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ORIGINAL ARTICLE

Cytocompatibility of calcium silicate-based sealers in a three-dimensional cell culture model

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Abstract
Objectives The aim of the present study was to evaluate cytotoxic effects and cytokine production of calcium silicate-based sealers (EndoSeal, EndoSequence BC Sealer, and MTA Fillapex) using an in vitro root canal filling model and three-dimensional (3D) cell culture. AH Plus as a reference was compared to contemporary calcium silicate cements regarding cell viability and cytokine production.
Material and methods Root canals of 30 human maxillary incisors were prepared using a single-file reciprocating technique. The samples were randomly distributed and canals filled with either AH Plus, EndoSeal, EndoSequence BC Sealer, and MTA Fillapex (n = 6). In the negative control group, the root canal remained unfilled. Sealers were placed into the canals along with a gutta-percha cone placed to working length. Balb/c 3T3 fibroblasts, cultured in a type I collagen 3D scaffold, were exposed to filling material and the respective root apex for 24 h. Cytocompatibility of the materials was evaluated using the methyl-thiazoldiphenyl-tetrazolium (MTT) assay. The production of IL-1β, IL-6, and IL-8 was analyzed using enzyme-linked immunosorbent assay (ELISA). One-way analysis of variance was performed, and when the F-ratios were significant, data were compared by Duncan's multiple-range test. The alpha-type error was set at 0.05.
Results EndoSeal, Endosequence BC Sealer and AH Plus showed cell viability that was similar to the negative control group ($P > 0.05$), while MTA Fillapex sealer was cytotoxic ($P < 0.05$). Varying production of IL-1β, IL-6, and IL-8 was detected in all samples.
Conclusions In an in vitro root canal filling model with 3D cell culture, AH Plus, EndoSeal, and EndoSequence BC Sealer were cytocompatible.
Clinical relevance These results may suggest that AH Plus, EndoSeal and EndoSequence BC Sealer may achieve better biological response when compared to MTA Fillapex.

Keywords Cytotoxicity · Calcium silicate-based materials · Root canal sealer

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Introduction

Laboratory and in vivo studies have demonstrated that mineral trioxide aggregate (MTA) has suitable biological and physical-chemical properties for the use in endodontics [1, 2]. As a consequence, this endodontic reparative material could be considered close to the ideal in terms of biocompatibility [1–3]. Nevertheless, despite its favorable characteristics, MTA does not exhibit the physical properties for its use as an endodontic sealer because of its working time, setting time, and difficult handling. EndoSeal (Maruchi, Wonju, Korea) and EndoSequence BC Sealer (Brasseler, Savannah, GA, USA) and MTA Fillapex (Angelus, Londrina, PR, Brazil)

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are examples of calcium silicate-containing endodontic materials recently developed for permanent root canal filling, with improved physico-chemical properties when compared to conventional MTA.

Root canal sealers may come in intimate contact with the periapical tissues for an extended period because of extrusion over the apex or because degradation products that may leach through lateral and accessory canal or apical foramina, reaching the surrounding tissues [4, 5]. Thus, for safety reasons, each sealer must have its biological properties comprehensively and independently first screened by in vitro tests before its unlimited clinical use in order to minimize the incidence of local and/or systemic adverse effects. Generally, cytotoxicity tests are evaluated using traditional two-dimensional (2D) culture [6].

It could be argued that although some sealers have significant toxic behavior in vitro, this may be of little relevance in the clinical situation mostly because of the difference between in vitro and in vivo conditions. Conventional 2D culture systems form a monolayer that might have contact inhibition among cells and hence not duplicate original characteristics of cell morphology and functionality [7]. Three-dimensional (3D) cell models, on the other hand, can mimic in vivo cellular conditions more appropriately because the 3D scaffold supports cell growth and cell functions, including morphogenesis, cell metabolism, and cell-to-cell interactions [8].

Therefore, the present study intended to assess, using such a 3D cell culture associated with an in vitro root canal filling model, the cytocompatibility of these calcium silicate-based sealers on Balb/c 3T3 fibroblasts. An often used root canal sealer, AH Plus (Maillefer Dentsply, Ballaigues, Switzerland), was used as reference material for comparison. Inflammatory cytokine expression was also evaluated. The null hypotheses tested were that there was no significant difference in cytotoxicity and cytokines synthesis between the different calcium silicate-based sealers.

