Cytotoxicity of a novel mineral trioxide aggregated-based root canal sealer

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INTRODUCTION

Root canal sealers are used to seal off the root canal system since root canal sealing cannot be achieved with gutta-percha alone. These materials may be categorized into different groups according to their main chemical composition, which usually comprises calcium hydroxide, zinc oxide and eugenol, or epoxy resin.

As root canal sealers are placed in close contact with the surrounding soft and hard tissues for long periods of time, there is always the possibility that elutable substances and degradation or corrosion products from root canal sealers may gain access to the periradicular tissues via dentinal tubules, lateral and accessory canals or apical foramina1,2. Although sealers should be confined within the root canal, their inadvertent extrusion beyond the apical foramen into the periradicular tissues may occur3. Thus toxic components of sealers, particularly in cases of open apex due to iatrogenic over-instrumentation, pathologic root resorption, or non-vital teeth with immature roots, would damage both the periodontal ligament and alveolar bone4, adversely affecting the treatment outcome. Therefore, sealers should have good biocompatibility and should be well tolerated by the periradicular tissues5. At present, none of the endodontic sealers satisfy all the ideal properties of an endodontic sealer listed by Grossman6. Hence, new sealers are constantly being developed.

Mineral trioxide aggregate (MTA) is an endodontic material that was first used as a root-end filling material7. The material has gained popularity over the last decade as numerous in vitro and in vivo studies confirmed its superior properties such as sealability8, biocompatibility9, and bioactivity10. Thereafter, MTA has been advocated as the material of choice in various clinical procedures such as perforation repair, indirect/direct pulp capping, pulpotomy, apexogenesis and apexification. Newer developments of MTA include its use as a root canal sealer. Recently, a novel root canal sealer based on MTA (EndoSeal, Maruchi, Seoul, Korea) has been developed in an attempt to enhance the biological properties of root canal sealers. However, limited information is available regarding the cytotoxicity of this new root canal sealer.

The purpose of this in vitro study was to evaluate the cytotoxic effects of EndoSeal in comparison with two commonly used sealers, AH Plus (Dentsply DeTrey, Konstanz, Germany) and Sealapex (SybronEndo, Romulus, MI, USA) on MG-63 cells and human gingival fibroblasts.

MATERIALS AND METHODS

Cytotoxicity assay

The endodontic sealers evaluated in this study were EndoSeal (Maruchi, Seoul, Korea; lot no. SA211091109), AH Plus (Dentsply DeTrey, Konstanz, Germany; lot no. 1105002820), and Sealapex (SybronEndo, Romulus, MI, USA; lot no. 1-1267). The composition of each sealer is shown in Table 1. The sealers were prepared according to the manufacturer’s instructions under aseptic conditions. For ensuring sterility, mixing spatula and pad were pre-sterilized with EO gas, and mixing of the sealers was carried out on a clean bench. The mixed material was transferred into a 1 mL syringe so as to dispense the same volume of the sealer (0.1 cc) into each well of 96-well culture plates. Twelve samples were prepared for each sealer. The mixed sealers were allowed to set at 37°C in 100% relative humidity for 24 h. Each well containing the set sealer received 200 µL of Dulbecco modified Eagle’s medium (DMEM) and was incubated for 24 h at 37°C in 100% relative humidity. One hundred microliters of eluate was aspirated from
Table 1  Constituents of the sealers used in this study

| Sealer   | Type         | Composition                                                                 | Manufacturer                  |
|----------|--------------|-----------------------------------------------------------------------------|--------------------------------|
| EndoSeal | MTA          | Sodium oxide, potassium oxide, calcium oxide, magnesium oxide, iron oxide,    | Maruchi, Seoul, Korea          |
|          |              | aluminium oxide, titanium dioxide, zirconium oxide, silicone dioxide          |                                |
| AH Plus  | Epoxy resin  | Paste A: bisphenol-A and -F epoxy resins, calcium tungstate, zirconium oxide,| Dentsply DeTrey, Konstanz,     |
|          |              | silica, iron oxide pigments                                                 | Germany                        |
|          |              | Paste B: dibenzylidiamine, aminoadamantane, tricyclodecane-diamine,          |                                |
|          |              | calcium tungstate, zirconium oxide, silica, silicone oil                      |                                |
| Sealapex | Calcium hydroxide | Catalyst: isobutyl salicylate resin, silicon dioxide, bismuth trioxide      | SybronEndo, Romulus, MI, USA   |
|          |              | titanium dioxide pigment                                                      |                                |
|          |              | Base: N-ethyl toluene sulfonamide resin, silicon dioxide, zinc oxide,         |                                |
|          |              | calcium oxide                                                                |                                |

each well, and the wells were replenished with fresh 100 µL DMEM every two days to obtain the eluates containing any toxic substances released from the sealers at 1, 3, 7 days.

