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Research Article

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Cytocompatibility and cell proliferation evaluation of calcium phosphate-based root canal sealers

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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Conceptualization: Mestieri LB, Zaccara IM. Data curation: Mestieri LB, Zaccara IM. Formal analysis: Mestieri LB, Zaccara IM, Pinheiro LS. Funding acquisition: Barletta FB, Kopper PMP, Grecca FS. Investigation: Mestieri LB, Zaccara IM. Methodology: Mestieri LB, Zaccara IM. Project administration: Barletta FB, Kopper PMP, Grecca FS. Resources: Barletta FB, Kopper PMP, Grecca FS. Software: Mestieri LB,

ABSTRACT

Objectives: This study aimed to evaluate the cell viability and migration of Endosequence Bioceramic Root Canal Sealer (BC Sealer) compared to MTA Fillapex and AH Plus.
Materials and Methods: BC Sealer, MTA Fillapex, and AH Plus were placed in contact with culture medium to obtain sealers extracts in dilution 1:1, 1:2 and 1:4. 3T3 cells were plated and exposed to the extracts. Cell viability and migration were assessed by 3-(4,5-dimethylthiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) and Scratch assay, respectively. Data were analyzed by Kruskal-Wallis and Dunn's test ($p < 0.05$).
Results: The MTT assay revealed greater cytotoxicity for AH Plus and MTA Fillapex at 1:1 dilution when compared to control ($p < 0.05$). At 1:2 and 1:4 dilutions, all sealers were similar to control ($p > 0.05$) and MTA Fillapex was more cytotoxic than BC Sealer ($p < 0.05$). Scratch assay demonstrated the continuous closure of the wound according to time. At 30 hours, the control group presented closure of the wound ($p < 0.05$). At 36 hours, only BC Sealer presented the closure when compared to AH Plus and MTA Fillapex ($p < 0.05$). At 42 hours, AH Plus and MTA Fillapex showed a wound healing ($p > 0.05$).
Conclusions: All tested sealers demonstrated cell viability highlighting BC Sealer, which showed increased cell migration capacity suggesting that this sealer may achieve better tissue repair when compared to other tested sealers.

Keywords: Root canal; Cell migration; Fibroblast; Endodontics

INTRODUCTION

Calcium silicate sealers have been developed aiming to find a material that has excellent biological properties and to associate different components that may improve the physical-chemical and mechanical properties. When in contact with tissue fluids, they produce calcium hydroxide ions, which are essential for the formation of hydroxyapatite [1,2], and have been termed bioactive endodontic materials [3]. However, the cytotoxicity of these sealers may cause cellular degeneration and delayed wound healing because of the direct contact of materials with periapical tissues.

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MTA Fillapex (Angelus S/A, Londrina, PR, Brazil) is composed of salicylate resin, resin diluent, natural resin, bismuth oxide, silica nanoparticles, and calcium silicate. It was created combining a material with excellent biocompatibility and bioactive potential such as MTA with synthetic resins with good physical properties. However, this blend has shown cytotoxicity [4,5]. The Brasseler Industry (Savannah, GA, USA) introduced the EndoSequence Root Repair Material; a premixed bioceramic material indicates to pulp capping/pulpotomy, perforation repair, apexification, and root-end filling. This material promoted cell migration and survival of human bone marrow-derived mesenchymal cells, periodontal ligament cells, and dental pulp stem cells [6]. As a root canal sealer, the EndoSequence Bioceramic Root Canal Sealer (BC Sealer, Brasseler) is a bioceramic-based material composed of tricalcium silicate, dicalcium silicate, calcium phosphates, colloidal silica, and calcium hydroxide; and uses zirconium oxide as the radiopacifier agent. It also contains water-free thickening vehicles, which enables the sealer to be presented as a premixed paste, facilitating its usage [7,8].

Several methodologies have been proposed for evaluating the cytocompatibility of endodontic sealers, 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) and Scratch assay are widely used methods for this purpose. MTT evaluates the enzymes mitochondrial succinate dehydrogenase, one of the enzymes responsible for cellular respiration, and has become the standard test for assessing cell viability [4-6]. For cell migration, the scratch assay mimics the extent of migration of cells *in vivo* during wound healing. In this assay, the rate of cell migration can be determined by creating an artificial gap on a confluent cell monolayer and, then, to observe cell migration until the gap is closed [9]. Thus, this study aimed to evaluate the viability and migration of 3T3 fibroblast cells when in contact with BC Sealer and compare it with AH Plus and MTA Fillapex sealers.

