In Vitro Cytotoxicity of Calcium Silicate–containing Endodontic Sealers

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Abstract

Introduction: The cytotoxicity of 2 novel calcium silicate–containing endodontic sealers to human gingival fibroblasts was studied. Methods: EndoSequence BC (Brasseler, Savannah, GA), MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil) and a control sealer (AH Plus; Dentsply DeTrey GmbH, Konstanz, Germany) were evaluated. Human gingival fibroblasts were incubated for 3 days both with the extracts from fresh and set materials in culture medium and cultured on the surface of the set materials in Dulbecco-modified Eagle medium. Fibroblasts cultured in Dulbecco-modified Eagle medium were used as a control group. Cytotoxicity was evaluated by flow cytometry, and the adhesion of the fibroblasts to the surface of the set materials was assessed using scanning electron microscopy. The data of cell cytotoxicity were analyzed statistically using a 1-way analysis of variance test at a significance level of P < .05. Results: Cells incubated with extracts from BC Sealer showed higher viabilities at all extract concentrations than cells incubated with extracts from freshly mixed AH Plus and fresh and set MTA Fillapex, especially for the high extract concentrations (1:2 and 1:8 dilutions). Extracts from set MTA Fillapex of 2 weeks and older were more cytotoxic than extracts from freshly mixed and 1-week-old cement. With extract concentrations of 1:32 and lower, MTA Fillapex was no longer cytotoxic. After setting, AH Plus was no longer cytotoxic, and the fibroblast cells grew on set AH Plus equally as well as on BC Sealer. Conclusions: BC Sealer and MTA Fillapex, the 2 calcium silicate–containing endodontic sealers, exhibited different cytotoxicity to human gingival fibroblasts. (J Endod 2015;41:56–61)

Key Words
Calcium silicate–containing sealer, cell attachment, cytotoxicity, flow cytometry, mineral trioxide aggregate

Mineral trioxide aggregate (MTA) is a calcium silicate–based hydraulic cement (1–3). MTA has gained wide acceptance in dentistry since its introduction in the early 1990s (4) because of its good biological and physical properties. It was initially used as a root-end filling material but is now used in a variety of challenging clinical situations such as pulp capping, pulpotomy, apexogenesis, apical barrier formation in teeth with open apaxes, repair of root perforations, and as a root canal filling material (1, 5). The excellent properties of MTA such as good biocompatibility, bioactivity, and osteoconductivity have encouraged scientists worldwide to develop other MTA-based endodontic materials. Recently, new calcium silicate–based endodontic sealers have been introduced, such as MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil). According to the manufacturer, it consists of 2 main components (MTA-like bioceramic mixture and resinous components), and after mixing it, contains MTA, salicylate resin, natural resin, bismuth oxide, and silica. It is also claimed by the manufacturer that MTA Fillapex has excellent radiopacity, easy handling, and good working time.

Another relatively new bioceramic root canal sealer, EndoSequence BC Sealer (Brasseler, Savannah, GA), was launched a few years ago. It is composed of calcium silicates, zirconium oxide, calcium phosphate monobasic, calcium hydroxide, filler, and thickening agents. It requires the presence of water for setting (6). BC Sealer has been reported to have antimicrobial activity possibly because of a high pH, hydrophilicity, and active calcium hydroxide diffusion (7). Both of these recently introduced endodontic sealers (MTA Fillapex and BC Sealer) contain calcium silicate, which is biocompatible (8) and reacts with water to generate a calcium silicate hydrate phase during setting (5). The development of new types of endodontic sealers containing calcium silicate is based on the pursuit of sealers with good biocompatibility that induce the formation of mineralized tissue and suitable physical properties including flow rate, sealing ability, manipulation, and faster setting reaction (9, 10).

Endodontic sealers are supposed to fill the irregularities between the dentinal walls and the gutta-percha core as well as the lateral or accessory canals and bond both to gutta-percha and dentin (11, 12). In addition to defined requirements of mechanical and physical properties (13), the biological compatibility of root canal sealers is important because they come into contact with periapical tissues (14) and the tissue response to the sealers may influence the final outcome of the root canal treatment (15). The sealers with good biocompatibility are beneficial to aid or stimulate the repair of injured tissues (14). In MTA Fillapex, calcium silicate is mainly combined with
a resins component, whereas in EndoSequence BC Sealer, calcium silicate is combined with an inorganic component, making it a true bio-ceramic cement. Several studies have been done to evaluate the cytotoxicity and physicochemical properties of MTA Fillapex (9, 10, 16–18) and BC Sealers (6, 19). However, to our knowledge, no studies that have made a direct comparison of the cytotoxicity between these 2 new types of calcium silicate–containing root canal sealers have been published. The purpose of this study was to compare the in vitro cytotoxicity of 2 calcium silicate–containing endodontic sealers (MTA Fillapex and BC Sealer) immediately after mixing and after complete setting. The results are compared with an epoxy resin–based sealer (AH Plus; Dentsply DeTrey GmbH, Konstanz, Germany), which has a long clinical track record and has been extensively studied (20–23).

