

Cytotoxicity of substances leached from a root canal sealer based on mineral trioxide aggregate

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ABSTRACT | *Aim:* Based on its biological and physicochemical characteristics, mineral trioxide aggregate (MTA) could be considered the most appropriate material for root canal obturation; nevertheless, handling of MTA is not easy. The MTA Fillapex (MTA-F) was created in an attempt to combine the physicochemical properties of a root canal sealer with the biological properties of MTA. However, the studies on the biological characteristics of MTA-F are still controversial. Thus, this study aimed to analyze the cytotoxicity of MTA-F. *Materials and Methods:* Cultured human gingival fibroblasts were grown in Dulbecco's modified Eagle Medium (DMEM) and submitted to a cell culture medium conditioned by MTA or MTA-F. The conditioned medium contained substances leached from the root canal sealers. Cells grown on a fresh medium served as a positive control. Cell viability was assessed by MTT assay at 1, 3, 5 and 7 days. Data was compared by ANOVA followed by Tukey's test ($p < 0.05$). *Results:* Cells submitted to media conditioned by MTA presented a cell growth curve similar to that of the control cells. For the MTA-F group, cell growth was not observed and cell viability was significantly lower than for the other groups during the entire experiment. *Conclusion:* Substances leached from MTA-F did not allow cell growth, indicating that this MTA-based root sealer is highly cytotoxic. The biocompatibility characteristic of MTA can be lost with MTA-F, and may compromise the endodontic treatment outcome.

DESCRIPTORS | Endodontics; Cell Culture Techniques; Biocompatible Materials.

RESUMO | **Citotoxicidade de substâncias liberadas por um cimento endodôntico à base do agregado de trióxido mineral** • *Objetivo:* Com base nas características biológicas e físico-químicas do agregado de trióxido mineral (MTA), este seria o material mais adequado para a obturação do canal radicular. No entanto, esse material apresenta baixo escoamento e, conseqüentemente, difícil manipulação. O MTA Fillapex (MTA-F) foi criado na tentativa de combinar as propriedades físico-químicas do cimento endodôntico com as propriedades biológicas do MTA. No entanto, os estudos sobre as características biológicas do MTA-F ainda são controversos. Dessa forma, este estudo teve como objetivo analisar *in vitro* a citotoxicidade do MTA-F. *Materiais e Métodos:* fibroblastos gengivais foram cultivados em *Dulbecco's modified Eagle Medium* (DMEM) e submetidos ao meio de cultura condicionado pelo MTA ou MTA-F. Esse meio condicionado continha substâncias liberadas pelos cimentos endodônticos. Células cultivadas em meio fresco serviram como controle positivo. A viabilidade celular foi avaliada por ensaio do MTT após 1, 3, 5 e 7 dias. Os dados obtidos foram comparados por análise de variância (ANOVA) seguida pelo teste de Tukey ($p < 0,05$). *Resultados:* As células submetidas ao meio condicionado pelo MTA apresentaram curva de crescimento celular semelhante à das células do grupo controle. Para o grupo MTA-F, não houve crescimento celular e foi observado um número de células viáveis significativamente menor do que o dos demais grupos durante todo o experimento. *Conclusão:* Substâncias liberadas a partir de MTA-F não permitiram o crescimento celular, mostrando que esse cimento endodôntico à base de MTA é altamente citotóxico. A característica de biocompatibilidade do MTA pode ser perdida com o MTA-F e comprometer o sucesso do tratamento endodôntico.

DESCRIPTORS | Endodontia; Técnicas de Cultura de Células; Materiais Biocompatíveis.

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INTRODUCTION

Mineral trioxide aggregate (MTA[®], Loma Linda University, Loma Linda, CA, USA) has been reported to be a biocompatible material. Moreover, MTA presents osteogenic, inductive and conductive activities on hard tissue formation. MTA is also able to improve the secretion of interleukin (IL-1 β) by neutrophils *in vitro*,¹ a cytokine secreted *in vivo* during the inflammatory process which is important for tissue repair.² This material has been used in several dental procedures and presents an advantage over other endodontic filling materials due to its physicochemical, bioactive and sealing ability properties.³⁻¹⁰ However, despite these favorable characteristics, MTA does not exhibit the physical properties needed to be used as a sealer, owing to its long setting time and difficult handling in the insertion of material into the canal.⁶⁻¹¹

To circumvent the handling difficulty of MTA, the industry has searched for compositions of MTA-based root canal sealers which combine the desirable physicochemical properties of endodontic sealers and the biological properties of MTA.¹² MTA Fillapex[®] (Angelus[®], Londrina, PR, Brazil) is an MTA-based root canal sealer introduced to the market with this proposal. However, there are few studies on the biological characteristics of this material and they are controversial.