MATERIALS AND METHODS

Sealer extracts preparation

BC Sealer, MTA Fillapex, and AH Plus were prepared according to manufactures' instructions. Each material was distributed to wells of two 12-well plates (314.0 mm^2 area and 3.0 mm height, $n = 8$ wells per sealer) (TPP Techno Plastic Products, Trasadingen, Switzerland) before setting. Extracts were obtained by filling of the well with Dulbecco's Modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA) and 1% penicillin and streptomycin (P/S; Gibco Life Technologies). The plate with the fresh extract was incubated at 37°C with 100% humidity for 24 hours. Extraction media were collected and passed through a 0.22-mm filter (Merck Millipore, Billerica, MA, USA). Thereafter, 1% of diluted extraction media were prepared with fresh DMEM.

3T3 fibroblasts (ATCC CRL-1658; American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C with 100% humidity and maintained in DMEM supplemented with 10% FBS and 1% P/S. At 80% confluence, the cells were treated with 0.25% trypsin-EDTA solution (Sigma-Aldrich) in the well plates (TPP Techno Plastic Products). Cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well. After 24 hours, the culture medium was removed, and the cells were incubated in the extracts for 24 hours at 37°C in 100% humidity and 5% CO_2. DMEM was used as the control.

Cell viability assay

Cell metabolism was determined by MTT assay, 6 hours, and 24 hours after the exposure of the cells. The 10 µL per well of a 5 mg/mL MTT solution (Sigma-Aldrich) was added in each well, followed by incubation for 3 hours at 37°C, 95% humidity and 5% CO_2. After that, the contents of the wells were removed and the colorimetric product solubilized in 100 µL of 0.04 N acidified isopropanol (Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Thermo Scientific Multiskan G0; Thermo Fischer Scientific Inc., Waltham, MA, USA) at 570 nm wavelength. The absorbance readouts were normalized with the absorbance of the group of cells exposed to DMEM (control group) and represented the activity of the viable cells. Results were expressed according to the control group (100% viability rate).

Scratch assay

Cell migration was evaluated by scratch assay. In order to obtain 90% confluence, 1×10^6 cells per well were plated. After 24 hours of incubation at 37°C, 95% humidity and 5% CO_2, using a pipette tip (TPP Techno Plastic Products), 2 defects were done in the cell monolayer, perpendicular to each other. The culture medium was replaced with DMEM containing extracts after 24 hours of the set at the dilution 1:8, defined by a pilot study. Using the Axio Observer Z1 microscope (Zeiss, Göttingen, Germany) coupled to a camera (Axiocammrn, Zeiss) with a 10× magnifying lens (Ecplan-NEOFLUAR 10×/0.3 aperture, Zeiss), After preparation of wound, and every 6 hours, images were taken in 4 different locations of the defects, until the full closure of the wound. The analysis was performed by measuring the areas obtained using Image J software (National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/).

Statistical analysis

Both MTT and Scratch assays were performed in triplicate and repeated 2 independent times. The results were statistically analyzed using Kruskal-Wallis and Dunn's tests, with 5% significance level, with the software program GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The MTT assay revealed greater cytotoxicity for AH Plus and MTA Fillapex at 1:1 dilution when compared to control ($p < 0.05$). At 1:2 and 1:4 dilutions, all sealers were similar to control ($p > 0.05$) and MTA Fillapex was more cytotoxic than BC Sealer ($p < 0.05$) (**Figure 1**).

Scratch assay demonstrated the continuous closure of the wound according to time. At 30 hours, the control group presented closure of the wound ($p < 0.05$). At 36 hours, only BC Sealer presented the closure when compared to AH Plus and MTA Fillapex ($p < 0.05$). At 42 hours, AH Plus and MTA Fillapex showed a wound healing ($p > 0.05$) (**Figures 2** and **3**).

