EMMANUEL JOÃO NOGUEIRA LEAL DA SILVA

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientador: Prof. Dr. Alexandre Augusto Zaia

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Este exemplar corresponde à versão final da dissertação defendido pelo aluno, e orientada pelo Prof. Dr. Alexandre Augusto Zaia

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"Em um determinado dia, em uma dada circunstância, você acha que você tem um limite. E então você busca esse limite e encosta nele, e então você pensa: ***'Certo, esse é o limite'****. Logo que você atinge esse limite, alguma coisa acontece e de repente você pode ir um pouco mais longe".*

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RESUMO

O cimento endodôntico obturador MTA Fillapex® foi criado numa tentativa de aliar as propriedades físico-químicas e a capacidade seladora dos cimentos resinosos às excelentes propriedades biológicas do agregado trióxido mineral (MTA). Entretanto, ainda existe uma escassez de trabalhos na literatura avaliando as suas características físico-químicas e biológicas. Dessa forma, os objetivos deste estudo foram: 1) avaliar propriedades físico-químicas (radiopacidade, escoamento e pH) e biológicas (citotoxicidade) do cimento endodôntico MTA Fillapex e comparar com o cimento AH Plus, 2) realizar um acompanhamento a longo prazo do efeito citotóxico em fibroblastos 3T3 de diferentes cimentos endodônticos contemporâneos, e 3) avaliar a partir de um ensaio multiparamétrico a longo prazo os efeitos citotóxicos do MTA Fillapex e do AH Plus em uma cultura primária de osteoblastos humanos. Os resultados do estudo mostraram que embora o AH Plus tenha apresentado radiopacidade estatisticamente maior que o MTA Fillapex ($P<0.05$), ambos os cimentos obtiveram os valores mínimos exigidos pela ISO 6876/2001. O MTA Fillapex apresentou um pH alcalino em todos os períodos experimentais, enquanto o AH Plus demonstrou um pH ligeiramente neutro, com diferenças estatisticamente significantes entre os cimentos ($P<0.05$). Com relação ao escoamento, ambos os cimentos apresentaram os valores mínimos exigidos pela ISO 6876/2001, no entanto o AH Plus apresentou valor estatisticamente mais baixo do que o do MTA Fillapex ($P<0.05$). Com relação a citotoxicidade, em todos os períodos testados o MTA Fillapex foi mais citotóxico do que o AH Plus ($P<0.05$). Quando comparado com diversos cimentos endodônticos, novamente o MTA Fillapex apresentou os maiores valores de citotoxicidade ($P<0.05$), permanecendo moderadamente citotóxico mesmo após 5 semanas de sua manipulação. Quando testados em um ensaio multiparamétrico utilizando culturas de osteoblastos humanos, ambos os cimentos foram citotóxicos sem apresentar nenhuma diferença significativa quando testados imediatamente após a manipulação ($P>0.05$). No entanto, uma semana após sua manipulação o AH Plus se tornou não citotóxico em todos os parâmetros avaliados. Por outro lado, o MTA Fillapex permaneceu citotóxico durante todo

o período experimental, mostrando diferenças estatísticamente significantes quando comparados com o AH Plus ($P<0.05$). Dentro da metodologia empregada e de acordo com os resultados apresentados, pode-se concluir que embora o MTA Fillapex tenha apresentado propriedades físico-químicas adequadas para a utilização na terapia endodôntica, o mesmo apresentou-se altamente citotóxico nas diversas condições testadas.

