

# Long-Term Cytotoxicity, pH and Dissolution Rate of AH Plus and MTA Fillapex

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The aim of the present study was to verify the long-term cytotoxic effects of the MTA Fillapex and to compare them with AH Plus. Dissolution rate and pH were also evaluated. Human osteoblast cells were incubated with elutes of fresh specimens from AH Plus and MTA Fillapex, and with elutes of the same specimens for 4 successive weeks. Elute's pH was evaluated at each time point. A multiparametric cell viability assay was performed. For dissolution rate, ISO methodology was used. The results were analyzed by one-way analysis of variance, complemented with the Tukey post-test ( $p < 0.05$ ). No significant difference was found among the materials when fresh mixed ( $p > 0.05$ ). After 1 week, AH Plus became non-cytotoxic on all three evaluated parameters. Conversely, MTA Fillapex remained severely and mildly cytotoxic over the entire experimental period ( $p < 0.05$ ). The dissolution rate of AH Plus was significantly lower than MTA Fillapex at all time points ( $p > 0.05$ ). The pH of AH Plus was significantly lower than MTA Fillapex at the second and third week ( $p < 0.05$ ). In the other tested time points no statistical difference was observed. In conclusion, MTA Fillapex remained cytotoxic after 4 weeks and its cytotoxicity may be related to the high dissolution rate of this material.

## Introduction

In the last decade, mineral trioxide aggregate (MTA), a calcium silicate-based material, became popular in endodontics as a root-end filling and pulp-capping material for the repair of root canal perforations and for apexification (1,2). MTA is a biocompatible, non-cytotoxic, non-mutagenic, non-genotoxic and non-carcinogenic material (2). In addition, MTA has shown antibacterial and excellent sealing properties (1,2). However, despite its favorable characteristics, MTA does not exhibit the physical properties required for an endodontic sealer, because of its working time, setting time and difficult handling (3).

MTA Fillapex (Angelus, Londrina, PR, Brazil), a sealer based on calcium silicate, was introduced recently. Its composition after mixing is basically MTA, salicylate resin, natural resin, bismuth oxide and silica. The manufacturer claims that it has excellent radiopacity, easy handling, a great working time and low solubility, providing sealing by its expansion during setting. A recent study showed suitable radiopacity, pH and flow of MTA Fillapex (4). However, concerning its cytotoxicity and biocompatibility, controversial results were disclosed (4-8). While some studies demonstrated suitable biocompatibility (5) and bioactivity (6), other studies demonstrated poor biocompatibility and cytotoxicity (4,7,8). The biocompatibility of endodontic sealers is an important factor in choosing the best material, because endodontic sealers are often placed in intimate contact with the periapical

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tissues for an extended period. Due to these controversial results, more studies about MTA Fillapex cytotoxic behavior should be performed before its clinical indication.

Thus, the aim of this study was to assess, by a multiparametric *in vitro* assay, the long-term cytotoxic effects of MTA Fillapex on primary human osteoblasts (hOB) cell line. AH Plus (Dentsply, Germany) was employed as the reference material for comparison. Moreover, the dissolution rate and pH of these sealers were tested. The null hypothesis was that there would be no differences between the tested endodontic sealers at any tested time point.

## Material and Methods

### Sample, Extract Preparation and pH Measurement

Two root canal sealers were evaluated: AH Plus and MTA Fillapex. The tested materials, product names, manufacturers and components are in Table 1.

The sealers were mixed according to the manufacturers' instructions. Nine discs of each sealer were fabricated under aseptic conditions in sterile cylindrical Teflon blocks with 5 mm diameter and 2 mm high. Excess flash material was removed with a sterile scalpel. Cytotoxicity of the sealers was assessed immediately after mixing and tested every week for 4 weeks. The extraction was made in cell culture medium using the surface area-to-volume ratio of approximately 50 mm<sup>2</sup>/mL between the surface of the samples and the volume of medium (20), for 24 h at 37 °C. After 24 h, the pH of the elute was measured with a pH meter (QM-400; Quimis,

São Paulo, SP, Brazil) previously calibrated with solutions with known pH (4.7,10). Between tests, the specimens were aseptically removed, rinsed twice with PBS and dry stored at 37 °C. Control samples containing only culture medium were treated similarly. Undiluted extracts were used for the tests.