DISCUSSION

Studies suggest that overfilling is associated with unfavorable outcomes [10,11]. Although endodontic sealer reabsorption occurs in many cases, this process is immune driven and thus suggests an inflammatory reaction. Sealer extrusion may occur unintentionally; therefore,

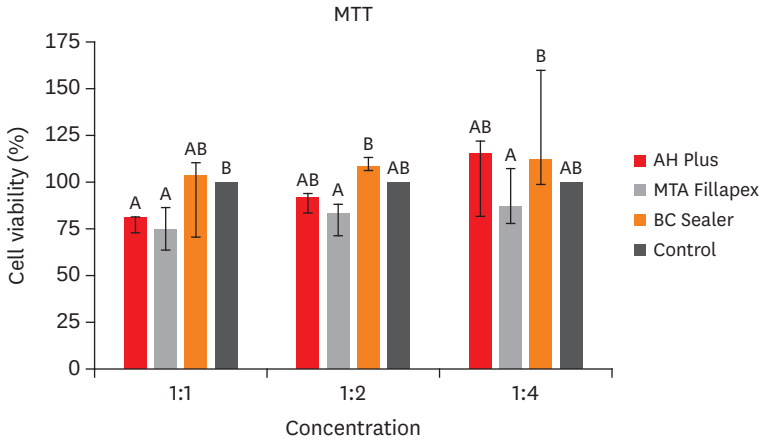
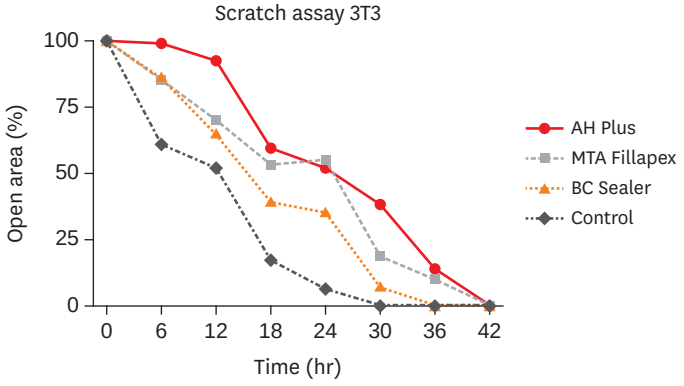


Figure 1. Cell viability results (%) by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in 3T3 cells exposed to 24 hours for AH Plus, MTA Fillapex and BC Sealer, at dilution of 1:1, 1:2 and 1:4, according to the control group (100% viability rate). Bars with different letters represent significant differences among groups in each dilution extract ($p < 0.05$).



Groups	Periods							
	0 hr	6 hr	12 hr	18 hr	24 hr	30 hr	36 hr	42 hr
Control	A	A	A	A	A	A	A	A
BC Sealer	A	AB	AB	A	B	A	A	A
MTA Fillapex	A	AB	AB	AB	B	B	B	A
AH Plus	A	B	B	B	B	B	B	A

Figure 2. Cell migration results by scratch assay in 3T3 cells exposed to set extracts of AH Plus, MTA Fillapex and BC Sealer at dilution of 1:8. Percentage of open area by scratch assay in 3T3 cells. Columns with different letters represent significant differences between groups in each evaluated period.

the clinicians should select materials that have adequate physical-chemical and biological characteristics [12].

Several methodologies have been used to evaluate the biocompatibility of endodontic sealers, including implantation into living tissues in animals and *in vitro* tests with different cell lines [4,5,12-16]. In this study, the 3T3 fibroblast cell line was chosen to perform the assays. Fibroblasts are the major constituents of connective tissue and the predominant cell type of the periodontal ligament that will be in contact with endodontic sealers [9].

At 1:1 dilution, AH Plus, and MTA Fillapex presented greater cytotoxicity when compared to control, and BC Sealer was similar to control. At 1:2 and 1:4 dilutions, MTA Fillapex was more cytotoxic than BC Sealer. As well as in a previous cell culture studies [4,5,13,14], MTA Fillapex has presented higher cytotoxicity, despite presenting calcium silicate.