Palavras-chave: Endodontia; Materiais dentários; Obturação do canal radicular

ABSTRACT

The endodontic sealer MTA Fillapex® was developed in an attempt to combine the physicochemical properties and sealing capacity of resin-based cements to the excellent biological properties of mineral trioxide aggregate (MTA). However, little information exists regarding MTA Fillapex physicochemical properties. Thus, the aims of the present study were: 1) evaluate the physicochemical (radiopacity, flow and pH) and biological (cytotoxicity) properties of MTA Fillapex and compare it with AH Plus, 2) investigate MTA Fillapex effects on the cytotoxicity during a period of 5 weeks in 3T3 fibroblasts and compare with 7 different endodontic sealers, and 3) verify, through a multiparametric in vitro assay, the long term cytotoxic effects in human osteoblasts of the MTA Fillapex and compare it with AH Plus. The results of the study showed that although AH Plus presented higher radiopacity than MTA Fillapex ($P<0.05$), both sealers showed ISO 6876/2001 minimum required values. MTA Fillapex presented alkaline pH in all experimental times, while AH Plus demonstrated slightly neutral pH ($P<0.05$). Both sealers showed ISO 6876/2001 required values for flow, however AH Plus flow was significantly lower than that of MTA Fillapex ($P<0.05$). In all tested periods, MTA Fillapex was more cytotoxic than AH Plus ($P < .05$). When compared to 7 different endodontic sealers, MTA Fillapex was associated with significantly less cell viability ($P<0.05$) even after 5 weeks of manipulation. When tested in a muliparametric assay using human osteoblasts, no significant difference was found among the materials when fresh mixed ($p>0.05$). After one week AH Plus become noncytotoxic, on all three parameters evaluated. Conversely, MTA Fillapex remained cytotoxic over the entire experimental period, showing significantly differences when compared to AH Plus ($P<0.05$). Within the employed methodology and according to the results can be concluded that although MTA Fillapex showed suitable physicochemical properties for use in endodontic therapy, it appeared highly cytotoxic in the different tested conditions.

Keywords: Endodontics; Dental materials; root canal obturation

SUMÁRIO

INTRODUÇÃO

O propósito do tratamento endodôntico é a remoção do tecido pulpar, a eliminação da infecção no canal radicular e o adequado selamento do canal. A obturação do canal radicular é a etapa do tratamento endodôntico que objetiva o total preenchimento do sistema de canais radiculares recém descontaminado, a fim de impedir a microinfiltração bacteriana do meio oral, dos tecidos apicais e periapicais para o interior dos mesmos (Ray & Trope, 1995; Cohen & Burns, 2000; Barthel *et al.*, 2001). Esse preenchimento é considerado uma das chaves do sucesso da terapia endodôntica (Schilder, 1967; Saunders & Saunders, 1994).

A maioria dos tratamentos endodônticos utiliza-se da guta-percha em combinação com algum cimento endodôntico. A principal função do cimento é preencher espaços existentes entre a guta-percha e as paredes do canal radicular. Atualmente os cimentos endodônticos são comercialmente disponíveis em diversas fórmulas tais como cimentos à base de óxido de zinco e eugenol; cimentos ionoméricos; cimentos contendo hidróxido de cálcio e cimentos resinosos, dentre outros (Silva *et al.,* 2008). Quando o comportamento dos diferentes cimentos endodônticos que buscam aliar propriedades físicas, químicas e biológicas foi avaliado, verificou-se que todos apresentam significantes limitações, mostrando vantagens e desvantagens (Bouillaguet *et al*., 2004).

Os esforços em se desenvolver materiais obturadores mais eficazes, aliados ao aperfeiçoamento das técnicas de obturação, fazem com que sejam desenvolvidos materiais

com propriedades favoráveis, tais com plasticidade, estabilidade dimensional, facilidade de inserção e remoção, radiopacidade e, principalmente biocompatibilidade (Siqueira-Jr. & Lopes, 2009). Durante a última década, o agregado trióxido mineral (MTA), uma material a base de silicato de cálcio, foi introduzido e ganhou muita popularidade em endodontia. O MTA foi desenvolvido na Universidade de Loma Linda e recebeu a aprovação do FDA para utilização em humanos no ano de 1998 (Parirokh & Torabinejad, 2010). O MTA é um pó que consiste de finas partículas hidrofílicas que tomam presa na presença de umidade. Este material é constituído principalmente de silicato tricálcico, óxido tricálcico, silicato dicálcico, óxido de silicato, aluminato tricálcico, além de pequenas quantidades de outros minerais que também são responsáveis pelas propriedades físico-químicas deste material, como por exemplo o óxido de bismuto adicionado para aumentar a radiopacidade do material (Parirokh & Torabinejad, 2010; Torabinejad *et* al., 1995; Asgary *et al*., 2005).