Materials and Methods

EndoSequence BC (batch #12003SP) and MTA Fillapex (batch #25612) were examined in the present study together with an epoxy resin–based sealer (AH Plus, batch #1210000912) used as a control material.

Cell Culture

In vitro cytotoxicity of the endodontic sealers was evaluated using human gingival fibroblasts. The fibroblasts were obtained from previously established stocks cultured from healthy patients who underwent oral surgery (7, 24). Standard protocols were followed in establishing and maintaining the cultures. Dulbecco-modified Eagle medium (DMEM) (Gibco, Grand Island, NY) supplemented with 100 μg/mL penicillin G, 50 μg/mL streptomycin, 0.25 μg/mL fungizone (Gibco), and 10% fetal bovine serum (Gibco) was used as the cell culture medium. Fibroblasts of the seventh to eighth passage were used for both cytotoxicity and cell adhesion assays.

Preparation of Specimens and Extracts

Three sealers (AH Plus, EndoSequence BC Sealer, and MTA Fillapex) were mixed in strict compliance with manufacturers’ instructions and shaped with 3-mm-thick nonreactive plastic molds with a diameter of 10 mm under aseptic conditions. Sealers that were used immediately after mixing were designated as fresh specimens, and those that were incubated (37°C, 100% relative humidity) for 3 times the setting time as given by the manufacturers were designated as set specimens. The set specimens were further exposed to ultraviolet light for 20 minutes on each surface tostandardize the settings and to determine background autofluorescence, respectively. The percentage distributions of viable and dead cells were determined by FlowJo software (Tree Star, Inc, Ashland, OR). The cell viability was expressed as a percentage of the mean of DMEM controls, which was taken to represent 100% cell viability. Experiments were performed in triplicate.

Cell Adhesion Assay

The morphology of human gingival fibroblasts attached to the surface of endodontic sealers after culturing for 1, 3, and 7 days was observed by scanning electron microscopy (Stereoscan 260; Cambridge Instruments, Cambridge, UK). The specimens of EndoSequence BC, MTA Fillapex, and AH Plus were shaped into 1.6-mm-thick disks of 5 mm in diameter using rubber molds under the same conditions as the cytotoxicity assay. Fifteen disks of each material were prepared and subdivided into 3 groups. Each group contained 5 parallel samples (n = 5). To remove the toxic byproducts, all disks were first incubated at 37°C in the wells of 24-well tissue culture plates (Sarstedt, Inc, Montreal, Canada) containing 1 mL distilled water that was changed daily for 5 days. The disks were then incubated in DMEM culture medium without cells for 7 days and then seeded with gingival fibroblasts (5 × 10⁴ cells/well with 1 mL DMEM) for 1, 3, and 7 days, respectively.

Specimens for scanning electron microscopic (SEM) examination were prefixed with phosphate buffered 2.5% glutaraldehyde (Sigma-Aldrich) for 30 minutes before further fixation in 1% osmium tetroxide (OsO₄) for 1 hour. The specimens were subsequently rinsed in PBS and dehydrated in sequential-graded concentrations of ethanol (50%, 70%, 80%, and 90%) for 5 minutes each and in pure ethanol (100%) for 10 minutes. The dehydrated specimens were dried using a critical point drier (Samdri-795; Tousimis Research Corporation, Rockville, MD) and then sputter coated with gold-palladium. Finally, specimens were examined with a scanning electron microscope at a magnification of 480–500 × and an accelerating voltage of 8–10 kV.

