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Cytocompatibility evaluation of hydroxyapatite/collagen composites doped with Zn^{+2}

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ABSTRACT

The cytocompatibility of synthetic hydroxyapatite/collagen composites alone or doped with Zn^{+2} was tested by using primary culture of osteoblasts. The hydroxyapatite (HAP) was synthesized having calcium hydroxide and orthophosphoric acid as precursors. A new HAP composite was developed adding 1.05 w% of $Zn(NO_3)_2.6H_2O$ forming HAPZn. The pure type I collagen (COL) was obtained from bovine pericardium by enzymatic digestion method. The HAP/COL and HAPZn/COL composites were developed and characterized by SEM/EDS. The cell viability and alkaline phosphatase activity in the presence of composites were evaluated by MTT assay and NBT-BCIP assay, respectively, and compared to osteoblastic cells of the control. Three individual experiments were accomplished in triplicates and submitted to the variance analysis and Bonferroni's post-test with statistically significant at $p<0.05$. The HAPZn/COL composite did not stimulate the proliferation and increasing of alkaline phosphatase activity of the osteoblastic cells. The tested composites did not alter the cellular viability neither caused alterations in the cellular morphology in 72 h showing adequate properties for biological applications.

Keywords: Biomaterials, hydroxyapatite, collagen, cytocompatibility, zinc.

Avaliação da citocompatibilidade dos compósitos hidroxiapatita/colágeno dopados com Zn^{+2}

RESUMO

A citocompatibilidade de compósitos sintéticos hidroxiapatita/colágeno dopados ou não com Zn^{+2} foi avaliada, usando cultura primária de osteoblastos. A hidroxiapatita (HAP) foi sintetizada tendo como precursores o hidróxido de cálcio e o ácido ortofosfórico. Um novo compósito de HAP foi desenvolvido adicionando 1,05% em peso de $Zn(NO_3)_2.6H_2O$, obtendo-se HAPZn. Colágeno tipo I puro (COL) foi obtido de pericárdio bovino pelo método da digestão enzimática. Os compósitos HAP/COL e HAPZn/COL foram desenvolvidos e caracterizados através de MEV/EDS. A viabilidade celular e a produção de fosfatase alcalina na presença dos compósitos foram avaliadas pelos ensaios de MTT e NBT-BCIP, respectivamente, e comparadas às células osteoblásticas do controle. Três experimentos individuais foram realizados em triplicatas e submetidos à análise de variância e pós-teste de Bonferroni com significância estatística $p<0,05$. Os compósitos HAPZn/COL não estimularam a proliferação e o aumento da produção de fosfatase alcalina das células osteoblásticas. Os compósitos testados não alteraram a viabilidade celular nem causaram alterações na morfologia celular em 72 h, mostrando propriedades adequadas para aplicações biológicas.

Palavras-chave: Biomateriais, hidroxiapatita, colágeno, citocompatibilidade, zinco.

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1 INTRODUCTION

Biodegradable polymers and bioactive ceramics are being combined in a variety of composite materials for tissue engineering scaffolds [1] with the objective of substitution and regeneration of hard tissues. Calcium phosphates (CaP)/collagen composites have been developed due to its similar composition with the bone tissue [2-4]. The main advantageous in collagen load with hydroxyapatite is the modulation of the adhesion process to osteoprogenitor cells migrate and differentiate in substratum [5].

Research groups have studied how to find biomaterials and techniques to impart appropriate biological properties to synthetic composites for replacement of the human skeleton. Zinc is an essential trace element with stimulatory effects on osteoblastic cell proliferation and bone formation *in vitro* and *in vivo*. It hold an inhibitory effect on osteoclastic bone resorption [6, 7]. Several researchers have attempted to doper materials with Zn^{+2} at low concentrations increasing the bioactivity of bone cells [4, 6-8] and decreasing and regulating the inflammatory reaction [9].

Evaluation of the citocompatibility of the composite is usually performed via *in vitro* citotoxicity test. It is a sensitive and reproducible screening method to detect cell death or other effects on cellular functions. The primary culture osteoblasts is a well established model to investigate biocompatibility evaluating the cellular viability due its proliferated capacity [8, 10, 11].

The present *in vitro* study developed hydroxyapatite/collagen composites doped with Zn^{+2} to attempt the materials association with adequate properties for biological applications in the recovery of the bone tissue by trauma or pathogenies.

