THE EFFECT OF BIOACTIVE GLASS NANOPARTICLES ON THE BEHAVIOR OF HUMAN PERIODONTAL LIGAMENT CELLS

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ABSTRACT

The periodontium is a complex tissue consisting of soft and mineralized structures. Several diseases affect the integrity of periodontium causing the destruction of the connective tissue and cells. In recent decades bioactive glass has been used in several studies in the dental field because of its excellent regenerative properties. It is known that the effect of bioactive glass can be enhanced in the nanoscale. In this study we isolated human periodontal dental ligament cells (HPDL) of third molar teeth with orthodontic indication for extraction. Bioactive glass nanoparticles (BGnp) were synthesized by modified Stöber method. The cell viability by Resazurin assay, cell proliferation by 5-Bromo-2-Deoxyuridine (BrdU) assay and (alkaline phosphatase) ALP activity assay were used to evaluate the behavior of HPDL cells in direct contact with particles. It was observed a significant increase in cell viability, cell proliferation and ALP activity in the presence of BGnp when compared to the control group. These results indicate that BGnp induces HPDL cells to proliferate, indicating that it is a potential material to be used for periodontal regeneration through tissue engineering.

Keywords: Periodontium, bioactive glass, nanoparticles.

INTRODUCTION

Periodontium is a complex anatomical structure composed of both hard and soft connective tissues. The structures comprising the periodontium include the gingiva, periodontal ligament, cementum and alveolar bone [1]. Several diseases affect the integrity of periodontal structures causing the destruction of the connective tissue matrix and cells, causing the loss of fibrous attachment and the resorption of alveolar bone. These changes often lead to tooth loss [2]. The biologic goal of periodontal regeneration is restoration of the periodontium to its original form and function. Bioactive glass is a tissue repairing material used for regeneration in many areas of dentistry. Bioceramics composition, crystallinity, particle size and porosity are characteristics that influence their dissolution rate and affect their material–tissue interaction [3-8]. An alternative to the synthesis of bioactive glass is in the form of nanometric particles, because is known that the effect of biomaterials can be enhanced in the nanoscale [9]. Therefore, in this work it was evaluated the effect of bioactive glass nanoparticles (BGnp) on HPDL cells behavior isolated from human periodontal tissue.

MATERIALS AND METHODS

Culture of HPDL cells. We isolated HPDL cells of third molar teeth with orthodontic indication for extraction. Then, they were immersed in a digestion solution, 20 mL of Dulbecco’s Modified Eagle Medium (DMEM) containing 2 mg/mL collagenase Type II and 0.25% trypsin/EDTA from Gibco (Grand Island, NY) at 37°C for 30 min. Five consecutive digestions were performed for 20 min each. The first digestion was
The solutions were centrifuged to collect released periodontal ligament cells. We obtained four cell populations that were named HPD cells. Primary cells were cultured in DMEM with 10% fetal bovine serum (FBS) from Gibco (Grand Island, NY), plus penicillin G sodium (10 units/mL) and streptomycin sulfate (10 mg/mL) all from Sigma (St. Louis, Mo) and in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. The culture medium was changed once every three days and cells at the second to fifth passages were used in the indicated studies.

**Synthesis of bioactive glass nanoparticles.** 200 ml of methanol was mixed with 0.46 ml of ammonium hydroxide 33% from Merck (Brazil) and 5.40 ml of water. Then 5.57 ml TEOS and 0.56 ml TEP all from Sigma (St. Louis, Mo) were added dropwise for 10 min. 3.46 g Ca(NO\textsubscript{3})\textsubscript{2}.4H\textsubscript{2}O from Merck (Brazil) was dissolved in the sol. The nanoparticles formed were separated by subsequently filtrations in a 0.22 and 0.11 mm Milipore. Then, the sol filtered was lyophilized. The powders obtained were thermally treated at 200°C for 40 min, with heating rate of 1°C/min [10].

**Biological assays.** Tests were performed according to ISO 10993. BGnp were mixed in the medium, with weight-to-solution volume ratio of 0.1 mg/mL, and soaked for 5 min. The mixture was placed directly over the HPDL cells culture on the plates and maintained at 37°C under 5% CO\textsubscript{2} atmosphere. It was used phosphate-buffered saline (PBS), Sigma-Aldrich USA, as positive control and chips of eppendorf (1 mg/ ml) sterilized under UV radiation as negative control. Control cultures and seeded materials were characterized at 72 hours for cell viability (Resazurine assay), at 72 hours for cell proliferation (BrdU assay) and alkaline phosphatase (ALP) activity after one week culture.