Cell viability data were expressed as the mean ± standard deviation. Differences between groups were analyzed statistically using 1-way analysis of variance (SPSS for Windows 11.0; SPSS, Chicago, IL) at a significance level of P < .05.
Results

Cytotoxicity of the Sealers

Representative flow cytometry histograms obtained from the stained human gingival fibroblasts after culture with various concentrations of extracts derived from AH Plus, BC Sealer, MTA Fillapex, and DMEM cultural medium for 3 days are shown in Figure 1. The percentage of viable cells was determined by flow cytometry analysis, and cell viabilities of various concentrations of extracts derived from both fresh and set calcium silicate–containing (MTA Fillapex and BC) and epoxy resin–based (AH Plus) endodontic sealers after culture for 3 days are summarized in Figure 2A–D. Fresh AH Plus was strongly cytotoxic at a high extract concentration (1:2), but the cytotoxic effect was reduced compared with the control at an extract dilution of 1:128. MTA Fillapex showed similar concentration-dependent cytotoxicity to AH Plus, but the cytotoxicity of MTA Fillapex was reduced to control levels when the extract was diluted to 1:8 (Fig. 2). BC Sealer exhibited mild toxicity at a high extract concentration, and there was no significant difference in the cell viabilities at various extract concentrations (P > .05). Cells incubated with extracts from BC Sealer showed the highest viabilities at all extract concentrations.

With sealers set for 2 weeks or more, extracts from AH Plus had no cytotoxic effect (P > .05). BC Sealer remained nontoxic throughout the 4-week testing period (fresh and set material), and there were no significant differences in viabilities of cells exposed to extracts from BC Sealer at all extraction times and extract concentrations (P > .05). Contrary to AH Plus and BC Sealer, extracts from MTA Fillapex set for more than 1 week at concentrations of 1:2 and 1:8 were significantly more cytotoxic than extract from fresh cement and 1-week-old cement (Fig. 2). With extract concentrations of 1:32 and lower, MTA Fillapex no longer was cytotoxic.

Cell Adhesion Assay

Representative morphologies of human gingival fibroblast adhesion on the surfaces of BC Sealer, MTA Fillapex, and AH Plus after culture in DMEM with the 3 sealers for 1, 3, and 7 days are shown in Figure 3. The morphology of the cells seeded on AH Plus and BC Sealer showed similar characteristics; the fibroblasts attached to and spread over the material surface displaying the typical spindle-shaped fibroblast morphology after an overnight culture (Fig. 3A and D). As the

Figure 1. Representative 2-dimensional dot plots of the flow cytometry data derived from CAM- and EthD-1 stained human gingival fibroblasts after exposure to extracts from set AH Plus, EndoSequence BC Sealer, MTA Fillapex (week 4), and DMEM control after culture for 3 days. The dot plots in each of the 13 images below represent the distribution of viable (right), early apoptotic (lower left), and dead cells (upper left), respectively.
culture time increased, the number of attached cells increased, and the cells began to connect with each other on the surface of sealers (Fig. 3B, C, E, and F). After culture for 7 days, the cells covered the entire surface of AH Plus, and the cell population density of fibroblasts grown on AH Plus was higher than those grown on BC Sealer (Fig. 3C and F). For MTA Fillapex, the cells were round and seemed weakly attached to the material surface at all determined time points (Fig. 3G–I).

The SEM images also revealed that the surface of both MTA Fillapex and BC Sealer showed uneven crystalline surface structures where fibroblasts grew, whereas AH Plus had a relatively smooth, noncrystallized surface.

**Discussion**

The cytotoxicity of endodontic sealers may cause cellular degeneration and delayed wound healing because of the direct contact of sealers with periapical tissues (26, 27). Most conventional endodontic sealers have shown inadequate biological activity and have been cytotoxic in cell

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**Figure 2.** Cell viability of extracts with various concentrations derived from the set (A) AH Plus, (B) EndoSequence BC Sealer, and (C) MTA Fillapex after culture for 3 days and (D) the fresh sealers. The extracts of set specimens were collected at different experimental periods (0, 1, 2, 3, and 4 weeks). The results show mean ± standard deviation of 3 parallel experiments performed in triplicate.