Apesar de não conter hidróxido de cálcio em sua formulação, após o endurecimento do MTA, é formado óxido de cálcio que ao reagir com os fluidos teciduais ou com água pode produzir hidróxido de cálcio. A presença de hidróxido de cálcio faz com que o cimento atinja um pH altamente alcalino (10.2~12.5), favorecendo as propriedades antimicrobianas desse material (Parirokh & Torabinejad, 2010; Lovato & Sedgley, 2011). Além dessas características, a alta biocompatibilidade (Torabinejad *et al*., 1995a; Parirokh & Torabinejad, 2010; Silva e*t al*., 2012), associada à baixa microinfiltração (Torabinejad *et al*., 1995b; Parirokh & Torabinejad, 2010b) e a capacidade de tomar presa, mesmo na presença de sangue ou de umidade (Torabinejad *et al*., 1994), estão entre as vantagens adicionais do material. Devido as excelentes propriedades biológicas do material, no ano de

1999, Holland *et al.* sugeriram a utilização do MTA como um cimento obturador endodôntico. No entanto, apesar das diversas características favoráveis, o MTA não apresenta as propriedades físicas necessárias para ser utilizado como um cimento endodôntico, principalmente devido as dificuldades de manipulação (Roberts e*t al.,* 2008).

Com base nas características biológicas favoráveis do MTA e buscando melhorar as desvantagens físicas da formulação do MTA convencional, novos cimentos obturadores a base de MTA foram lançados no mercado odontológico. Dentre essas novas formulações, um novo cimento que contém MTA em sua formulação, chamado de MTA Fillapex (Angelus, Londrina, PR, Brasil) foi introduzido recentemente. Segundo o fabricante sua composição é basicamente uma mistura do MTA, resina salicilato, resina diluente, resina natural, óxido de bismuto, sílica nanoparticulada e pigmentos. Segundo o fabricante o material é biocompatível apresentando alta radiopacidade, excelente escoamento, tempo de trabalho adequado e baixa solubilidade, proporcionando vedação através da expansão durante a presa. No entanto, até agora poucas são as publicações independentes sobre as propriedades físico-químicas e biológicas do MTA Fillapex e seu possível uso na prática clínica. Portanto o objetivo do presente estudo foi avaliar diferentes propriedades fisico-químicas e biológicas do MTA Fillapex.

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CAPÍTULO 1

Evaluation of Cytotoxicity and Physicochemical Properties of Calcium Silicate-based Endodontic Sealer MTA Fillapex

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Evaluation of Cytotoxicity and Physicochemical Properties of Calcium Silicate-based Endodontic Sealer MTA Fillapex

Abstract

Introduction: The aim of the study was to evaluate the cytotoxicity, radiopacity, pH and flow of a calcium silicate-based and an epoxy resin-based endodontic sealer: MTA Fillapex and AH Plus, respectively. **Methods:** Cytotoxicity, radiopacity and flow evaluation were performed following ISO requirements. pH was measured at periods of 3, 24, 72, 168 hours. Cytotoxicity was evaluated by MTT assay to check the Balb/c 3T3 cells viability at 1-4 week periods. Data were statistically analyzed by ANOVA and Tukey test with a significance level of 5%. **Results:** In all tested periods, MTA Fillapex was more cytotoxic than AH Plus ($P < .05$). Although AH Plus presented higher radiopacity than MTA Fillapex ($P < .05$), both sealers showed minimum required values. MTA Fillapex presented alkaline pH in all experimental times, while AH Plus cement demonstrated slightly neutral pH. and a flow significantly lower than that of MTA Fillapex ($P < .05$). **Conclusions:** Although MTA Fillapex was more cytotoxic than AH Plus, it demonstrated suitable physicochemical properties for an endodontic sealer.