Materials and methods

Selection and preparation of specimen

This study was approved by an internal review board. Thirty human maxillary incisor teeth were selected. Only roots with angle of curvature <10° and an initial apical size equivalent to a size 10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) were selected for the study. Straight-line access cavities were performed and apical patency was determined by inserting a size 10 K-file into the root canal until its tip was visible at the apical foramen. The working length (WL) was set 1 mm shorter of this measurement. After that, the foramen diameter of all specimens was standardized to a size 15 K-file (Dentsply Maillefer).

Root canals were shaped using Reciproc R40 files (VDW, Munich, Germany) 1 mm coronal to the foramen. The canals were irrigated with 10 mL of freshly prepared 5.25 % NaOCl and received a final flush of 3 mL of 17 % EDTA (pH 7.7) for 3 min. The canals were dried with R40 paper points (VDW) and sterilized at 135 °C for 35 min. Thereafter, roots were randomly distributed with the aid of a computer algorithm (http://www.random.org) in order to create three equal experimental groups and two control groups, with six teeth each.

Root canal filling procedures

Root canals were filled under sterile conditions in a laminar flow hood. All procedures were performed by the same operator using a single-cone technique with one of the three following sealers (n = 6): EndoSeal, Endosequence BC sealer, and MTA Fillapex. AH Plus was used as a reference material and teeth with unfilled root canals were used as negative controls. Sealers were prepared following the manufacturers' instructions. A R40 (VDW) gutta-percha cone was coated with one of the tested sealers and placed in the canal to the full working length. Then, a heated instrument was used to remove excess coronal gutta-percha. Filled roots were immediately exposed to cell culture (Fig. 1).

3D cell culture

Balb/c 3T3 fibroblasts cells (ATCC®, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), 100 μg/mL of streptomycin, and 100 mg/mL of penicillin at 37 °C at 100 % humidity in an incubator under air

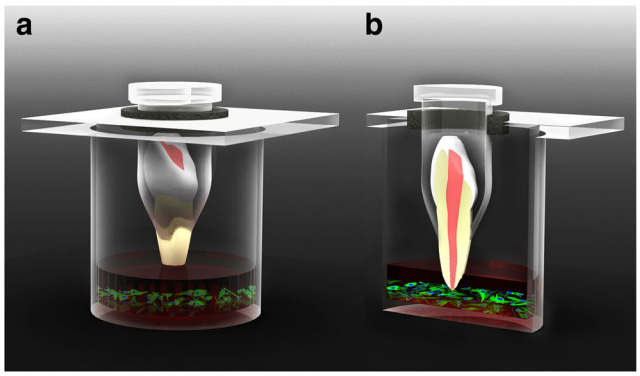


Fig. 1 In vitro root filling model apparatus. The bottom of Eppendorf tubes was cut to allow the protrusion of the tooth apex through this opening and placed into a six-well cell culture plate using a rubber O-ring (**a**). Three millimeters of the root apex was placed in contact with the 3D cell culture for 24 h (**b**)

atmosphere containing 5 % CO_2 at ambient pressure. When cells reached confluence, trypsin/ethylenediaminetetraacetic solution was used to remove adherent cells.

For the 3D cell culture, rat tail collagen type I (Gibco) was diluted to 1 mg/mL at 4 °C and neutralized with 0.1 M NaOH and 10× DMEM as recommended by the manufacturer. Six-well plates culture dishes were prechilled and then precoated with 1 mL collagen solution mixed with 3×10^1 cell. Then, plates were immediately transferred to an incubator for 20 min to allow polymerization of the collagen. After polymerization of the collagen, culture media (2 mL) was added on the top of the collagen gel. Cells were cultured for 7 days and then exposed to the filled roots. The medium was changed daily.

In vitro root canal filling model

The root model applied on this work has been described previously in detail [9]. In brief, 24 1.5-mL polypropylene Eppendorf tubes were cut 3 mm from the bottom to receive the roots with the apex protruding 5 mm through the opening. A rubber O-ring (Ø 0.8 cm) was inserted into the apex of each tube and its position adjusted so that the calcium silicate-based sealers dipped into a six-well cell culture plate well containing the 3D cell culture. With this apparatus, 3 mm of the root apex stayed in contact with the cells for 24 h (Fig. 1).