### Culture of Osteoblastic Cells

Human osteoblasts on second passage (hOB) from the Rio de Janeiro Cell Culture Bank were subcultured for 24 h at 37 °C on 96-well culture plates (1×10<sup>4</sup> cells per well). After 24 h, the medium was gently removed from each well and replaced by 200 µL of one of the selected test media (AH Plus and MTA Fillapex extracts) in triplicate and was incubated for further 24 h. A medium not exposed to filling materials was used as control of culture conditions. During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### Cytotoxicity Assay

After 24 h of cell exposure to each extract medium, cytotoxicity was evaluated by a commercial kit (In Cytotox; Xenometrix, Allschwil, Switzerland), which allows the use of three different tests of cell survival and integrity in the same sample: XTT, neutral red (NR) and crystal violet dye elution (CVDE). The XTT test is based on the ability of mitochondrial dehydrogenase enzymes to convert the yellow water-soluble tetrazolium salt XTT into orange-colored soluble compounds of formazan, measured by their absorbance at 480 nm using a microplate UV/Vis spectrophotometer (PowerWave MS2; BioTek Instruments, Winooski, VT, USA). NR is a survival/viability test based on the ability of living cells to incorporate NR dye on their lysosomes, where it accumulates on intact membrane cells; the amount of dye incorporated can be measured at 540 nm. CVDE is a simple assay that evaluates cell density by staining DNA; after elimination of the excess dye, the absorbance at 540 nm is proportional to the amount of cells in the well (7).

### Dissolution Rate

Endodontic sealer discs were manipulated and inserted into 7 mm x 1 mm molds. During insertion of the prepared sealers into the mold, a piece of dental floss was attached to one side of the specimen. After the post-manipulation

periods, samples were removed from the molds, and after removal of residues or loose particles, they were weighted in

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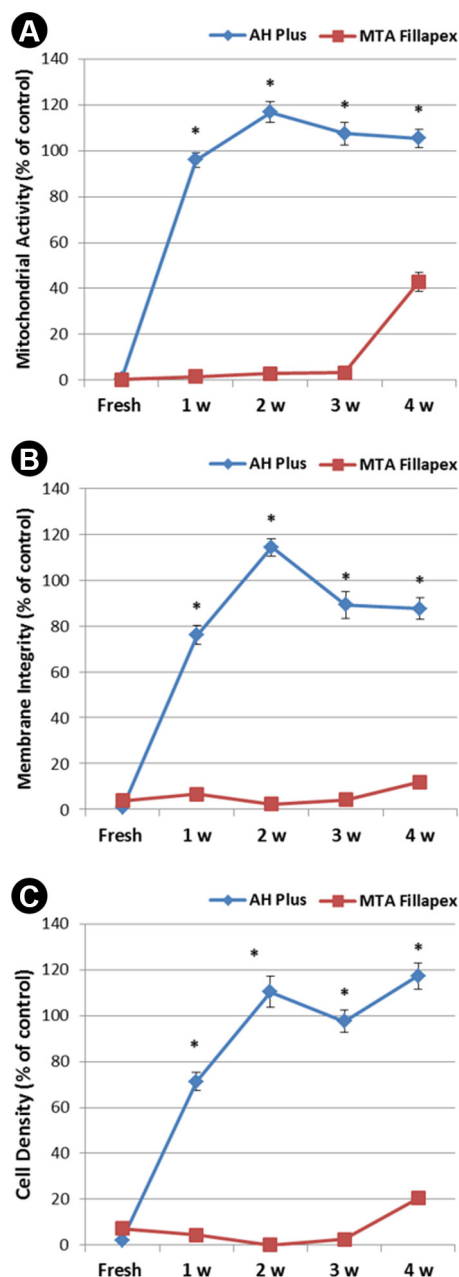


Figure 1. Multiparametric long-term cytotoxicity assay. Cytotoxic effects of endodontic sealers on hOB by (A) XTT, (B) NR and (C) crystal violet tests, expressed as percentage of control group (cells exposed only to DMEM). (\*) indicates significant difference between the groups (p<0.05).

Table 1. Composition of the materials and their manufacturers

| Root Canal Sealer                      | Components   |
|--|--|
| AHPlus, Dentsply De Trey GmbH, Germany | Paste A: Epoxy resins, calcium tungstate, zirconium oxide, silica, iron oxide pigments, aerosol<br>Paste B: Adamantane amine, N,N-Dibenzyl-5-oxanonane, TCD-diamine, calcium tungstate, zirconium oxide, aerosil |
| MTA Fillapex, Angelus, Brazil          | Salicylate resin, diluting resin, natural resin, bismuth trioxide, nanoparticulated silica, mta, pigments  |

a precision scale (FGH, Fujian Scale, China), with an accuracy of 0.001 g, to obtain the initial mass.