Key Words

MTA Fillapex; AH Plus; Physicochemical Properties; Cytotoxicity; Root Canal Sealer

INTRODUCTION

The characteristics and physicochemical properties of endodontic sealers are fundamental to allow hermetic sealing, which with an adequate coronal restoration will avoid bacterial leakage (1, 2). Mineral trioxide aggregate (MTA) is a material consisting of tricalcium oxide and other mineral oxides such as tricalcium silicate and silicate oxide. The pH of the material has been determined as 12.5 when set, which is comparable with that of calcium hydroxide (3–6). MTA biocompatibility, low cytotoxicity, antimicrobial properties (3, 7), low microleakage (4, 8), and its ability to set in the presence of blood or moisture are among the material's additional advantages. Because of these properties, it has been used for several dental applications such as root-end filling, root repair materials, and pulp capping (3–5). However, despite its favorable characteristics, MTA does not exhibit the physical properties needed to be used as an endodontic sealer because of its working time, setting time, and difficult handling (9).

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 through expansion during setting. However, up to now, there are limited independent publications about the physicochemical and biological properties of MTA Fillapex and its possible use in clinical practice. Thus, the aim of the present study was to evaluate the cytotoxicity, radiopacity, pH, and flow of MTA Fillapex sealer and to compare them with those of AH Plus (Dentsply, Konstanz, Germany).

MATERIALS AND METHODS

Cytotoxicity evaluation was performed according to ISO 10993-5 specifications (10). Radiopacity and flow evaluations were performed according to ISO 6876/2001 specifications (11). The pH level was measured at 3, 24, 72, and 168 hours. AH Plus cement was mixed according to the manufacturer's instructions for all the tests. MTA Fillapex is premixed, so further manipulations were unnecessary. The composition of the evaluated sealers is shown in Table 1.

TABLE 1. Sealers Tested and Their Composition

Material	Composition	Manufacturer
AH Plus	**Epoxy paste:** diepoxy, calcium tungstate, zirconium oxide, aerosol, and dye **Amine paste:** 1-adamantane amine, N.N'dibenzyl-5 oxanonandiamine-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosol, and silicon oil	Dentsply (Konstanz, Germany)
MTA Fillapex	Salicylate resin, diluting resin, natural resin, bismuth oxide, nanoparticulated silica, MTA, pigments	Angelus (Londrina, Brazil)

Cytotoxicity

Discs of each sealer were fabricated under aseptic conditions in sterile cylindric Teflon blocks with a 5-mm diameter and a 2-mm height. Excess flash material was removed with a sterile scalpel. Cytotoxicity of the sealers was assessed immediately after mixing and during 4 succeeding weeks. The extraction was made in cell culture medium using a surface area to volume ratio of approximately 150 mm^2/mL between the surface of the samples and the volume of the medium (8). The extraction vials were agitated for 24

hours in a water bath at 37°C. Control samples containing only culture medium were treated similarly. Undiluted extracts were used for the testing.

Balb/c 3T3 cells (American Tissue Type Collection, Manassas, VA) were cultured in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma Chemical Co, St Louis, MO), 100 mg/mL streptomycin, and 100 mg/mL penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 minutes, and aliquots of separated cells were subcultured. Cells were seeded in 24-well plates ($1x10^5$ cells/well). After overnight attachment, cells were treated with various extracts of sealers (500 µL/well) for a total of 4 weeks.

Cell viability was determined every week (for 4 weeks) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After the removal of culture medium from each well, the cells were gently washed with 1.0 mL phosphate-buffered saline. The wash was replaced with an MTT-succinate solution (1 mg/mL; Sigma-Aldrich, St Louis, MO) for 4 hours. After aspiration of the solution, the cell monolayers were rinsed with double-distilled water, and then the water was completely removed. Formazan crystals produced within the cells using a succinate dehydrogenase reduction of MTT were dissolved using distaining solution (isopropanol–10%NP40-0.4N HCl). 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). The formazan content of each well was computed as a percentage of the control group (untreated cells). All assays were repeated 3 times to ensure reproducibility.

Cytotoxicity responses were rated as severe (30%), moderate (30%–60%), slight (60%–90%), or noncytotoxic (>90%) (12).