MG-63 cells (KCLB 21427) and human gingival fibroblasts (ATCC CRL-2014) were used in this study. Two types of cells were seeded separately at a density of 1×10^4 cells in a 96-well culture plate containing 100 µL culture medium per well, and incubated for 24 h at 37°C and 5% CO₂ to achieve cell attachment before addition of the extracts. One hundred microliters of the eluate from the sealer was applied to the cells. Following incubation for 24 h, cell viability was measured by the water-soluble tetrazolium salt (WST-1) assay (Roche, Basel, Switzerland) to evaluate the cytotoxic effects of 1-, 3- and 7-day eluates of sealers on MG-63 cells and human gingival fibroblasts. Twenty microliters of the WST-1 reagent was added into each well and the cells were incubated at 37°C for an additional 2 h. The spectrophotometric absorbance at 450 nm was then measured using ELISA analyzer (Spectra MAX 384, Molecular Devices Co., Sunnyvale, CA, USA). Cells cultured without eluates served as controls.

Analyses were all performed using JMP 5.0.1 statistical software (SAS Institute, Cary, NC, USA). The mean absorbances were statistically analyzed by Kruskal-Wallis test for comparison among groups at a significance level of 5%. Post hoc comparisons were made by using the Tukey-Kramer test.

Cell adhesion assay
EndoSeal and AH Plus were placed into polyvinyl siloxane molds with an inner diameter of 6 mm and a thickness of 2 mm. After 24 h, the discs of EndoSeal and AH Plus were removed from the molds and incubated in the 24-well culture plate containing 200 µL DMEM at 37°C in 100% relative humidity for 24 h. Every two days, 100 µL of the eluate was removed, and each well was replenished with fresh 100 µL DMEM as in the cytotoxicity assay. After 7 days of extraction, MG-63 cells and human fibroblasts were seeded separately at a cell density of 1×10^4 cells in each well, and further incubated for 1 day and 7 days at 37°C. Specimens for SEM examination were prefixed with 2.5% phosphate-buffered glutaraldehyde for 10 min before further fixation in 1% osmium tetroxide for 1 h. The specimens were then dehydrated in increasing concentrations of ethanol. The dehydrated specimens were dried and sputter-coated with platinum, and examined by using scanning electron microscope (SEM) (Hitachi S-4700, Tokyo, Japan).

RESULTS

Cytotoxicity assay
The optical density values obtained from spectroscopy were linearly related to the number of viable cells. Hence, there was an inverse correlation between the optical density values and the degree of cytotoxicity of root canal sealers.

The cytotoxic effects of the tested sealers on MG-63 cells are shown in Fig. 1 and Table 2. All tested root canal sealers were very cytotoxic on day 1. No statistically significant difference was observed among the tested sealers on day 1 (p>0.05). On days 3 and 7, EndoSeal and AH Plus were less cytotoxic than Sealapex (p<0.05), and the difference in the cytotoxic effect between EndoSeal and AH Plus was not statistically significant (p>0.05). EndoSeal and AH Plus exhibited a gradual decline in their cytotoxic activity against MG-63 cells on days 3 and 7, while Sealapex retained its cytotoxic activity throughout the observation period of 7 days.

Figure 2 and Table 3 represent the cytotoxic effects of the tested sealers on human gingival fibroblasts. EndoSeal demonstrated a significantly lower cytotoxicity than AH Plus and Sealapex on days 1, 3, and 7 (p<0.05), and a gradual decline in its cytotoxic activity throughout the 7-day elution period. AH Plus and Sealapex retained their cytotoxic activity against human gingival fibroblasts throughout the observation period of 7 days. The difference in cytotoxic effects between Sealapex and AH Plus was not statistically
**Table 2** Optical density units of EndoSeal, AH Plus, and Sealapex on MG-63

|                  | Control     | EndoSeal     | AH Plus     | Sealapex     | Multiple Comparisons (Tukey-Kramer) |
|------------------|-------------|--------------|-------------|--------------|-----------------------------------|
| **Day 1**        | 3.274 (0.363)\(^aA\) | 0.254 (0.156)\(^aB\) | 0.089 (0.008)\(^aB\) | 0.118 (0.037)\(^aB\) | Control ≠ EndoSeal, AH Plus, Sealapex |
| **Day 3**        | 3.111 (0.155)\(^aA\) | 0.745 (0.631)\(^aB\) | 1.029 (0.758)\(^aB\) | 0.110 (0.047)\(^aC\) | Sealapex ≠ EndoSeal, AH Plus |
| **Day 7**        | 3.507 (0.097)\(^aA\) | 2.977 (0.888)\(^aB\) | 2.953 (1.396)\(^aB\) | 0.071 (0.014)\(^aB\) | Sealapex ≠ EndoSeal, AH Plus |

Within columns, groups with same lowercase letters are not significantly different (\(p<0.05\)).
Within rows, groups with same uppercase letters are not significantly different (\(p<0.05\)).