Cytotoxicity evaluation

Cytotoxicity of endodontic sealers in 3D culture was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 h of contact. After the removal of culture medium from each well, the 3D structures were gently washed two times with 1.0 mL phosphate-buffered saline. The wash was replaced with 1 mL MTT-succinate solution (1 mg/mL; Sigma-Aldrich, St Louis MO) for 4 h at 37 °C. Then, the 3D structures were washed two times with 1.0 mL phosphate-buffered saline. The blue formazan precipitate was solubilized using 0.3 mL dimethyl sulfoxide on a shaker at room temperature for 30 min. Three aliquots (100 μL) of the solution were then transferred from each well to a 96-well plate, and the absorbance was measured at 490 nm using a microplate reader (Urit 660, Urit, Guillin Guanxi, China). Percentage cell viability was calculated by dividing the absorbance values of experimental wells by those of negative control group wells and multiplying by 100.

Cytokine production

After 24 h of cell exposure to the root model, the production of IL-1β, IL-6, and IL-8 was analyzed. Cytokines were measured in triplicate in culture supernatants by enzyme-linked immunosorbent assay kit (BD Biosciences, San Jose, CA, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was performed, and when the F-ratios were significant, data were compared by Duncan's multiple range test. The alpha-type error was set at 0.05. SPSS 11.0 (SPSS Inc., Chicago) and Origin 6.0 (Microcal Software, Inc., Northampton, MA) were used as analytical tools.

Results

Figure 2a shows cell viability measured by MTT after 24 h in 3D culture with AH Plus, EndoSeal, EndoSequence BC sealer and MTA Fillapex. AH Plus, EndoSeal and Endosequence BC Sealer had similar cell activity to the negative control group ($P > 0.05$), indicating no cytotoxic effects. A significantly stronger cytotoxicity effect was identified for MTA Fillapex sealer ($P < 0.05$). The production of IL-1β, IL-6, and IL-8 was detected in all samples; while there were no differences for IL-8, both IL-1β and IL-6 were significantly elevated with MTA Fillapex compared to the other sealers and the negative control (Fig. 2b).

Discussion

The first results of the present study revealed that MTA Fillapex showed higher cytotoxic effects when compared to AH Plus, EndoSeal, and EndoSequence BC sealer ($P < 0.05$). Therefore, the first null hypothesis was rejected. Cytotoxic effects of MTA Fillapex have indeed been well documented in endodontic literature [10–12]. Components present in MTA Fillapex formulation, such as salicylate resin, diluting resin, and silica, may be related to the results. Moreover, MTA Fillapex probably has an unbalanced ratio among resin and MTA, with higher values for salicylate resin. It has been observed that to take set, a ratio of 1:1 is necessary (MTA:salicylate resin) [13]. Thus, this unbalanced resin/MTA ratio and the difficulty to set may explain the higher cytotoxicity and the properties of extended setting time and working time, excessive flow ability, and solubility as disturbance in calcium silicate properties, which has been reported in previous studies [10, 13–16].

The cytotoxicity results of AH Plus, EndoSeal, and EndoSequence BC sealer did not differ from those observed for the control group (unfilled canal), suggesting no cytotoxic effects for these sealers under the present experimental

conditions. These results are in apparent contrast with some previously published studies [10–12, 17–21] that showed some amount of cytotoxicity for those sealers; however, it is important to emphasize that in the present study, a 3D cell culture and an in vitro root model was used; these methodological differences could explain the differences in the studies.

Another consideration for the level of cytotoxicity is that, under clinical conditions, the concentration of toxic substances may be reduced by tissue fluid [22]. This fact reduces the direct transferability of the current results to the clinic but could be mitigated but pretreatment of the set test material with simulated tissue fluid according to Widbiller et al. 2016 [23]. Using this approach, further research may provide additional data points regarding the cytotoxicity of MTA Fillapex.

The cytotoxicity potential of dental materials is commonly assessed using 2D cell monolayer structures, in line with the International Organization for Standardization 10-993 [24]. In comparison with the traditional 2D cell culture model, considered a simple model without most in vivo characteristics and natural tissue architecture, the 3D model may be a more physiologically relevant, with more typical cell behavior, greater stability and longer cell lifespans [25].

