCP was found to remain nearly constant with immersion time.

3.4. pH measurements

The pH of unreacted cement is the same as α-TCP (Fig. 6), before the dissolution reaction and the formation of hydroxyapatite, which was approximately 9.3 (initial composition) in water solution, quickly dropped when the cement composition contains the reaction accelerator, Na_2HPO_4 (composition 0D), related to the different phases present in the cement at the time of measurement, as shown in Fig. 5. From this figure, the initial composition is only α-TCP, while the composition 0D is a mixture of 57 wt% CDHA, 29 wt% α-TCP and 4 wt% α-TCP (Fig. 5). A reaction accelerator is commonly used in this type of calcium phosphate cement composition due to the long setting time. This accelerator favors rapid precipitation of CDHA, with the suppression of the induction period of the reaction [5].

The α-TCP has a high pH in water, 9.3, which slowly decreases to values close to 7.0 [24], due to the transformation reaction $\alpha\text{-}Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)(PO_4)_5OH$. This reduction is gradual and is related to the hydration process of α-TCP [25]. However, because α-TCP has a smaller Ca/P ratio than that of hydroxyapatite, H_3PO_4 can be formed during hydrolyzation ($10Ca_3(PO_4)_2 + H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 2H_3PO_4$). This explains the pH of cements immersed in SBF (compositions 1D and 7D), below the neutral value (7.0). The higher pH observed in the CPCp in water after 7 days of immersion in SBF than after 24 h is probably a result of the reaction of H_3PO_4 with calcium ions from the SBF solution, again producing hydroxyapatite, since this is the least soluble calcium phosphate compound in $pH \geqslant 4.2$ [26], after which the pH stabilized at close to 6.8. The pH values obtained for CPCp for compositions 1D and 7D cannot lead to a conclusion about any deleterious reaction of the material implanted in vivo since, apart from the fact that the pH was close to the physiological level, the extra-cellular fluids have buffer characteristics that prevent a stronger reaction.

The pH of the cement is the principal controlling factor of the Ca and P concentrations. The most important parameters that affect pH variations during cement setting are the chemical composition of the main components of the cement, the proportion of the reagents in the mixture, the relative particle size of the main cement components, the particle size of the material used as crystallization seed, the use of accel-

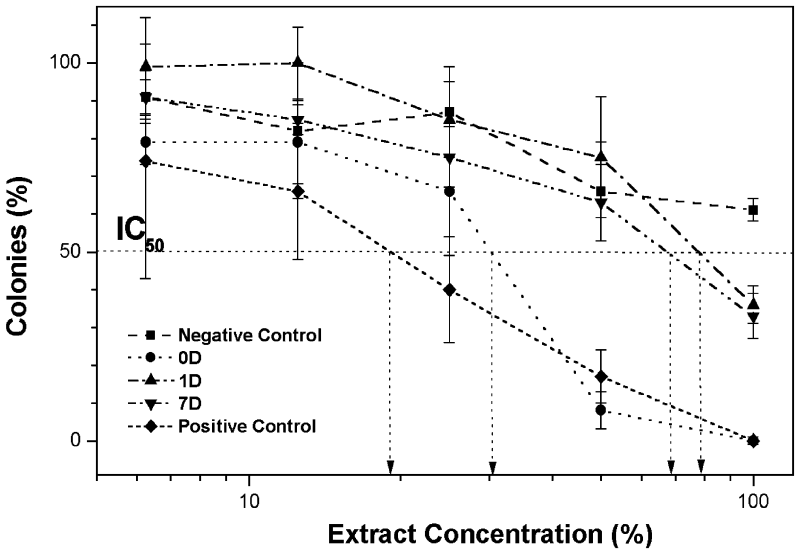

Fig. 7. Colony suppression curves in the cytotoxicity test.

erators or delayers in the liquid phase and their relative concentration, the liquid/powder ratio, and temperature [25].