After that, each specimen was held by the dental floss and placed in a plastic container with 20 mL of deionized water, preventing the sealer disc from touching the inner walls of the container. Specimens were placed in an oven at 37 °C, where they remained for 1, 7, 14 and 28 days. After each time point, the samples were removed from the solutions, rinsed with deionized water, blotted dry and placed in an incubator at 37 °C for 48 h and then re-weighted until a constant weight (dry weight). The dissolution rate (percentage weight variation) was calculated according to the following equation:

$$\text{Dissolution rate} = \left[ \frac{\text{Dry weight at each time point} - \text{Initial weight}}{\text{Initial weight}} \right] \times 100$$

### Statistical Analysis

Data were statistically analyzed by analysis of variance (ANOVA) and the Tukey's test using SPSS software 15.0 (SPSS Inc, Chicago, IL, USA). The significance level was set at  $p < 0.05$ .

## Results

The results of the hOB viability, as measured by the multiparametric cytotoxicity assay over the entire time periods, are collectively represented in Figure 1 as a percentage of the control group (cells exposed to unconditioned medium). As seen in panels A, B and C, both sealers showed strong cytotoxic effects during the first 24 h after mixing, as measured by all three employed methods. No

significant difference was found among the materials in this time point ( $p > 0.05$ ). After one week, AH Plus became non-cytotoxic on all three evaluated parameters. Conversely, MTA Fillapex remained severely and mildly cytotoxic over the entire experimental period. At the end of the fourth week, this sealer exhibited a toxicity level that was significantly more severe than the AH Plus at all tested time points ( $p < 0.01$ ), except in the fresh conditions ( $p > 0.05$ ) in all evaluated parameters.

Table 2 shows the dissolution rate of the materials at 1, 7, 14 and 28 days after manipulation. Dissolution rate increased at the time-points for both sealers. Both materials showed the highest values of dissolution rate at 28 days. The dissolution rate of AH Plus was significantly lower than MTA Fillapex for all tested time points ( $p < 0.05$ ).

Table 3 shows the pH of the materials eluted in cell culture medium. The pH of AH Plus was significantly lower than MTA Fillapex in the second and third week ( $p < 0.05$ ). In the other tested time-points, no statistical difference was observed between the sealers.

## Discussion

In the present study, cytotoxicity of MTA Fillapex and AH Plus was tested employing some *in vitro* methodological strategies on these materials that differ from most previous works. First, three different parameters were evaluated on the same sample using a multiparametric assay that evaluated: (1) mitochondrial metabolism and respiratory toxicity, (2) lysosomal integrity and membrane permeability and (3) the presence of DNA and cell proliferation. This methodology increases the chances of cytotoxic effects detection, allows correlation of different parameters and sometimes provides hints about the mechanisms of toxicity (7).

A long-term cytotoxicity evaluation was also used in the present study. Cytotoxicity testing of freshly mixed sealers is relevant since they are placed into the root canal system in a freshly mixed and incompletely polymerized stage. Nevertheless, it is important to evaluate sealers over extended time periods after setting because, it is probable that during some period after clinical application, changes in cytotoxicity levels may be observed after diffusion of toxic components from the materials into the surrounding environment. This could be confirmed because the tested sealers showed different degrees of toxicity reduction after repeated testing at extended periods. This long-term evaluation is also superior to previous strategies that assessed cytotoxicity for the shorter term, because it enables the establishment of distinct toxicity

Table 2. Means and standard deviations of dissolution rate values at the different time points

| Material     | 1 day                     | 7 days                     | 14 days                    | 28 days                    |
|--------------|---------------------------|----------------------------|----------------------------|----------------------------|
| AH Plus      | -0.34 ± 0.07 <sup>A</sup> | -0.41 ± 0.12 <sup>A</sup>  | -0.46 ± 0.09 <sup>A</sup>  | -0.71 ± 0.16 <sup>A</sup>  |
| MTA Fillapex | -5.10 ± 1.12 <sup>B</sup> | -12.72 ± 3.51 <sup>B</sup> | -18.37 ± 5.03 <sup>B</sup> | -24.64 ± 6.13 <sup>B</sup> |

Values followed by different superscript letters indicate statistically significant differences according to ANOVA ( $p < 0.05$ ) in comparison between sealers at the same experimental time period.