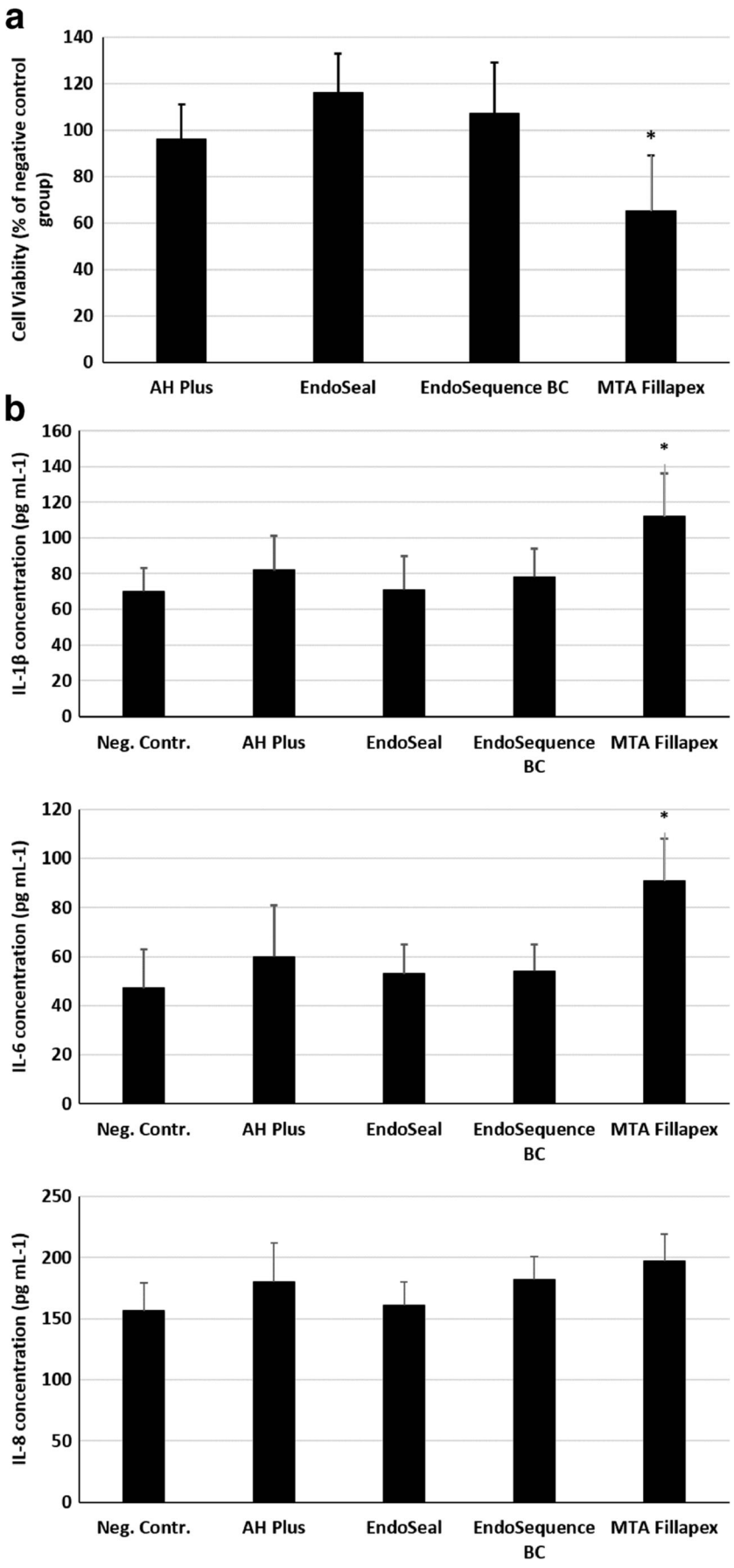
It has been recently demonstrated that endodontic sealers have higher cytotoxic effects in the 2D cell culture model than the 3D cell culture model [26]. This discrepancy was put down to the use of non-physiological 2D cell monolayers which does not simulate the 3D structure of the in vivo tissue [27]. These differences could be explained by the extensive cell-cell and cell-to-matrix interactions occurring in the 3D cell aggregates and the decreased capability of sealers extracts to penetrate within the 3D cell aggregates. Longer time points should be considered in further studies. Another argument for less cytotoxicity in 3D culture compared to 2D is the fact that biocompatibility testing in conventional cultures mostly uses material eluates and therefore provides a continuous stimulus, whereas most 3D models are created with only an initial stimulus. Collagen type I was chosen as scaffold material as it represents the major component of extracellular matrix in dental pulp. The material is highly cytocompatible and biodegradable, promotes cell adhesion, and allows for cell migration. A recent study confirmed using confocal images the equal distribution of cells within this carrier material [23]. Regardless of the type of cell culture, any dentin in contact with cultured cells in vitro must be sterilized first to avoid microbial contamination. In the present study, this was done with heat sterilization which is likely to denaturize some of the proteins contained in dentin such as TGF-β; it has been shown that acids and alkaline compounds such as EDTA and $Ca(OH)_2$ may release these sequestered compounds and thus modify the biologic response [23]. Future studies should be conducted using gamma-ray and ethylene oxide sterilized samples and compare the results with those obtained by heat sterilization.