**Resazurin assay.** HPDL cells were plated in 96 well culture plates (10\textsuperscript{3} cell/mm\textsuperscript{2}) cultured in DMEM containing 10% FBS, penicillin G sodium (10 units/mL) and streptomycin sulfate (10 mg/mL). After 72 hours, 20\textmu l of Resazurine (0.01 mg/ml) from Sigma (St. Louis, Mo) was added to each well and incubated for 24 hours at 37°C under 5% CO\textsubscript{2}. After, the absorbance was measured at spectrophotometer ADAP 1.6 (Netherlands) with 570nm and 595 dual filters.

**BrdU assay.** Cell proliferation was measured by BrdU assay from Roche (Brazil) according to the manufacturer’s instructions. HPDL cells were plated and cultured as above and 72 hours afterwards the cells were treated with BrdU labeling solution. The cells were then fixed, and anti-BrdU antibody was added (1:100). A colorimetric substrate was used, and BrdU incorporation was measured with a multiplate reader ADAP 1.6 (Netherlands) with 405nm filter.

**Alkaline phosphatase activity.** HPDL cells were plated in 24 well culture plates (1\times10\textsuperscript{4} cells/mm\textsuperscript{2}) and cultured in DMEM containing 10% FBS, penicillin G sodium (10 U/ml) and streptomycin sulfate (10 mg/ml) all from Sigma (St. Louis, Mo). After one week, 200 ml of BCIP-NBT solution by Invitrogen (Carlsbad, Ca), was added to each well. After 2 h of incubation, insoluble purple precipitates were solubilized with 210 ml of SDS 10% HCl and incubated for 18 h. The optical density was measured at spectrophotometer ADAP 1.6 (Netherlands) with 595nm filter.

**Results and discussion.** In this work we investigated the effect of bioactive glass nanoparticles on HPDL cells viability, cell proliferation and ALP activity. The particles presented a regular round shape with particle size around 100 nm. The EDS analysis
shows the presence of Si, Ca and P in the glass composition, as expected giving, a semi quantitative chemical composition of 79% SiO$_2$, 19% CaO and 2% P$_2$O$_5$. The mitochondrial activity was assessed by Resazurin analysis. This assay is specifically used to evaluate mitochondrial function and cell viability. Cells treated with the glass nanoparticles after 72 hrs showed a 20% increase in cell viability when compared with the control group (Figure 1.A). Experiments were performed with n = 5 (p < 0.05 statistical analysis: One way / ANOVA / Bonferroni / GraphPad Prism). To confirm the importance of bioactive glass nanoparticles in the proliferation of HPDL cells we performed the BrdU assay. In this study the quantification of BrdU on cells, after 72 hours of treatment with the bioactive glass nanoparticles, was about 33% larger when compared with control group (Figure 1.B). Experiments were performed with n = 5 (p < 0.05 statistical analysis one way / ANOVA / Bonferroni / GraphPad Prism). Together, BrdU and Resazurin results suggest the importance of bioactive glass nanoparticles for HPDL cells proliferation in cell cycle. We also investigated ALP activity in the cells populations. ALP expression is considered an early differentiation marker of the mineralized tissue cells phenotype. Cells treated with the glass nanoparticles after 1 week showed a 177% increase in ALP activity when compared with the control group (Figure 1.C). Experiments were performed with n = 3 (p < 0.05 statistical analysis one way / ANOVA / Bonferroni / GraphPad Prism). Results show that there is an increased production of ALP, thus suggesting an increase in cell differentiation.

Figure 1. Cell viability, cell proliferation and alkaline phosphatase activity. HPDL cells were incubated with bioactive glass nanoparticles for 72 hours and cell viability, ALP activity and cell
proliferation rate was evaluated in comparison to control conditions. (A) Mitochondrial activity by Resazurin assay. We observed an increase of $20 \pm 1.63$ in HPDL cells viability when cells were exposed to bioactive glass nanoparticles, compared with control group ($p < 0.05$, $n = 5$). (B) Cell proliferation by BrdU analysis. We observed an increase of $33 \pm 3.0$ in cells proliferation when cells were exposed to bioactive glass nanoparticles, compared with control group ($p < 0.05$, $n = 5$). (C) ALP activity. It was observed an increase in ALP activity of $177 \pm 2.9\%$ in HPDL cells compared with control group ($p < 0.05$, $n = 3$).

**Conclusions.** In this work, it was observed a significant increase in cell viability, cell proliferation and ALP activity in the presence of BGnp. These results together suggest that the bioactive glass nanoparticles induce proliferation and differentiation of human periodontal tissue cells, indicating that it is a potential material to be used for periodontal regeneration through tissue engineering.

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