The biological aspects of MTA Fillapex[®] (MTA-F) have been tested *in vivo*^{13,14} using an animal model, applying the sealer in rat subcutaneous tissue and *in vitro* using cells in culture.¹⁵⁻²¹ Initial studies were carried out in rat subcutaneous tissue. MTA-F evoked a tissue response similar to that of Angelus MTA[®], leading to the conclusion that both materials are biocompatible, bioactive and stimulate mineralization.¹³ On the other hand, Zmener *et al.*¹⁴ concluded that the material remained toxic until 90 days after implantation in rat subcutaneous tissue. Reports on the *in vitro* cytotoxicity of MTA-F have shown effects regardless of testing time.^{15,18} More-

over, it causes cell death and micronucleus formation in cultured cells.¹⁶ On the other hand, another *in vitro* study observed that, once set, the material cytotoxicity decreases, resulting in suitable bioactivity.¹⁷ In these studies the MTA-based sealer was applied to the cultures at different times after handling. Knowing that the substances leached from root canal sealers during setting are important in determining the initial periapical tissue response, the present study aimed to analyze the cytotoxicity of substances leached from an MTA-based canal sealer during its setting on human cultured fibroblasts and discuss current knowledge about the biological properties of MTA-F.

MATERIALS AND METHODS

Cell culture

This study was approved by the Human Research Ethics Committee of the School of Dentistry, University of São Paulo (CAAE 0116.0.017.000-11). Human gingival fibroblasts (FMM1 cells) grown between the fifth and the tenth passages were used. These cells were retrieved from the files of the cell bank, Department of Restorative Dentistry, School of Dentistry, University of São Paulo. The cells were cultured in high glucose Dulbecco's modified Eagle Medium (DMEM, LGC Biotecnologia, Cotia, SP, Brazil) supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and a 1% antibiotic-antimycotic solution (Penicilin-Streptomycin, LGC Biotecnologia, Cotia, SP, Brazil). The cell growth was monitored daily using phase contrast microscopy, the medium was changed every other day, and the cells were maintained in an incubator at 37°C in a humid atmosphere containing 5% CO₂ and 95% air.

Conditioned medium

The mineral trioxide aggregate endodontic sealer (MTA[®], Loma Linda University, Loma Linda, CA, USA), White MTA (MTA) and MTA Fillapex (MTA-F) were prepared according to the manu-

facturers' instructions and immediately applied to the bottom of 50 mL centrifuge tubes. The material was weighed and fresh medium was added to the tubes to produce a proportion of 0.02 g of MTA-F for each mL of medium.

Experiments

Prior to the experiments, cells were plated (2×10^3 cells/well) in 24-well culture plates and maintained in an incubator for 24 h. Then, the culture medium of each well was replaced by the experimental medium (i.e. fresh medium for the control group and conditioned media for the test groups). The conditioned media remained in contact with the cells for 1, 3, 5 and 7 days. Every other day, half of the medium of each well was replaced by fresh medium, in order to simulate the solubility of canal sealers in periapical tissues. All the experimental groups were tested in triplicates.

Cytotoxicity analysis

After exposure of the cultured cells to the conditioned media, the cell viability of all groups was measured. This analysis was based on a measurement of cell mitochondrial activity using the MTT-based (Invitrogen, Eugene, OR, USA) cytotoxicity assay. This assay involves the conversion of water-soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to insoluble formazan. This salt is then solubilized in order to measure its concentration by optical density (570 nm, Biotrak II, Biochrom Ltd., Eugendorf, Austria). According to Freshney,¹⁸ this assay indirectly determines cell viability. Thus, the percentage of cell viability was calculated by using the mean absorbance data of the control group as 100%. Viabilities smaller than 70% are considered cytotoxic according to ISO 10993-5:2009.²²

Experimental groups

The experimental groups were as follows:

- **Control Group (CG):** Cells grown in fresh medium;
- **MTA-F:** Cells grown in medium conditioned by MTA Fillapex root canal sealer;
- **MTA:** Cells grown in medium conditioned by White MTA cement.

