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Research Article

Cytotoxicity and Genotoxicity of PLA and PCL Membranes on Osteoblasts

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Abstract

The objective of this study was to analyze the behavior of MG-63 osteoblasts in contact with poly (lactic acid) (PLA) and polycaprolactone (PCL) porous resorbable membranes, incorporated with calcium silicate ceramic fibers ($CaSiO_3$), aiming application in bone regeneration guided in periapical lesions. Four experimental groups were used, from the concentration of; PLA + 5% $CaSiO_3$; PLA + 10% $CaSiO_3$; PCL + 5% $CaSiO_3$; PCL + 10% $CaSiO_3$, and the control group. Cell viability and genotoxicity were evaluated. Data were analyzed by ANOVA and Tukey ($p < 0.05\%$). The results showed that no experimental group was cytotoxic, but the PLA 5% and PCL 10% groups presented higher cell viability with a statistical difference between PCL 5% and PLA 10% ($p < 0.05$). In the genotoxicity test, the experimental groups were not genotoxic, since they had similar or smaller numbers of micronuclei to the control group ($p > 0.05$). It was concluded that the polymer membranes associated with $CaSiO_3$ are biocompatible materials once did not present any cytotoxicity and genotoxicity on osteoblasts *in vitro.*

Keywords: Biomaterial; Polymers; Biocompatibility

Introduction

The success in developing a biomaterial depends on physical, chemical, biological, clinical, and technological factors. The physiological response due to the biological interaction depends on the physicochemical characteristics and the surface properties of the material, and it is necessary to identify and control these factors in order to guarantee the cellular adhesion at the interface, as well as to enhance this interaction [1,2].

A biomaterial may be constituted by an inert animal or synthetic substance; not presenting toxicity, neither being carcinogenic or radioactive. They can be used temporary or permanently, degradable and absorbable, in a way that allows bone growth and repairs [3-6].

Poly (lactic acid) (PLA) and polycaprolactone (PCL) polymers are synthetic, biocompatible and biodegradable polymers from

to the aliphatic polyester family and have controllable degradation time [6]. PLA has a rapid *in vivo* degradation rate while PCL is slower degraded and is ideal for medical conditions that require an extended time for bone regeneration [7-10], but this polymer has a low mechanical resistance [11-13] restricting the application in bone regenerative medicine.

In order to solve this low mechanical resistance of these polymers, it is suggested the incorporation of calcium silicate fibers, which can improve the mechanical strength and biocompatibility [14-17].

The objective of this *in vitro* study was to analyze the biocompatibility of porous resorbable membranes of PLA and PCL, reinforced with calcium silicate fibers used for guided bone regeneration.

Materials and Methods

The development of porous polymer membranes reinforced with calcium silicate ($CaSiO_3$) ceramic fibers was prepared experimentally in collaboration with the Federal State University of São Paulo (Institute of Science and Technology, UNIFESP).

For this study, four study groups were considered, consisting of porous absorbable membranes of poly (lactic acid) (PLA) and polycaprolactone (PCL), with dimensions of 9 mm in diameter.

For the determination of biocompatibility, the materials in contact with the MG-63 osteoblasts was evaluated utilizing a cytotoxicity test with the Alamar Blue® colorimetric test (Thermo Scientific) and the genotoxicity verified by the micronucleus assay.

Four experimental groups were used, from the concentration of; PLA + 5% $CaSiO_3$; PLA + 10% $CaSiO_3$; PCL + 5% $CaSiO_3$; PCL + 10% $CaSiO_3$, and the control group.

Cytotoxicity test

MG-63 cells were obtained from the Laboratory of Cell Studies (LEIC, ICT-Unesp, São José dos Campos, SP, Brazil). The cells were cultured in DMEM medium (Cultilab, São Paulo, Brazil), supplemented with 10% fetal bovine serum (FBS), 50 µg/mL gentamicin, 0.3 µg/mL fungisone, 10^{-7} M dexamethasone (Sigma), 5 µg/mL ascorbic acid, 7 mM beta-glycerophosphate (Sigma), at 37°C and 5% CO_2. The culture medium was renewed every 3 days and the cells were subcultured until reaching subconfluency (85%).

For cytotoxicity testing, samples of the PLA and PCL polymers were placed in direct contact with the MG-63 osteoblasts. For this, PLA and PCL samples were placed on 24-well plates and osteoblasts ($2x10^4$) were cultivated on the samples and incubated for 3 days (5% CO_2 at 37°C). A control group was performed, where only cells were cultured in the 24-well plates. After this time, the supernatant of the wells was removed, and the plate was washed three times with 1 mL of sterile PBS to discard dead cells and residues. After washing, 20 µL of 10% Alamar Blue reagent was added in 500 µL culture medium (DMEM) in each well. The plates were covered with aluminium foil and incubated for 4 hours (5% CO_2 at 37°C). During this time, non-fluorescent blue resazurin was reduced to fluorescent pink resofurin. Subsequently, the supernatants of the 24-well plates were transferred to 96-well plates and the absorbance reading was performed on a microplate spectrophotometer (570 nm). Cytotoxicity was expressed as the percentage in relation to the control group (= 100%). This test was performed in duplicate.