**Table 3** Optical density units of EndoSeal, AH Plus, and Sealapex on Human gingival fibroblasts

|                  | Control     | EndoSeal     | AH Plus     | Sealapex     | Multiple Comparisons (Tukey-Kramer) |
|------------------|-------------|--------------|-------------|--------------|-----------------------------------|
| **Day 1**        | 2.883 (0.466)\(^aA\) | 0.355 (0.189)\(^aB\) | 0.035 (0.008)\(^aC\) | 0.073 (0.014)\(^aC\) | Control ≠ EndoSeal, AH Plus, Sealapex |
| **Day 3**        | 3.178 (0.424)\(^aA\) | 0.980 (0.219)\(^aB\) | 0.042 (0.006)\(^aC\) | 0.046 (0.015)\(^aC\) | Sealapex ≠ EndoSeal, AH Plus |
| **Day 7**        | 3.054 (0.350)\(^aA\) | 2.252 (0.526)\(^aB\) | 0.042 (0.006)\(^aC\) | 0.155 (0.101)\(^aC\) | Sealapex ≠ AH Plus, Sealapex |

Within columns, groups with same lowercase letters are not significantly different (\(p<0.05\)).
Within rows, groups with same uppercase letters are not significantly different (\(p<0.05\)).

Cell adhesion assay

The SEM images of MG-63 cells and human gingival fibroblasts after overnight and 7-day incubation on AH Plus and EndoSeal are shown in Figs. 3 and 4, respectively.

When the cells were compared at the same magnification between the two materials, the size of MG-63 cells seeded on EndoSeal was much larger than that of MG-63 cells seeded on AH Plus. After overnight incubation, both types of cells seeded on AH Plus appeared to be detached from the materials and had very smooth round cell margins, while cells seeded on EndoSeal showed irregular margins and cell attachment with growing cellular processes. After 7 days of incubation, although both types of cells showed stronger adhesion and obvious spreading on EndoSeal and AH Plus discs, cells seeded on EndoSeal were generally larger in size.

**DISCUSSION**

This study was designed to investigate the cytotoxicity...
Fig. 3  SEM images of MG-63 cells (a, c), human gingival fibroblasts (b, d) on AH Plus. After overnight incubation (a, b). After 7 days of incubation (c, d). Magnification ×2,500

Fig. 4  SEM images of MG-63 cells (a, c), human gingival fibroblasts (b, d) on EndoSeal. After overnight incubation (a, b). After 7 days of incubation (c, d). Magnification ×2,500
of a novel MTA-based root canal sealer named EndoSeal by comparing with two commonly used sealers with different compositions (AH Plus, Sealapex) on MG-63 cells and human gingival fibroblasts using the WST-1 cytotoxicity assay and the SEM adhesion assay.

Since root canal sealers may be exposed to the periradicular tissue, biocompatibility of root canal sealers is of prime importance to minimize the possible local and systemic side effects. Evaluating the biocompatibility of a material using an in vitro cell culture assay, and attempting to predict in vivo tissue responses based on in vitro results is controversial\(^{11}\). It should be emphasized that currently there is no sealer that satisfies all the ideal criteria.

As sealers are made of a mixture that hardens through a chemical reaction, the release of toxic material during the chemical setting reaction makes the sealer less biocompatible\(^{12}\). The cytotoxicity of endodontic sealers has been assessed inconsistently due to differences in the methodologies for testing different cells. In vitro cytotoxicity assays have the advantages of being simple, reproducible, cost-effective, and suitable for the evaluation of basic biological aspects related to biocompatibility\(^{13}\).

Eluates of the sealers have been used in the cytotoxicity assay since they contain toxic substances released from the sealer and they offer the advantage to evaluate the effect of materials on the surrounding cells. WST-1 is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability. It is based on the conversion of the tetrazolium salt to formazan by mitochondrial dehydrogenase, which only occurs in viable cells. Unlike the MTT, the WST-1 assay does not require a solubilization step owing to its water solubility. Other advantages of the WST-1 assay include convenient storage conditions, and a faster and more sensitive method than MTT.

AH Plus is an epoxy resin-based sealer, and is the successor to AH 26. AH Plus maintains the advantageous properties of the precursor product AH 26 such as high radiopacity, low solubility, and little shrinkage. As the toxicity of AH 26 can be, to some extent, related to the release of a small amount of formaldehyde during chemical setting process\(^{14,15}\), AH Plus was subsequently introduced in the market to overcome the drawbacks of AH 26 such as the tendency to discoloration and the release of formaldehyde. According to the manufacturer, AH Plus has superior clinical properties than AH 26 and the formulation does not release formaldehyde. However, moderate to severe cytotoxicity of AH Plus immediately after mixing is well documented\(^{16,17}\). It has been reported that this new formulation could also release a minimal amount of formaldehyde\(^{15,17}\).