Table 3. Means and standard deviations of pH values at the different time

| Material       | Fresh                    | 1 week                   | 2 weeks                  | 3 weeks                  | 4 weeks                  |
|----------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| AH Plus        | 7.31 ± 0.12 <sup>A</sup> | 7.08 ± 0.09 <sup>A</sup> | 7.12 ± 0.10 <sup>A</sup> | 7.04 ± 0.02 <sup>A</sup> | 7.07 ± 0.04 <sup>A</sup> |
| MTA Fillapex   | 7.47 ± 0.22 <sup>A</sup> | 7.42 ± 0.17 <sup>B</sup> | 7.27 ± 0.12 <sup>B</sup> | 7.10 ± 0.05 <sup>A</sup> | 7.10 ± 0.03 <sup>A</sup> |
| DMEM + 10% FBS | 7.00                     | 7.00                     | 7.00                     | 7.00                     | 7.00                     |

Values followed by different superscript letters indicate statistically significant differences according to ANOVA ( $p < 0.05$ ) in comparison between sealers in the same experimental time period.

profiles characteristic of each sealer (9,10).

Different immortalized cell lines have been used to address endodontic sealers cytocompatibility, especially because they multiply rapidly and have an unlimited lifespan, allowing a higher reproducibility of results. However, biocompatibility assessments through primary cell culture are appealing, because the biomaterials will interact with such kind of cells after *in vivo* implantation (11). Use of human primary cells of a relevant type to the study of endodontic materials, has been pointed out previously (12), in view of several expected differences in the responses of immortalized cells. For this reason, hOB cells have been considered closest to the ideal cells for cytocompatibility assays, because the direct interaction of these cells with biomaterials could play a critical role in a clinical setting (13). It was also reported that hOB cells provide a useful tool able to help predicting the effects of biomaterials on regenerative capability of periapical tissues (14). It is important to mention that the used cell line has been previously characterized (15).

Well-known and extensively used ISO standards cytotoxicity tests were used in the present study. However, the area-to-volume ratio between the surface of the samples and the volume of medium, recommended by ISO standards, may be superior to the real exposition during root canal treatment. A root experimental model was suggested to test cytotoxicity of endodontic materials (14). This kind of model presents some advantages over assessments performed with isolated sealers, because more realistic material amounts are used, and the interaction between sealer and the surrounding dentin is also taken into consideration (14). However, a recent editorial published in the International Endodontic Journal (16) contraindicated this kind of methodology emphasizing the use of international standards in the cytotoxicity screening. Our results revealed that both materials, AH Plus and MTA Fillapex, exhibited strong cytotoxic effects in freshly mixed conditions. This results is in agreement with previous studies that also observed severe cytotoxic effect of AH Plus and MTA Fillapex immediately after mixing and in the first days after mixing (4,7,10). After one week and in the succeeding weeks, AH Plus became non-cytotoxic, but MTA Fillapex toxicity did not decrease over time. Therefore, it may be assumed that MTA Fillapex does not have the claimed biological advantages over other available products. The results suggest the correlations among the components present in MTA Fillapex formulation, like salicylate resin, diluting resin and silica with the cytotoxic effects. Salicylate resin has stimulated the process of apoptosis in human fibrosarcoma and has caused the fragmentation of cell genetic material, determining its precipitation in the cytoplasm (17). Arsenic, a heavy metal found as a possible contaminant in MTA (18) could be also related to MTA Fillapex cytotoxicity. Arsenic reacts with protein thiols and exposure to high

concentrations of this element may induce genotoxicity (6). Even if these materials have high arsenic content it does not necessarily indicate a greater release of arsenic. These materials have a higher content of ferric salts, which stabilize the arsenic (10). This may explain why the release of arsenic from various MTA preparations was minimal in previous studies (18,20) and somehow eliminates the possibility of arsenic inducing cytotoxicity in MTA Fillapex.