2 MATERIAL AND METHODS

Hydroxyapatite (HAP) was synthesized having calcium hydroxide and orthophosphoric acid as precursors [12]. A new composite of HAP was developed adding 1.05 w% of $Zn(NO_3)_2.6H_2O$ during the synthesis obtaining HAPZn [4]. The pure type I collagen (COL) was obtained from bovine pericardium by enzymatic digestion method [13]. The composites HAP/COL and HAPZn/COL were developed with the ratio 80/20 (CaP/COL, weight %) [4].

The materials were covered with a fine layer of gold (Au) and the morphology and semi-quantitative elementary analysis of the sample microareas were obtained by X-ray energy dispersive spectroscopy (EDS) and scanning electron microscopy (SEM) thought JSM model 6360LV - JEOL equipment, using 10 to 15 KeV. The results were determined for the analysis of three different areas from each sample.

2.1 Culture of Osteoblasts

Osteoblasts were isolated from the calvaria of 1-5 days old Wistar rats by enzymatic digestio method [14]. Briefly, after cut into small pieces, the calvaria bone was digested with 1% trypsin and four times with 2% collagenase. The supernatant of the three last washes were centrifuged 1000xg for 5 min and the pellet was ressuspended in 5 mL of RPMI-1640 (Sigma, St Louis, USA) medium supplement with 10% fetal bovine serum (FBS) (GibcoBRL, NY, USA) and 1% antibiotic-antimycotic solution (GibcoBRL, NY, USA). After confluence the cells were replicated and used on passage 2.

Osteoblasts were plated at the density 1 x 10^5 cells and incubated with granules of the different composites. The same culture medium containing osteoblasts without the presence of the composites was use as a negative control. The experiments were performed 72 h after incubation.

2.2 Cellular Viability and Alkaline Phosphatase Activity

Osteoblast viability was evaluated by MTT assay in the presence of composites. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) is based on the capacity of viable cells to metabolize tetrazolium to formazan crystals, a purple dye that can be solubilized and measured by optical density. After incubation for 18 h the plates were measured at 595 nm.

Alkaline phosphatase production of osteoblasts was analyzed by NBT-BCIP (indolylphosphate-nitroblue tetrazolium) assay. Nitroblue tetrazolium is hydrolyzed by the alkaline phosphatase secreted by osteoblasts and the intensity of enzyme activity was measured at 595 nm. The morphology of the osteoblasts in contact with the composites and in the control group was observed by transmitted light microscopy and photographies were obtained.

Data were analyzed statistically using variance test (ANOVA) and Bonferroni's post-test. Differences were considered significant at $p<0.05$

3 RESULT AND DISCUSSION

Human bone is mainly composed of hydroxyapatite crystals and collagen fibers. Calcium phosphate (CaP)/COL composites are a biodegradable artificial bone developed to engineer the organized bone structure, mimicking the biological conditions [5]. These composites are considered one of the most promising biomaterials to replace autologous bones due to structural and biological similarity to this tissue and excellent biocompatibility [1, 3, 5]. The composite development is one alternative that is being considered and studied to combine the typical bioactive behavior and mechanical properties such as elastic modulus and toughness of some materials and to produce composites with properties closer to bone tissue [3, 5].

The morphology and semi-quantitative composition of present chemical elements in the HAP/COL sample can be observed in Fig. 1. The scanning electron micrograph (SEM) of HAP/COL surface showing a homogeneous aspect with HAP particles into COL matrix (Fig. 1A) and the energy dispersive spectroscopy (EDS) showing peaks of high intensity of Ca, P and C, and residual elements of its synthesis, Na and Cl (Fig. 1B). The qualitative chemical composition of the composites showed similar characteristics to their constituent materials.

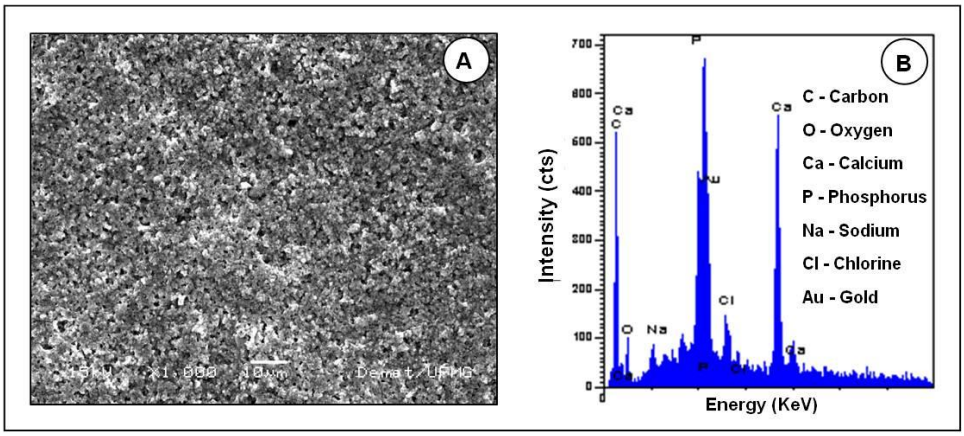

Figure 1: Scanning electron micrograph at 1,000x magnification (A) and energy dispersive spectroscopy (B) of HAP/COL composite.