**Figure 3.** Scanning electron micrographs of the morphology of human gingival fibroblasts attached on the surface of (A–C) AH Plus, (D–F) EndoSequence BC Sealer, and (G–I) MTA Fillapex after culture in DMEM culture medium for (A, D, and G) 1, (B, E, and H) 3, and (C, F, and I) 7 days.
cultures, especially when freshly mixed (27). Calcium silicate cements have exhibited good biocompatibility, bioactivity, and osteoconductivity (5); therefore, it has appeared interesting to develop endodontic sealers based on calcium silicate hydraulic cements. Consequently, new types of calcium silicate–containing root canal sealers have been introduced, such as MTA Fillapex and BC Sealer. However, there are some limitations resulting from the physical characteristics of calcium silicate cements such as their long setting times (28). Such drawbacks can be overcome by adding other components to the sealers to adjust their physical properties. Thus, it is important to investigate the influence of the added components on the biocompatibility of the newly developed endodontic sealers. Therefore, in the present study, the cytotoxicity of MTA Fillapex, which is a combination of calcium silicate with a resin component and a pure bioceramic sealer, and the cytotoxicity of BC Sealer, which is a combination of calcium silicate with an inorganic component, were evaluated with an epoxy resin–based sealer (AH Plus) used as a control material.

In the present study, the in vitro cytotoxicity assessment of both freshly mixed and set sealers over a period of 4 weeks was performed using cell culture and sealer extracts in various concentrations. The extracts obtained by incubating the freshly mixed materials with culture medium were used to evaluate the short-term cytotoxic effect because clinically the sealers are applied in this form (29). The extracts collected by incubating the set materials with culture medium over the 4-week period were used to assess the cytotoxicity of set sealers. In addition, under the clinical condition, it is assumed that the apical and periradicular tissues come into contact with progressively lower concentrations of the leachable cytotoxic compounds because of the setting reaction and because the leached components are continually cleared by extracellular fluids (29). Therefore, the cytotoxicity of the extracts with various concentrations was investigated in the present study.

Among the methods and strategies available for cytotoxicity assay of materials, flow cytometry provides a fast, cost-effective, safe, and sensitive assessment to the cytotoxic events (15). The determination of cell viability depends on the type of material, culture medium, and incubation time to which the cells are exposed. Calcium silicate is 1 of the main components in both MTA Fillapex and BC Sealer; it reacts with water to form C-S-H gel and contributes to good biocompatibility (5). However, fresh MTA Fillapex was clearly more cytotoxic than BC Sealer, and the set Fillapex was more cytotoxic than both BC Sealer and AH Plus. This indicates the different influence of constituent components other than calcium silicate on the cytotoxicity of the sealers. In agreement with earlier studies, BC Sealer showed excellent biocompatibility at all extract concentrations as both fresh and set material (Figs. 1–3). There was no significant difference of cell viabilities exposed to extracts of various concentrations and extraction times. The significantly higher cytotoxicity of MTA Fillapex may be caused by the resin component or by some other component of the sealer. A previous study found that both MTA Fillapex and BC Sealer produced an alkaline pH after being freshly mixed and set (30), suggesting that the pH does not explain the difference in cytotoxicity. AH Plus is an epoxy resin–based sealer; however, after initial cytotoxicity of the freshly mixed AH Plus sealer in high extract concentrations, set AH Plus was no longer cytotoxic, unlike MTA Fillapex (Figs. 2 and 3).

The 3 sealers each had their own, characteristic timeline of cytotoxicity. BC Sealer was not cytotoxic at any stage of the setting (from fresh to 4 weeks after mixing). The freshly mixed AH Plus was cytotoxic in a concentration-dependent manner. This is in agreement with previous studies that have documented the moderate to severe cytotoxic effect of AH Plus immediately after mixing (20–22). This initial cytotoxicity has been attributed to a minimum formaldehyde release from amines added to accelerate the epoxy polymerization (21–25), which decreases after setting (21, 23), and the epoxy resin component (21, 23). Two weeks after mixing, no cytotoxic effect was measured from AH Plus extracts. In fact, the growth of gingival fibroblasts on set AH Plus sealer was comparable with that on BC Sealer (Fig. 3), which was perhaps somewhat unexpected and further proof of the biocompatibility of set AH Plus. MTA Fillapex was cytotoxic throughout the 4-week test period. It can be speculated that the initial cytotoxicity is caused by the resin component and the long-lasting cytotoxic effect is caused by other substances released from the sealer, such as lead (31). SEM images of cell culture experiments revealed round, damaged fibroblasts on the surface of the MTA Fillapex sealer (Fig. 3).

Conclusions

Within the limitations of the present study, freshly mixed and set BC Sealer showed better biocompatibility to human gingival fibroblasts than MTA Fillapex. The long-lasting cytotoxic effect by MTA Fillapex may be caused by lead released only from the set sealer. AH Plus was cytotoxic only as freshly mixed sealer and allowed growth of gingival fibroblasts on the surface of the set material.

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The authors deny any conflicts of interest related to this study.

References