Bioceramic sealers can produce an alkaline pH after mixing, due to the presence of calcium hydroxide on its composition, calcium release is important for the bioactivity of this sealer class [1,17], however, it might negatively influence cell's metabolism, decreasing the

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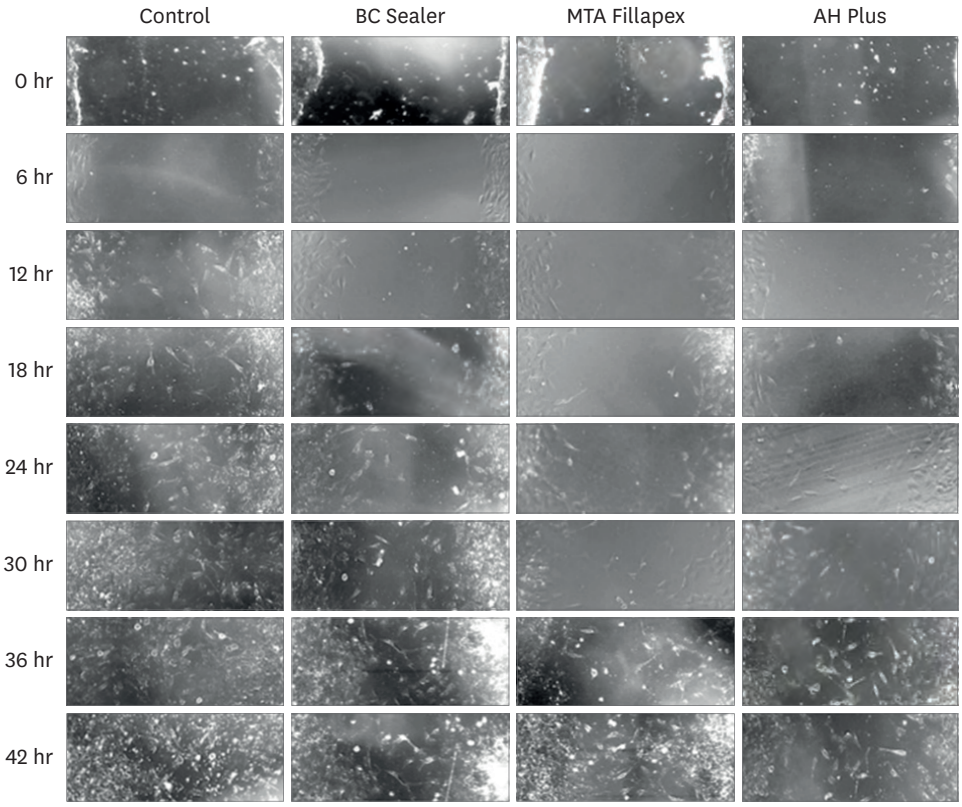

Figure 3. Illustrative image of scratch assay in 3T3 cells.

viability rate [18], However, the results of this study showed that besides these effects, BC Sealer showed a higher viability. As sealers might inadvertently extrude through the apical constriction during placement [19], the presence of BC Sealer in the apical tissues would promote less cytotoxicity than the other sealers.

The analysis of different cell parameters to evaluate the potential toxicity of the materials is necessary [20]. The scratch assay was first described by Liang *et al.* [9], reporting that it could mimic the extent of migration of cells *in vivo* during wound healing. The findings of this assay demonstrated that the closure of the wound occurred for all sealers, being more time consuming for AH Plus and MTA Fillapex. AH Plus and MTA Fillapex present resin in its composition, which can negatively influence the results [21], as also seen in the viability results. Mahdi *et al.* [22] suppose that MTA Fillapex's genotoxic effect might be associated with the release of the resinous components. On the other hand, the reparative and bioactive capacity of MTA-based materials, such as BC Sealer, was demonstrated when evaluated in connective tissue and osteoblastic cell line of rats [12,16] corroborating the results of this study. This ability may be explained by the presence of particles in their formulation responsible for maintaining a highly alkaline pH around 9, similar to MTA powder [23].

As ISO 10993-514 [24] accepts as cytocompatible sealers, materials that have viability values superior to 70%, it is possible to affirm that the tested materials provided positive results, emphasizing BC Sealer that presented a greater percentage of cellular viability and wound closure faster than the other sealers tested.

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CONCLUSIONS

Considering the limits of the methods used herein, it can be concluded that the sealers have cytocompatibility, highlighting BC Sealer which showed increased cell migration capacity. These results may suggest that BC Sealer may achieve better tissue healing when compared to MTA Fillapex and AH Plus. However, further *in vitro* and *in vivo* studies are required to confirm the suitability of BC Sealer for clinical application.

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