The composites tested by MTT assay showed no significant differences in absorbance when compared with the control (p>0.05). The cell viability in composites doped with Zn^{+2} (HAPZn/COL) or undoped (HAP/COL) showed no difference (Fig. 2).

The alkaline phosphatase production (Fig. 3) of the osteoblasts in presence of the Zn^{+2} doped and undoped composites was comparable to alkaline phosphatase production of the control cells. The osteoblasts in contact with HAPZn/COL and HAP/COL composites produced alkaline phosphatase comparable to control cells.

The presence of Zn^{+2} in low concentrations can induce a greater metabolization and can produce a greater alkaline phosphatase production of the osteoblasts. The literature studies related that zinc released from the ZnCaP stimulates osteoblastic activities [6, 7, 9]. Nevertheless, zinc is known as a potent inhibitor of the crystal growth of apatite, which is a controversial issue. Sogo *et al.*, 2004, reported that cytocompatibility test using preosteoblastic of mouse calvaria showed no significant differences between pure αTCP and αZnTCP with a zinc content of 0.11 wt%. Webster *et al.*, 2004, reported that the adhesion of human osteoblasts was greater on HA doped with Zn^{+2} than undoped HA. Kawamura *et al.*, 2003 related that the optimum content of zinc was found to be 0.316 wt% in the composite ceramic of βZnTCP and hydroxyapatite (HA) for promoting bone formation *in vivo*, and a zinc content of 0.63 wt% was excessive for both ceramics.

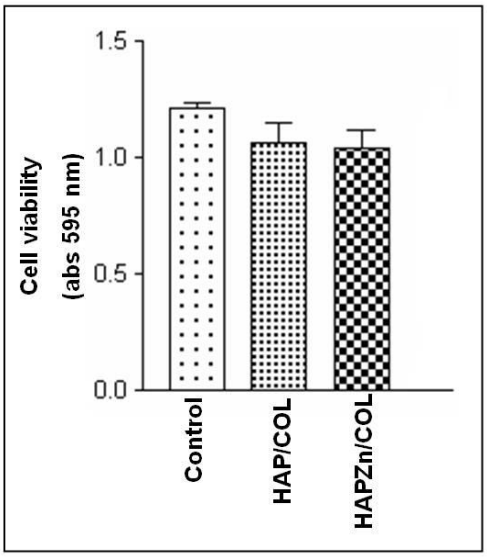

Figure 2: Cell viability after 72 h of incubation: osteoblasts showed no significant difference proliferation in presence of the composites when compared to control. Results represent mean ± SD of three triplicates from three separate experiments (p<0.05).

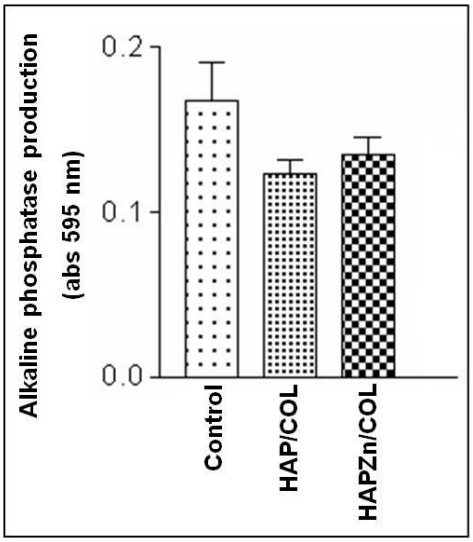

Figure 3: Alkaline phosphatase production of osteoblasts after 72 h of incubation: cells showed no significant difference in presence of the composites when compared to control cells. Results represent mean ± SD of three triplicates from three separate experiments (p<0.05).