Fig 2. **a** Cytotoxic effects after 24 h exposure to in vitro root models in 3T3 fibroblast cells ($n = 6$). Optical density measurements of negative control group (0.477 ± 0.024) served as reference and were set to 100 %. Results are expressed as means and standard deviations. The *asterisk* indicates significant differences ($P < 0.05$) to control group; **b** mean production of IL-1β, IL-6, and IL-8 after 24 h exposure to root models in 3T3 fibroblasts cells ($n = 6$). Results are expressed as means and standard deviations. *Asterisks* indicate significant differences ($P < 0.05$) to the control group

Another methodological issue that could contribute to the nontoxic results of AH Plus, EndoSeal, and EndoSequence BC sealer is the use of root model in the present study, simulating the in vivo situation for endodontic sealers. In this root model, the contact area of the root canal sealer with the cell culture is small compared to standard 2D tests, likely decreasing the toxic effects of the root canal sealers. This model has been previously recommended and has several advantages when compared to assessments performed with sealers alone: (a) in this model, more realistic material amounts are used, and (b) the interaction between sealer and the surrounding dentin is also taken into consideration [9, 28, 29]. Based on this, the combination of 3D cell culture and the root model may be used as an alternative in vitro experimental model to provide reliable information on endodontic sealer toxicity under conditions more closely related to the physiological scenario found in real-life tissue microenvironments.

The inflammatory process is initiated and maintained by upregulation of a network of chemokines (e.g., IL-8) and proinflammatory mediators (e.g., IL-1β and IL-6) that play distinct or shared biological activities [30]. IL-1β and IL-6 are pivotal in periapical disease development, stimulating osteoclastic differentiation and bone resorption as well as contributing to inflammation by inducing synthesis of other cytokines [31].

In the present study, contact to MTA Fillapex led to upregulation of IL-1β and IL-6 expression compared to the other sealers and the negative control. This observation suggests that MTA Fillapex may be associated with acute inflammation after its use during root canal filling. However, chemokine IL-8 was not upregulated by exposition to either sealer. This may be related to the fact that no interaction with immune cells occurred in this model. Future studies could include a co-culture system with neutrophils or macrophages, to study a possible interaction [32].


a
Cell Viability (% of negative control group)
140
120
100
80
60
40
20
0
*
AH Plus
EndoSeal
EndoSequence BC
MTA Fillapex
b
IL-1β concentration (pg mL-1)
160
140
120
100
80
60
40
20
0
*
Neg. Contr.
AH Plus
EndoSeal
EndoSequence BC
MTA Fillapex
IL-6 concentration (pg mL-1)
120
100
80
60
40
20
0
*
Neg. Contr.
AH Plus
EndoSeal
EndoSequence BC
MTA Fillapex
IL-8 concentration (pg mL-1)
250
200
150
100
50
0
Neg. Contr.
AH Plus
EndoSeal
EndoSequence BC
MTA Fillapex

Collectively, the data suggest that in an in vitro model with 3D cell culture, AH Plus, EndoSeal, and EndoSequence BC Sealer showed better cytocompatibility compared to MTA Fillapex.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study, formal consent is not required.

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