Statistical analysis

The data was presented as optical density mean \pm standard error of the mean (SEM) or cell viability percentages. Data was compared by analysis of variance (ANOVA) complemented by Tukey's test. The significance level was 5% ($p \leq 0.05$).

RESULTS

Figure 1 graphically illustrates the cell growth curves of all the experimental groups. The control group showed continuous growth. The amount of viable cells was significantly higher at the end of the experiment than at the beginning ($p < 0.01$). A similar result was observed for the MTA group ($p < 0.01$). The cells in the MTA-F group did not show cell growth. The number of viable cells in this group was significantly smaller than that of the other groups during the entire experiment ($p < 0.01$).

Figure 2 shows the percentage of cell viability of all the experimental groups during the entire experiment. Only the MTA-F group showed cell viabilities smaller than 20%.

DISCUSSION

The characteristics of mineral trioxide aggregate (MTA) such as biocompatibility, bioactivity, and osteoconductivity^{6,23,24} are the most desirable biological properties of an endodontic sealer. However, handling of MTA is not easy. So, in an attempt to combine the physicochemical properties of a resinous root canal sealer with the biological properties of White MTA (MTA), MTA Fillapex (MTA-F) was created. The biological properties of this material have been studied with controversial results.

Figure 1 | Graphic representation of mean (\pm standard error of the mean, SEM) cell viability (MTT units in optical densities, OD) as a function of experimental time (hours). **CG:** Control Group; **MTA:** White MTA cement; **MTA-F:** MTA Fillapex canal sealer.

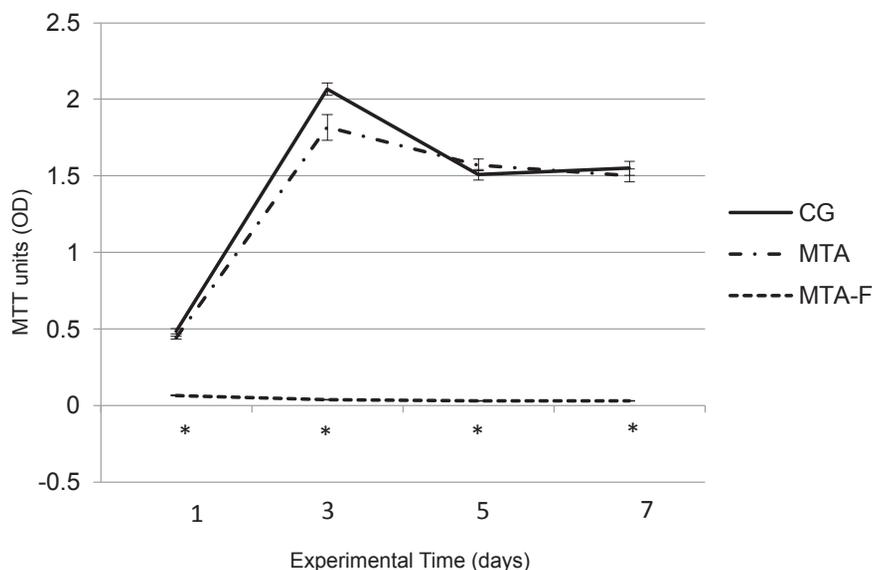
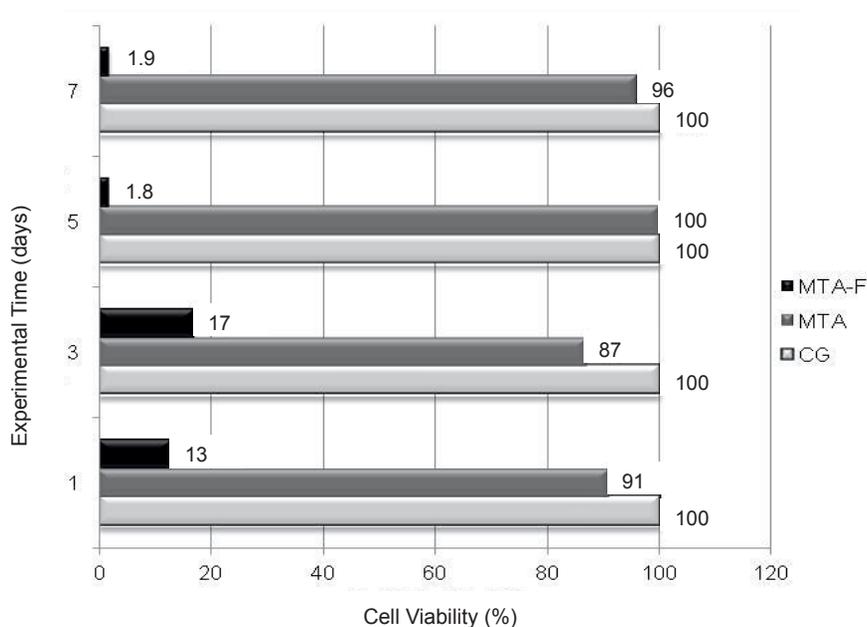


