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pH Evolution and Cytotoxicity of [Alpha]-Tricalcium Phosphate Cement with Three Different Additives

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Abstract. An application of calcium phosphates is as bone cements, among which the system based on alpha-tricalcium phosphate (α-TCP) exhibits excellent properties. The aim of this study is to analyze pH evolution and cytotoxicity of α-TCP cement with three different additives. Changes on the pH were measured at intervals of 12h during seven days. But initial measurements were executed at each 15 minutes. Indirect cytotoxicity test was performed according to ISO (10993-5, 1992) employing CHO-k1 cells and RPMI 1640 as culture medium. It was used a colorimetric method which uses the tetrazolium compound. The additives used on the liquid phase were disodium hydrogen phosphate (Na_2HPO_4) and/or citric acid ($C_6H_8O_7$) and/or tannic acid ($C_{76}H_{52}O_{46}$). The results indicate that the cement without additives does not have requirements to be applied like bone cement, while the other cements composition exhibit different responses in the pH and the cytotoxicity test. In conclusion, due to the presence of additives it was possible to control pH evolution during setting and cytotoxic response. However, further investigation is necessary in order to determine the influence of these additives, mainly tannic acid, on the in vivo behavior of these bone cements.

Introduction

The calcium phosphate cements (CPC) are biomaterials made from a powder mixed with an aqueous solution. Although excellent biological properties of CPC, they are indicated just to applications which do not require high mechanical resistance [1]. Among them, the cement based on α-TCP system has an extensive use due to its conversion into CDHA (calcium deficient hydroxyapatite) and excellent biological properties [2]. However, the synthesis of high purity α-TCP is difficult. Contamination with β-TCP is common due mainly to the presence of Mg on the precursors reagents [3-5].

In order to increase CPC mechanical properties, the use of additives in the liquid phase has been studied exhaustively. This is a low coast and an efficient method to change CPC properties. Many additives have been employed [6,7], among them disodium hydrogen phosphate (Na_2HPO_4) and citric acid ($C_6H_8O_7$) have their use fully explored.

Na_2HPO_4 is a well-known additive used to accelerate the setting reaction. CPC without this additive in its composition present very slow setting reaction kinetics and does not exhibit the minimal properties for clinical application. No volumetric change and heat liberation, biocompatibility, continuous acquisition of mechanical resistance since the start of the reaction and

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transformation into CDHA plates are some of the excellent properties of α-TCP cement with Na_2HPO_4 as additive [8,9]. The citric acid is other additive responsible to improve CPC properties. Its fluidificant effect decreases the liquid/ powder ratio and, consequently, promotes an increase on the final mechanical properties of the cement [10].

One additive which is not well explored is tannic acid ($C_{76}H_{52}O_{46}$). CPC with this composition present good results, mainly in the biological properties [11,12]. In the studies presented, in vitro citotoxicity an in vivo histocompatibility are evaluated. The biological responses are excellent without prejudice in the setting time. But in these works, the tannic acid was used with combination only with citric acid. Beside this, the powder used was a composition of α-TCP with or without barium sulfate to application as root canal sealer.

However, the use of each one of these additives can change the CPC biological properties, one of the most important properties of this material [13-15]. The main objective of this study is to evaluate the influence of these three additives on pH evolution and in the cytotoxicity response of the α-TCP cement.

Materials and Methods

α-TCP Powder (α-$Ca_3(PO_4)_2$). It was obtained by solid state reaction of $CaCO_3$ and $CaHPO_4$ in stoichiometric proportion at 1300ºC. Heating rate was 5°C.min^{-1}. After the dwell time of 6 hours the furnace was shut off and the samples were cooled down to room temperature naturally.

The reactants employed were synthesized by aqueous solution precipitation in the presence of EDTA in order to eliminate Mg [3]. Afterwards, the powder was ball milled for 2 days and characterized by dynamic light scattering and X-ray diffraction (Malvern), XRD (Rigaku, DMAX 2200, CuKα, Ni filter, 20kV, 20mA, 0.01º.s^{-1}).

