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Cytotoxicity Test for Bioceramics of Calcium Phosphate

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Abstract. Calcium and phosphor compounds have been used as biomaterials in the medical and odontological field to replace bone tissue. Calcium pyrophosphate ($Ca_2P_2O_7$), synthetic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and natural hydroxyapatite from bovine bone were obtained by powder process, milled and characterized by chemical and crystallographic techniques. The toxic effect of these compounds on cells was evaluated by a cytotoxicity test, a quantitative method of colony suppression assay using Chinese Hamster Ovary (CHO) cultured cells in contact with diluted extracts of the bioceramics. The results showed that only natural hydroxyapatite from bovine bone presented cytotoxic effect in the *in vitro* test of biocompatibility.

Introduction

Since calcium phosphates started to show possible clinic solution to repair bone anomalies and losses, researchers and technologists with multidisciplinary knowledge in Materials Science and Health area studied the correlation between the biofunctional behavior and the physico-chemical properties of these compounds.

In 1983, a pioneer review offered information about phase compositions of mineral compounds in calcified tissues, their formation, histological characteristics and medical uses [1]. The most explored material during the last ten years, for bone devices purpose, has been hydroxyapatite (HA) due to its structural stability and compatibility with body metabolism. HA is the most important compound identified in hard tissues of the human body, although some others as calcium hexametaphosphate and calcium pyrophosphate had been identified to nucleate sites of natural HA crystals in the bone structure [2].

The purpose of the present study is to determine the cytotoxic levels of natural HA, synthesized HA and synthesized calcium pyrophosphate, due to their applications as biomaterials in medical and odontological field to replace bone tissue [3].

Materials and Methods

Calcium and phosphor compounds used in this study were obtained as follows: The hydroxyapatite (HAS) was obtained by precipitation method in aqueous solution, pressed and sieved to 250-450 μm range. The precipitation process started from calcium hydroxide and 0.3 M orthophosphoric acid solution under temperature below 100 °C and 7.5 to 8.5 pH range. The HAS powder was pressed at 100 MPa and the pellets were sintered at 1200 °C, and then sieved to the desired particles size range. Natural hydroxyapatite (HAN) has been prepared using trabecular bone slices from the femoral condyle of bovine femur that were cleaned in water vapor at 0.5 MPa prior to soaking in NCS-Tissue Solubilizer (Searle) for 48 h. After that, the slices were soaked in oxygen peroxide before be cleaned again in autoclave. The dried slices were sieved and classified to the 250-450 μm particles size range.

The calcium pyrophosphate (CPP) was obtained by solid state reaction at 870 °C and normal atmosphere from a mixture of tricalcium phosphate and orthophosphoric acid solution. The block reacted material was sieved and classified to the 250-450 μm particles size range.

Crystallinity and chemical purity were verified by X-ray diffraction and X-ray fluorescence (WDXRF) methods, respectively. The toxicity of the bioceramic powders was evaluated by an *in vitro* test with cells.

Cytotoxicity test [4, 5]: Chinese Hamster Ovary K-1 cells were obtained from American Type Culture Collection (ATCC) bank. The cytotoxicity test was carried out with dilution of the bioceramics extracts in contact with CHO cell culture. The cytotoxicity potential can be quantitatively determined by cytolethality using colony suppression assay that is expressed as cytotoxicity index [$IC_{50(\%)}$]. Phenol solution (0.02%) and alumina extracts were used as a positive and negative control, respectively.

Preparation of extracts: 6 g of each material: calcium pyrophosphate, synthetic HA, HA from bovine bone and alumina was placed in a 120 mL capacity screw capped glass bottle and sterilized by autoclaving at 120 °C for 20 min. 60 mL of RPMI-FCS (RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin solution) was added in each bottle. The bottles were shaken and incubated stationary at 37 °C for 48 hours. After incubation the supernatants were filtered in 0.22 μm Millipore membrane and the extracts were serially diluted with RPMI-FCS.