Figure 2 | Graphic representation of cell viability (in percentage) as a function of experimental time (hours). **CG:** Control Group; **MTA:** White MTA cement; **MTA-F:** MTA Fillapex canal sealer. Note that only MTA-F showed viabilities lower than 20% during the entire experimental time.



For this reason, this study compared the MTA-F and MTA root canal sealers by analyzing the effects of substances leached from these materials on cell viability. The method chosen was the analysis of mitochondrial activity, the MTT assay, against cultured media conditioned by the endodontic sealers.

The MTA group presented viability and cell growth similar to that of the control group. This would explain the favorable response of the peri-

apical tissues to MTA.^{15,22} This material has been confirmed over the years as a promising endodontic material for root canal filling, perforation repair, vital pulp therapy, apical barrier formation for teeth with necrotic pulps and open apices, and internal and external root resorptions.^{4,25-27} Nevertheless, MTA presents disadvantages such as handling difficulty and long setting time.^{3,6,11}

The present study showed that MTA-F is highly

cytotoxic, because it prevented cellular growth during the entire experimental time. This finding corroborates previous *in vitro*^{15,16,18,19,21} and *in vivo*¹⁴ studies suggesting that this material is cytotoxic, even a long time after exposure. A possible explanation for the high level of MTA-F cytotoxicity could be related to the presence of toxic products from resin and/or unpolymerized resin monomers of the paste-paste MTA-F system. Concurring with this hypothesis, Zmener *et al.*¹⁴ reported a severe inflammatory reaction *in vivo* that persisted until the end of the experiment (90 days) with MTA-F in rat subcutaneous tissue.

The cytotoxic effect of MTA-F persisted even 7 days after exposure. On the other hand, a study by Salles *et al.*¹⁷ showed that this cytotoxicity effect occurred only up to day 7 in culture, after which cell viability recovered. The differences in methodology of these two studies could explain this disagreement. In fact, these authors placed polymerized MTA-F in direct contact with osteoblasts, whereas, in the present study, the contact was indirect, using cultured medium that was conditioned by MTA-F during the polymerization process. Thus, this conditioned medium would contain MTA-F byproducts (e.g. unpolymerized resin monomers or other byproducts) that leached from the MTA-F during the polymerization process. Another *in vivo* study also showed that the cytotoxicity of MTA-F was only observed in the early periods of analysis, and that, after 2 weeks, the MTA-F and the MTA produced similar tissue reactions.¹³ Thus, further research must be conducted to better understand the tissue response to MTA-F before polymerization, by mimicking the clinical situation in which unpolymerized material remains in contact with periapical tissues until complete polymerization occurs.

Clinically, an adequate apical seal is considered an important factor for improving endodontic success.^{28,29} Moreover, the material must not impair periapical tissue repair.³ Although MTA does not impair periapical tissue repair,^{24,30} when resin is incorporated, as in MTA-F, this biocompatible characteristic of MTA is lost, thus jeopardizing the endodontic treatment.

The present study confirmed the cytotoxicity of MTA-F. Only a few authors have studied this material and some of them showed similar results;^{15,16,18,19,21} however, confirmation of these results is important to support this piece of evidence. An interesting finding of this study is that there was a higher cytotoxic effect of MTA-F at 5 and 7 days despite the continuing dilution of the conditioned media. This data may suggest that, besides affecting cell viability, MTA-F may affect important biological aspects of the surviving cells that impaired their continued growth. Taken together, the available data allow us to suggest that the hypothesis that MTA-F would combine the physicochemical properties of a resin root canal sealer with the biological properties of MTA was wrong. Thus, future studies are warranted to understand the degree of cellular changes caused by the soluble components of MTA-F to living cells. Additionally, *in vivo* studies must be conducted using MTA-F in the dental root canal system in order to observe the response of periapical tissues when tissue conditions are quite different from those of the *in vitro* studies and even *in vivo* studies conducted in rat subcutaneous tissue.

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