As per our results, EndoSeal and AH Plus exhibited the most potent cytotoxicity after 1 day against both cells. This was in accordance with the generally recognized cytotoxic profile wherein most sealers are clearly more cytotoxic when freshly mixed in comparison to set specimens\(^{9}\). The cytotoxicity of AH Plus declined gradually over time only against MG-63 cells, while EndoSeal exhibited a gradual reduction in its cytotoxic activity against both MG-63 cells and human gingival fibroblasts. AH Plus exhibited a severe cytotoxic response on human gingival fibroblasts, while its cytotoxic effect on MG-63 cells was comparable to that of EndoSeal. Further evaluation is required to explain the related factors responsible for greater sensitivity of human gingival fibroblasts to AH Plus when compared to MG-63 cells.

The cytotoxicity of AH Plus can be attributed to the release of small amounts of formaldehyde from the sealer or to the release of the sealer's amine and epoxy resin components\(^{17,18}\).

Sealapex is a calcium hydroxide-based root canal sealer. Calcium hydroxide sealers are generally known to have good cytocompatibility\(^{19,20}\). Regarding Sealapex, our results were not in agreement with those of previous studies, which showed that this material was non-cytotoxic at 24 h after mixing\(^{21}\). In the present study, Sealapex exhibited a high cytotoxic effect on MG-63 cells and human gingival fibroblasts and retained its cytotoxicity throughout the observation period of 7 days. The higher cytotoxicity of Sealapex can be attributed to the difference in the volume of sealers and medium used. In this study, each well containing 0.1 cc set sealer received 200 µL of DMEM for preparation of the eluate of the sealer, whereas in the other published studies\(^{20,21}\) the eluates of the sealers were prepared by placing the sealer material in a greater amount of culture medium. When preparing the eluates of the sealers, the sealers were mixed and allowed to set at 37°C in 100% relative humidity for 24 h. However, Sealapex did not set after 1 day, and it was reported that Sealapex sets in 2 to 3 weeks in 100% relative humidity\(^{22}\). This is the reason why Sealapex was not included for SEM evaluation as the disc specimens of Sealapex were partially set even after 24 h and thus they failed to achieve their original disc form during removal from the silicone molds. Considering greater cytotoxicity of unset material, a longer setting time required for Sealapex may also explain the lower cellular viability as more cytotoxic components from the unset Sealapex may have leached into the medium.

Cell adhesion and morphology were analyzed by SEM. It is recognized that both surface chemistry and surface topography are responsible for diverse cell behavior including cell adhesion, spreading, proliferation, and differentiation\(^{23}\). Adhesion and spreading of the cells are the initial phases of cellular function where the attachment of cells to the substratum, radial growth of filopodia, cytoplasmic webbing, and the resultant flattening of the cells take place\(^{24}\). On the other hand, the persistence of rounded cells with little or no spreading suggests that the surface material might be toxic\(^{25}\).

Based on the SEM results, EndoSeal demonstrated a favorable response to both MG-63 cells and human gingival fibroblasts than AH Plus. Despite the fact that
pronounced cytoplasmic webbing or elongation was not observed after overnight incubation, both types of cells seeded on EndoSeal were much larger due to their adhesion to the substrate. Contrarily, both types of cells seeded on AH Plus were smaller and had very smooth surfaces, indicating that the cells were detached from the substrate. However, 7 days of incubation allowed stronger cell adhesion and spreading of both types of cells.

MTA has been shown to be the most biocompatible dental material. Sealers based on MTA have been reported to be biocompatible, to stimulate mineralization[21], to encourage apatite-like crystalline deposits along the apical and middle thirds of the canal walls[20].

EndoSeal showed the most favorable effect on both types of cells compared to AH Plus and Sealapex possibly due to the superior biological properties of MTA. Although EndoSeal is another type of MTA-based root canal sealer, the composition and particle size of various MTAs are not the same. Furthermore, in EndoSeal, bismuth oxide is replaced with zirconium oxide as the radiopacifier. Although the results of this study showed that EndoSeal appears to be a very promising sealer in terms of cytocompatibility using the WST-1 cytotoxicity assay and SEM cell adhesion analysis, further studies should be conducted to evaluate the biocompatibility of this endodontic sealer in comparison with other commercially available sealers based on MTA, as well as to assess the physical and biological properties of this novel root canal sealer before any specific recommendations can be made.

CONCLUSION

Within the limitations of the present in vitro study, all sealers seem to release most of the cytotoxic byproducts during the early period after setting. EndoSeal, a novel MTA-based sealer, demonstrated a gradual decline in its cytotoxic activity over time, and showed the lowest cytotoxicity against MG-63 cells and human gingival fibroblasts.

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