In this study, the result showed no significant difference between the experimental groups. A zinc amount of 1.05 w% in the HAP materials did not indicate an efficient content of this divalent metal to stimulate effects on proliferation and increasing of alkaline phosphatase activity of the bone cells in the presence of HAPZn/COL composite. Probably, the amount of the dopant Zn^{+2} 1.05 wt% in HAPZn was insufficient just after mixed with the collagen during the synthesis of the HAPZn/COL composite. Thus, the tested composites did not promote this effective stimulation.

Osteoblasts in direct contact with the HAP/COL (Fig. 4A) and HAPZn/COL (HAPZn/COL (Fig. 4B) composites showed typical morphology and produced alkaline phosphatase comparable to the control cells (Fig. 4C).

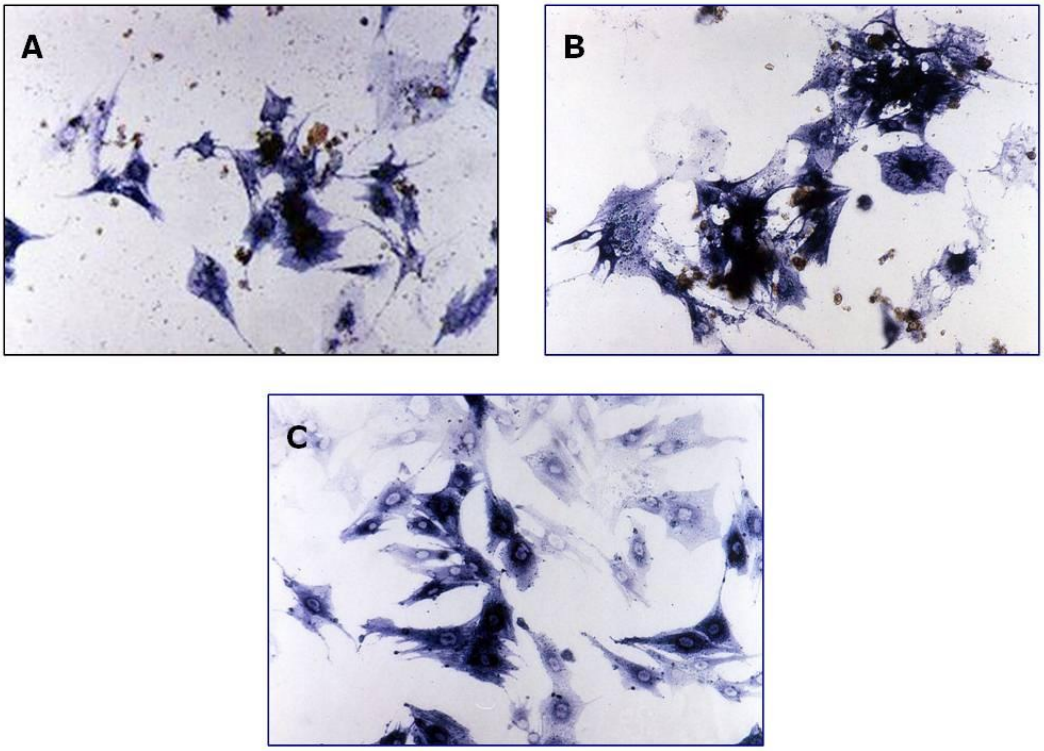

Figure 4: Osteoblasts dyed with NBT-BCIP evidencing the alkaline phosphatase production in the HAP/COL composite (A), HAPZn/COL composite (B) and control cells (C).

Osteoblastic cells are the basic structural and functional units in bone growth and metabolism and its behavior in the presence of materials is a suitable experimental model for biocompatibility evaluation [7]. The osteoblasts produced free alkaline phosphatase in the matrix that was colored by BCIP-NBT. Only normal osteoblastic cells can metabolize and transform the tetrazolium salt in blue crystals observed in the cells (Fig. 4). This evidences the presence of alkaline phosphatase already metabolized that will begin the mineralization.

The doped and undoped composites with a zinc content of 1.05 w% showed cytocompatibility. The osteoblasts grew well in contact with the composites and showed normal morphology in 72 h. These findings are in accord with the literature that describe the citocompatibility of Zn^{+2} within a nontoxic level [16].

4 CONCLUSION

The tested HAP/COL composite doped with Zn+2 in amount of 1.05 w% did not stimulate effects on proliferation and increasing of alkaline phosphatase activity of the osteoblastic cells. The HAP/COL composites doped and undoped with Zn+2 showed acceptable cytocompatibility presenting adequate properties for biological applications. Further investigation is important to determine the effective amount of Zn+2 in these composites.

5 ACKNOWLEDGEMENTS

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