Liquid Phase Solution. The additives used on the liquid phase were disodium hydrogen phosphate (Na_2HPO_4, Synth, 117366) and/or citric acid ($C_6H_8O_7$, Synth, 101707) and/or tannic acid ($C_{76}H_{52}O_{46}$, Synth, 93992). The solutions prepared, as well their respective concentrations and cement samples labels are exhibited on Table 1.

Table 1. Solutions, liquid phase additives and cements.

Solution	Additives (w%)	Cement
S1	-	CS1
S2	2.5% Na_2HPO_4 + 1.5% $C_6H_8O_7$	CS2
S3	2.5% Na_2HPO_4 + 5.0% $C_{76}H_{52}O_{46}$	CS3
S4	2.5% Na_2HPO_4 + 1.5% $C_6H_8O_7$ + 5.0% $C_{76}H_{52}O_{46}$	CS4

Cement Preparation. The powder and the respective liquid phase were mixed during 1 minute. The liquid/powder ratio was 0.38 mL.g^{-1}. The cement paste was molded into 6x12mm cylinders. After 15 minutes, the samples were immersed in Ringer's solution.

pH Evolution. 2g of α-TCP powder were immersed in 10 mL of each one the four solutions. The mixture was maintained at 37ºC with periodic stiring. The pH variations were measured in 12h intervals during seven days (Micronal, B474). It is known that the more abrupt changes in the CPC pH occur in the beginning of the reaction. Then, in the first hours of reaction, the intervals between pH measurements were smaller, after every 15 minutes. Solutions' pH evolution was also monitored for comparison. .

Cytotoxicity Test. Cement samples' cytotoxicity was determined as described previously [16]. Chinese hamster ovary cell line (CHO-k1) was used. Cells were maintained in RPMI 1640 medium supplemented with antibiotic and antimicotic (100 units/mL penicillin, 100 mg/mL streptomycin, and 0.025 mg/mL amphotericin B), 2 mM glutamine, and 10% fetal bovine serum, at 37°C in a humidified 5% CO2 atmosphere until they reached confluence. For subculturing and for experiments, cells were harvested using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline, pH 7.4.

A colorimetric method which uses the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) (MTS) and phenazine methosulphate (PMS) was employed for determining the number of viable cells in proliferation. Microplates of 96 wells were prepared with 50 µL of extracts diluted from 100 to 6.25% in RPMI 1640 medium in quadruplicates. A suspension of CHO-k1 with 6 X 104 cell/mL was prepared and 50 µL/well was pipetted in the microplates and incubated for 72 h at 37°C in a humidified 5%CO2 atmosphere. Blank and control of the cells were also prepared. The cell viability was measured by adding 20 µL of MTS/PMS (20:1) solution in the humidified 5% CO2 incubator followed by incubation for 2 h at 37°C. The microplates were read in a spectrophotometer reader at 495 nm. The test was compared with a negative control of commercial powder hydroxyapatite and a positive control of phenol 0.3% in saline 0.9% solution. The Cytotoxicity Index for 50% of cell viability (CI50) was graphically determined.

Results and Discussions

Solutions' pH can be viewed on Table 2.

Table 2. Aqueous solutions' pH.

Solution	pH
S1	6.2
S2	5.7
S3	7.8
S4	5.2

The solutions that have citric acid on their composition (S2 and S4) presented the most acid pH, while the solution with additives, but without citric acid, presented pH value more similar to physiological one. CPC pH evolution during the seven days analyzed can be observed on Figure 1.