Genotoxicity assay

The micronucleus assay consists of the visualization of a small nucleus coupled to the true nucleus that appears from the displacement of chromosome fragments of the main nucleus, these micronuclei arise by spontaneous genetic alterations or are induced by genotoxic agents. In this study, MG-63 ($2x10^4$) osteoblasts were cultivated in 24-well plates in direct contact with the PLA and PCL samples, and incubated for 3 days (5% CO_2 at 37°C). A positive control group (ethylmethanesulfanate, EMS) and a negative control group were also performed. After three days, each well was washed with 1 mL sterile PBS and 500 µL of the 4% formalin-fixing solution (Sigma-Aldrich) was added for 10 minutes to fix the cells. Then, the wells were washed with 1 mL of PBS and 200 µL of PBS and one drop of fluoroshield with DAPI (Sigma Aldrich, Brazil) was added and shaking for 5 minutes under light protection. The images were recorded with a fluorescence microscope (Axiovert 200, Zeiss, Jena, Germany) and the micronuclei wasquantified in every 2,000 cells counted using the Image J software.

The statistical analysis was performed by ANOVA and Tukey test, with significance level of 5% ($p \leq .05$).

Results

Cytotoxicity test

Data analysis showed that PLA and PCL membranes were not cytotoxic by Alamar Blue® test. The percentage of absorbance, related to the cell viability in contact with the materials, was not lower than 70% compared to the control group, which was considered as 100%.

PLA and PCL membranes presented significant statistical difference to control group, showing a p value of 0.001 and 0.006 for PLA 5% and 10%, respectively. PCL 5 and 10% showed p value of 0.001 and 0.005, respectively.

PLA 5% presented higher cell viability than PLA 10% ($p < .05$). In contrast, PCL 5% showed lower cell viability compared to PCL 10% ($p < .05$) (Figure 1).

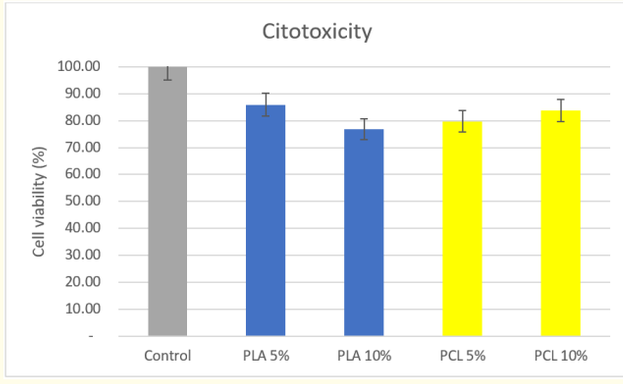

Figure 1: Percentage of cell viability of PLA and PCL membranes and control group (100%) after 3 days in contact with osteoblasts.

Genotoxicity assay

The results of the genotoxicity assay showed that no PCL or PLA groups was genotoxic for the cells (Table 1). However, all groups were statistically significant in relation to the positive control group (EMS) ($p < 0.05$). In figure 2, it was observed the formation of micronuclei in the positive control group.

Groups	Mean of micronuclei
PLA 5%	10
PLA 10%	11
PCL 5%	9
PLA 10%	10
EMS (positive control)	62
Control	8

Table 1: Mean of number of micronuclei in 2,000 cells.

Figure 2: Cell nucleus stained by fluorescent DAPI dye. (A) without micronuclei (10X); (B) micronuclei indicated by arrows (10X); (C) Nucleus e Micronuclei (60X).

Discussion

PLA and PCL polymers have been widely using in tissue engineering due to its present satisfactory properties [8,10,18,19].

In the present study, the results demonstrated that PLA or PCL membranes were not cytotoxic for MG-63 osteoblasts; however, there was a difference between 5% and 10% of calcium silicate concentrations for the two membranes. The PLA 5% membranes and PCL 10% membranes presented higher cell viability, showing a dose-dependent difference.

According to our results, some authors verified that PLA membranes did not present cytotoxicity *in vitro* and also promoted proliferation of osteoblasts [6,18,20] or others cell lineages [21].

It can also be observed in the literature that PCL membranes showed adequate physical, mechanical and biological properties to be used in contact with living tissues. Lu., *et al.* [22] and Dziadek., *et al.* [17] found that PCL membranes associated or not to bioglass were biocompatible on osteoblasts and adipocytes, respectively. In an agreement, Rowe., *et al.* [6] report that both PLC and PLA membranes associated with bioglass promoted adhesion and proliferation of osteoblasts.

In the present study, PLA and PCL membranes at 5% and 10% concentrations of calcium silicate showed no genotoxicity, indicating that they cannot cause mutagenic changes in osteoblasts under the conditions of this study.

Accordingly, Uzun., *et al.* [21] found that PLA membranes were not genotoxic by comet and micronucleus tests. Filipović., *et al.* [23] verified that PCL nanospheres did not cause mutagenic changes by the comet test, demonstrating their low genotoxic potential. Huang., *et al.* [24] also did not observe genotoxicity by the micronucleus assay on Chinese Hamster cells [25].

Conclusion

It is concluded that the PCL and PLA membranes associated with calcium silicate presented great biocompatibility since they did not cause cytotoxicity or genotoxicity on osteoblasts *in vitro.* Our results showed a preliminary study indicating that PCL and PLA membranes have the potential to be used in bone regeneration. However, an *in vivo* complimentary study is essential to confirm this hypothesis.

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