Another possible explanation may be related to the physicochemical properties of MTA Fillapex. In the present study, MTA Fillapex high dissolution rate was demonstrated in all tested periods. Similar results have been previously reported (21) showing that this material has a higher dissolution rate than the 3% recommended by ISO specification (ISO 10993-5). This higher dissolution rate may account for a greater sealer particles release during the elution in DMEM, resulting in a higher exposition of MTA Fillapex and a mechanical aggression to the cell culture. One important aspect to be mentioned is that even after four weeks, MTA Fillapex particles were observed during the elution of the material in the cell culture medium (Fig. 2). The high pH of MTA Fillapex may be related to this material's cytotoxicity, as previously reported (4). However, in the present study the sealer was eluted in cell culture medium, which contains several buffers. As a result, just a slight increase in pH was observed in the

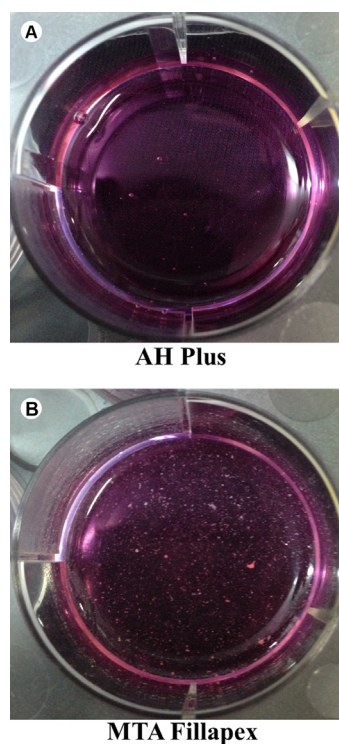


Figure 2. Images of AH Plus (A) and MTA Fillapex (B) elutes 4 weeks after manipulation. Observe a higher particle amount in the MTA Fillapex group. These particles may be related to the higher MTA Fillapex cytotoxicity results observed in the present study.

present study (Table 3). The authors do not believe that this slight change could affect the cytotoxicity of the tested endodontic sealers.

Despite the obvious cell destructive defect of MTA Fillapex sealer, a recent animal experimental study reported more limited cell destruction, followed by tissue repair activity and mineralization (5). On the other hand, a recent study showed intense and extensive inflammation in response to MTA Fillapex implanted in rat subcutaneous tissues (8). The conflicting results among the studies are probably related to details of the experimental procedures. In another study, MTA Fillapex showed clearly the ability to stimulate nucleation sites for the formation of apatite crystals in human osteoblast-like cell culture, using non-freshly mixed conditions that could have masked the cytotoxicity effects of the sealer (6). In the present study was tried to test the mineralization potential of MTA Fillapex (data not shown), but the high cytotoxic results from this sealer did not allow the cells to adhere to the culture plate, invalidating the test. Thus, further studies are needed for a better understanding of the cytotoxic effects of freshly mixed MTA Fillapex, and if this cytotoxicity may be related to some impairment of the endodontic treatment. In conclusion, AH Plus and MTA Fillapex had similar levels of cytotoxicity in fresh conditions. AH Plus became non-cytotoxic after one week and MTA Fillapex remained cytotoxic after 4 weeks. The high dissolution rate of MTA Fillapex may be related to the cytotoxicity of this material.

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## Resumo

O objetivo do presente estudo foi verificar os efeitos citotóxicos de longo prazo da MTA Fillapex e comparar com os do AH Plus. Solubilidade e pH também foram avaliados. Osteoblastos humanos foram incubados com elutos de amostras frescas de AH Plus e MTA Fillapex, e com elutos dos mesmos espécimes pelas 4 semanas seguintes. O pH foi avaliado a cada semana. Um ensaio multiparamétrico de viabilidade celular foi realizado. Para solubilidade foi utilizada metodologia ISO. Os resultados foram analisados por ANOVA, complementada com o pós-teste de Tukey ( $p < 0,05$ ). Nenhuma diferença significativa foi encontrada entre os materiais frescos quando avaliados em relação a citotoxicidade ( $p > 0,05$ ). Depois de uma semana, o AH Plus tornou-se não-citotóxico em todos os três parâmetros avaliados. Por outro lado, MTA Fillapex permaneceu citotóxico durante todo o período experimental ( $p < 0,05$ ). A solubilidade do AH Plus foi significativamente menor do MTA Fillapex para todos os períodos avaliados ( $p > 0,05$ ). O pH da AH Plus foi significativamente menor do que o MTA Fillapex na segunda e na terceira semana ( $p < 0,05$ ). Nos outros períodos testados não houve diferença estatística. Em conclusão, o MTA Fillapex permaneceu citotóxico após 4 semanas e a sua citotoxicidade pode estar relacionada à elevada solubilidade deste material.

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