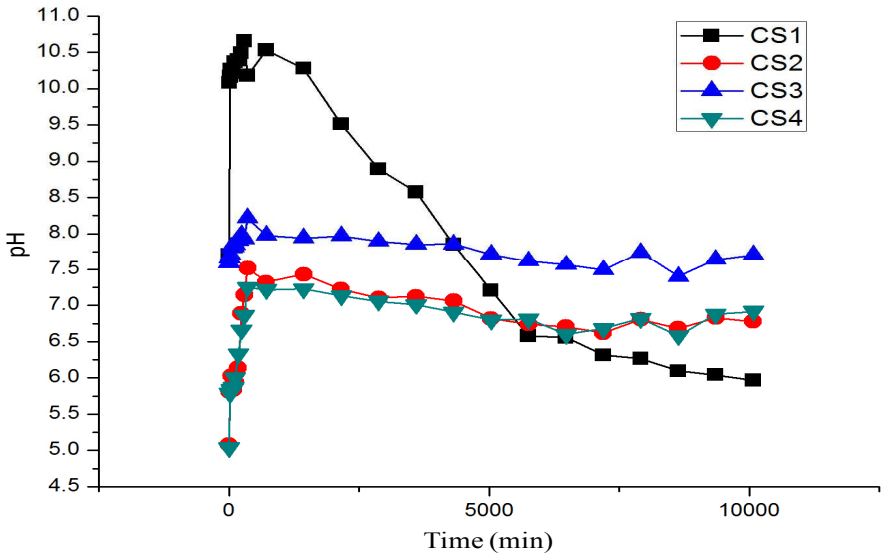

Figure 1. pH evolution during the 168h of setting reaction.

In the four combinations studied, just CS3 does not exhibit abrupt changes on its pH. CS3 pH is always next to 7.5, excellent behavior for clinical application. CS2 and CS4 presented similar behaviors during all the period. After 6h of immersion, CS2 and CS4 pH does not increase, achieving neutral values at the end of the experiment. CS1 pH does not increase its value after 6h too, but pH keeps decreasing even after the period studied. Cs1 pH does not stabilize.

More abrupt changes happen in the initial time of the reaction, as can be analyzed on Figure 2.

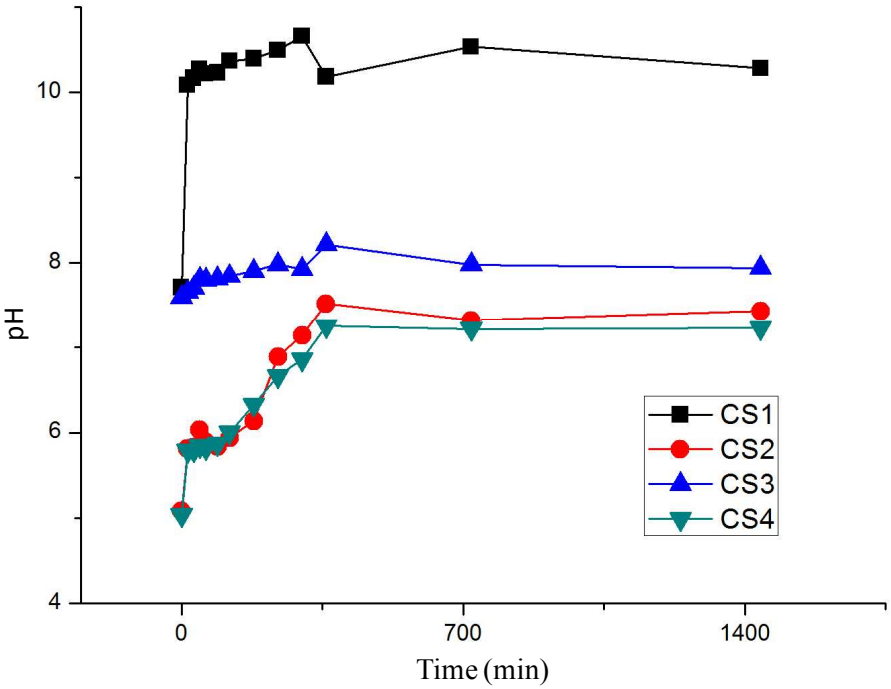

Figure 2. pH evolution in the first 24h of setting reaction.