The pH exerted a strong influence on the cytotoxicity test, determining the test results. The alteration of the pH in the extraction medium was illustrated by its changed color, which went from dark pink to yellowish shades when the CPCp compositions evaluated were added to the culture medium MEM-BFS. The lower the pH of CPCp in water, the more yellowish the MEM-BFS became. This effect was produced by the presence of pH indicators in the MEM solution, which were introduced for qualitative evaluation of the pH of solution. The pH of the MEM-BFS solution containing CPCp tends not to be modified in time, as seen by the lack of change in extraction medium color. Despite the dissolution of α-TCP, CDHA should not precipitate since the amount of liquid is extremely high, preventing the medium from being saturated in Ca and P.

The effect of maintaining the pH when the material is powdered and mixed in a large amount of liquid also appears to occur in the case of the calcium phosphate cement system based on TTCP (tetracalcium phosphate). Ishikawa et al. [27] demonstrated that several powdered calcium phosphates (TTCP, DCPA (anhydrous dicalcium phosphate) and TTCP + DCPA) implanted in mice produced an inflammatory reaction after one week, which did not occur, however, when shaped cement (TTCP + DCPA) was used. Miyamoto et al. [28] reported the same phenomenon using calcium phosphate cement with slow setting times in subcutaneous rat tissue, demonstrating that the setting reaction must take place in order to preclude an inflammatory response. Nonetheless, the 78% IC_{50} for the cement immersed in SBF for 24 h (composition 1D) revealed an existing, nevertheless not so heavy cytotoxicity, of this material, which remained almost constant immersed for 7 days in the cement. According to XRD analysis (Fig. 5) there is no difference in phase composition between 1D and 7D samples. Moreover, even in the negative control, the percentage of colonies decreased as the concentration of extract increased (Fig. 7), confirming the need for other types of cytotoxicity tests or in vivo tests for more conclusive results.

3.5. Cytotoxicity results

Fig. 7 shows the percentage of visible colonies on the plates for different concentrations of the extracts plotted on a graph. The cytotoxic potential was evaluated by the index of citotoxicity, IC_{50} (%), which represents the concentration of the extract of the analyzed material that inhibits the formation of colonies by 50%. When tested, the negative control should produce no cytotoxic response, as can be observed in the alumina ($IC_{50} > 100\%$), and the positive control should produce a positive cytotoxic response, as in the case of the phenol solution, which presented $IC_{50} = 18\%$. It is interesting to note that increased concentrations of extract in the positive control lead to a gradual reduction in the number of colonies formed. This behavior helps to control the cytotoxicity test by showing up possible errors during testing. There is also a gradual reduction of colonies for negative control, for which the authors have no explanation.

The CPCp extracts showed an increase in IC_{50} (%) with longer cement immersion times, from a 30% IC_{50} for the cement maintained at 100% relative humidity but without immersion in SBF to a 78% IC_{50} for the material maintained for 24 h in SBF at 37°C. The

material kept in SBF for 7 days showed a slight increase in cytotoxicity, i.e., decrease of IC_{50} ($IC_{50} = 68\%$). However, considering the standard deviation, the values were very similar. In fact, XRD analysis (see Fig. 5) shows no phase difference between samples 1D and 7D. The changes in IC_{50} (%) values may be explained by phase and pH alterations, both are related, in the material after immersion in SBF.

4. Conclusions

- The α-TCP-based cement tested, evaluated through its extracts, is cytotoxic.
- The level of the α-TCP cement's citotoxicity was determined, from which IC_{50} values ranging from 30% to 78% were obtained, depending on the time of cement immersion in SBF.
- The pH of the calcium phosphate cement in water varied according to the time of its immersion in SBF, a factor that appeared to be directly linked to the level of cytotoxicity. Direct pH, Ca and P concentration measurements on extracts are necessary to confirm this effect.
- The relatively high IC_{50} values of the compositions immersed for 24 h and 7 days in SBF do not preclude the use of this material in grafting procedures. However, "in vivo" tests are still needed to better evaluate the material's cytotoxicity.

Acknowledgements

The authors gratefully acknowledge the financial support of the Brazilian research funding institution FAPESP (Process # Us. 98/11691-2 and 98/00563-3). Special thanks are also due to the Third World Academy of Sciences (JRP 18/95) and to Fábio S. Fukazawa for his laboratorial contribution.

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