Preparation of cell culture dishes: CHO cells were grown in RPMI-FCS in plastic tissue culture flask, at 37 °C in a humidified 5% CO_2 air incubator. After a confluent monolayer propagation, the culture medium was removed and the cells were washed with calcium and magnesium free phosphate saline buffer (PBS-CMF). For detachment of the cells from the culture tissue flask the cells were treated with 0.25% trypsin solution. After trypsinization the cells were transferred to a screw capped plastic centrifuge tube and washed twice with PBS-CMF. The cells were re-suspended in RPMI-FCS and adjusted to give $1x10^2$ cells/mL. Two milliliters of this cell suspension was seeded to each 60 mm diameter assay culture dish and incubated for about 5 hours for adhesion of the cells. The medium then was removed and replaced with 5 mL of fresh RPMI-FCS as control, and undiluted or serial diluted extract of test materials. Each concentration of extract was tested in triplicate. The culture dishes were incubated in humidified 5% CO_2 atmosphere at 37 °C for colonies formation. After 7 days the medium was removed from the dishes, the colonies were fixed with 10% formalin in 0.9% saline and stained with Giemsa. The number of visible colonies on each dish was counted and compared with the number of colonies in CHO control dish.

Results and Discussion

The relative percentage of visible colonies number in different concentration of extracts was calculated and presented in Table 1. The cytotoxic potentials of the bioceramics can be quantitatively expressed as $IC_{50\ (\%)}$ which is easily determined plotting the Table 1 data on semi-logarithmic graphic. $IC_{50\ (\%)}$ is the concentration of the extract necessary to kill half of the cell population or the extract concentration that suppress colony formation to 50% of the control value. The negative control should not present toxic effect as observed with alumina ($IC_{50(\%)} > 100$) and the positive control should present cytotoxic effect, like 0.02% phenol solution ($IC_{50(\%)}=56$). That means that in the positive control 50% of the cells were killed by a concentration of 56%, as shown in Fig. 1.

Table 1. Cytotoxicity Test of Calcium Pyrophosphate (CPP), Synthetic Hydroxylapatite (HAS) and Natural Hydroxylapatite (HAN): percentage of visible colonies number and concentration of extract of bioceramics.

	% Number of Colonies				
Extract conc. (%)	Negative control	Positive control	HAS	HAN	CPP
6.25	109	91	94	97	105
12.5	101	106	102	101	96
25	94	82	103	102	98
50	107	56	101	62	90
100	94	6	82	0	71

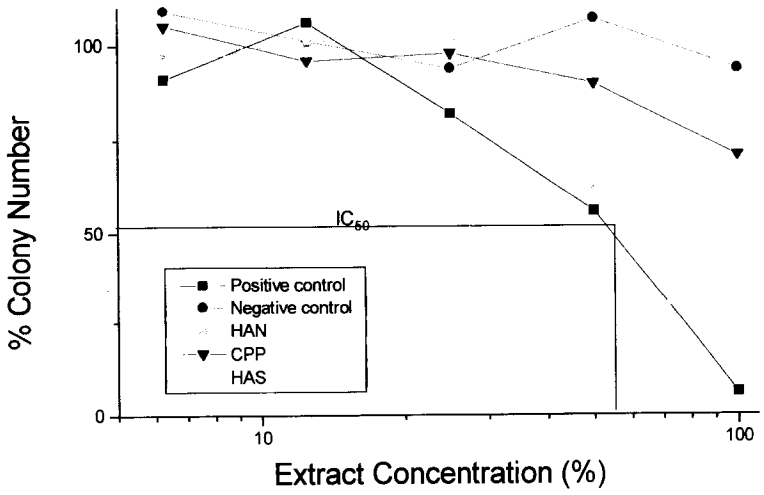

Figure 1: Colony suppression curves of Calcium Pyrophosphate (CPP), Synthetic Hydroxylapatite (HAS) and Natural Hydroxylapatite (HAN).

Fig. 1 shows that HAS and CPP are not cytotoxic, $IC_{50(\%)} > 100$, but for HAN, $IC_{50(\%)}=60$, i.e., HAN is cytotoxic. A strong alkali solution in toluene (NCS-Tissue Solubilizer) was used to purify HAN from bovine bone and probably this procedure let some residuals that showed to be toxic in the culture cell assay.

Conclusion

The results showed that synthesized hydroxyapatite (HAS) obtained by precipitation method and calcium pyrophosphate (CPP) obtained by solid state reaction are not cytotoxic. On the other hand, the procedure to obtain natural hydroxyapatite (HAN) from bovine bone showed not be adequate, giving a cytotoxic final product.

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