Additives in the liquid phase that accelerate the reaction are used to decrease setting time. They act solubilizing calcium phosphate crystals; the medium gets its saturation faster with consequent CDHA precipitation. Without additives, the only medium achieves the necessary saturation to CDHA precipitation later.

CS2 and CS4 (cements with citric acid) exhibit more acids pH, while CS3, even with Na_2HPO_4 on its composition, does not present a significant decrease on pH. Probably, the citric acid changes the ionic equilibrium substantially. The solubility constants are more modified, reflecting in abrupt changes on pH equilibrium. As smaller are the pH changes promoted by a material, better it is for its application as biomaterial.

On the cytotoxicity assay (Figure 3), CS2 presented the most satisfactory response. CS2 cell viability is similar to the negative control; however, on the contrary of what was expected by the pH evaluation assay, CS3 was the cement which had produced the most cytotoxic response (approximately 20% of cell viability). One possibility to unsatisfactory CS3 behavior is tannic acid high concentration. It was used 5 wt% of tannic acid, while other additives were used on smaller concentrations. Minor tannic acid concentrations may exhibit the same performance without prejudices in cement biocompatibility.

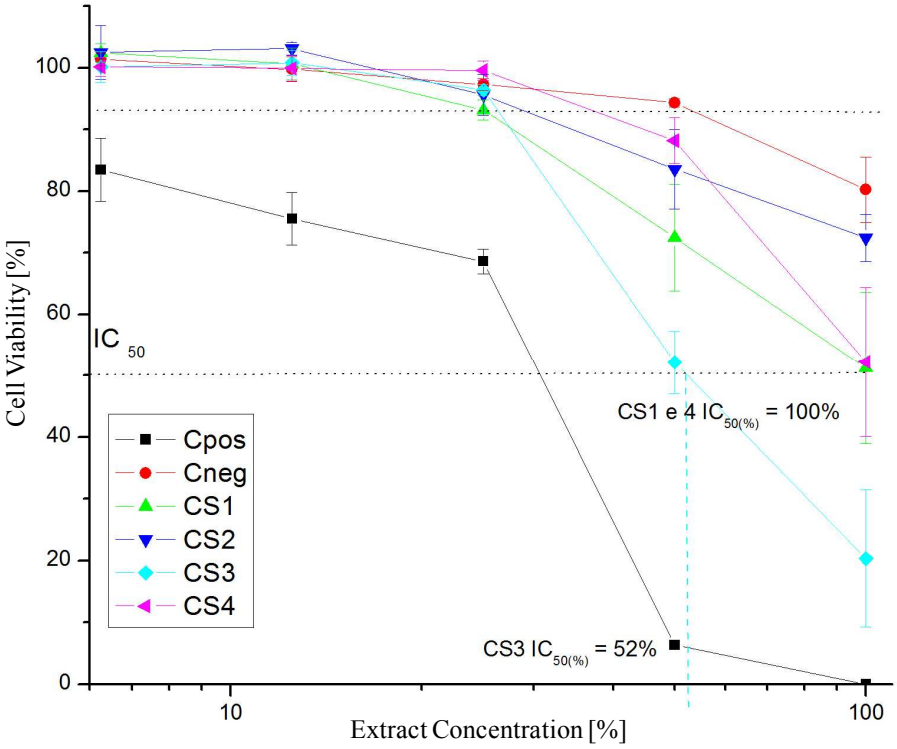

Figure 3. Cell viability as function of extract concentration.

Conclusions

By the results obtained in this work, the cement without additives does not present the ideal conditions for application as bone cement since its pH did not stabilize and it is cytotoxic. Moreover, due to the presence of additives it was possible to control pH evolution during setting and cytotoxic response; however, further investigation is necessary in order to determine the influence of these additives, mainly tannic acid, on the in vivo behavior of these bone cements. The results exhibits an ambiguous behavior in the techniques used to study its biological development.

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