Radiopacity Test

Cylindric samples from each material were manufactured by pouring the manipulated cements into metallic rings measuring 10 mm in diameter and 1 mm in thickness. Five samples of each material were prepared. The filled rings were kept at 37°C until the cements were completely set. The specimens were then removed, and the thickness was checked with a digital caliper (700–126; Mitutoyo MTI Corp, Tokyo, Japan). All sealers were placed on 5 occlusal films (Insight; Kodak Company, Rochester, NY) along with an aluminum step wedge graduated from 1 to 10 mm Al (in 1-mm increments). Radiographs were taken by using a radiographic unit (XR 6010; Gnatus, Ribeirão Preto, SP, Brazil) operating at 60 kV and 10 mA, with the exposure set at 0.3 seconds and a focus-film distance of 30 cm. After processing, the radiographs were digitized by using Canon EOS XSi with the Canon 100-mm macro lens (Canon, Tokyo, Japan) and imported into Digora 1.51 for Windows software (Orion Corporation Soredex, Helsinki, Finland). The radiopacity value was determined according to the radiographic density, which was also converted into millimeters of aluminum. Conversion was performed as described previously by Húngaro Duarte et al (13).

pH Analysis

Shortly after manipulation, the sealers were carefully placed in plastic tubes (polyethylene) measuring 1.0 mm in internal diameter and 10.0 mm in length with only 1 open end with the aid of a lentulo spiral. Periapical radiographs were taken to confirm the

complete filling of tubes and the absence of bubbles. Eight samples were used for each material. After being filled and weighed, each specimen was immediately immersed in test glass tubes containing 10 mL deionized water (Permution, Curitiba, PR, Brazil), which were then sealed with Parafilm (American National Can, Menasha, WI) and placed in oven at 37°C. The pH was measured with a pH meter (QM-400; Quimis, São Paulo, SP, Brazil) previously calibrated with solutions of known pH (4, 7, 10). Before the immersion of specimens, the pH of the deionized water was verified to be 6.5. After the removal of the specimens, the test tubes were shaken for 5 seconds before pH measurement. pH evaluations were performed always in fresh tubes containing deionized water at each evaluation period.

Flow Analysis

A final volume of 0.05 mL cement was prepared and put on a glass plate using a tuberculin syringe of 1.0 mL. At 180 ± 5 seconds after the onset of mixing, the second glass plate (50 x 50 x 3.2 mm and 20-g weight) was carefully and centrally placed on top of the sealer followed by weighting of approximately 100g to make a total mass on the plate of 120g. Ten minutes after the onset of mixing, the weight was removed, and the maximum and minimum diameters of the compressed sealer disks were measured with a digital caliper. Two conditions were necessary to validate the tests: the difference between the maximum and minimum diameters should not exceed 1.0 mm, and the compressed disk should have a uniform shape. If these conditions were not met, the test was repeated. Five samples for each sealer were used, and the mean of 3 measurements for each sample, expressed to the nearest millimeter, was taken as the sample flow. According to ISO

6876/2001 specifications (11) for the flow test, a disk with at least a 20-mm diameter should be obtained.

Statistical Analysis

Data were statistically analyzed by analysis of variance (ANOVA) and Tukey test by using SPSS software 15.0 (SPSS Inc, Chicago, IL). The significance level adopted was $P < .05$.

RESULTS

Cytotoxicity

The results of the MTT assay over all the time periods are represented in Figure 1. AH Plus was moderately cytotoxic in fresh conditions, was mildly cytotoxic after 1 week, and became noncytotoxic after 2 weeks. Conversely, MTA Fillapex remained severely cytotoxic over the entire experimental period. In all tested periods, MTA Fillapex was more cytotoxic ($P < .05$) than AH Plus.

Radiopacity Test

The mean values for radiopacity (mm Al) of the sealers were as follows: AH Plus = 8.59 and MTA Fillapex = 7.06. Data were compared with minimal requirements as specified in ISO 6876/2001. Although ANOVA and the Tukey test showed statistically significant differences between sealers ($P < .05$), both sealers showed values complying with the previously mentioned requirements (minimum equivalent to 3 mm Al).

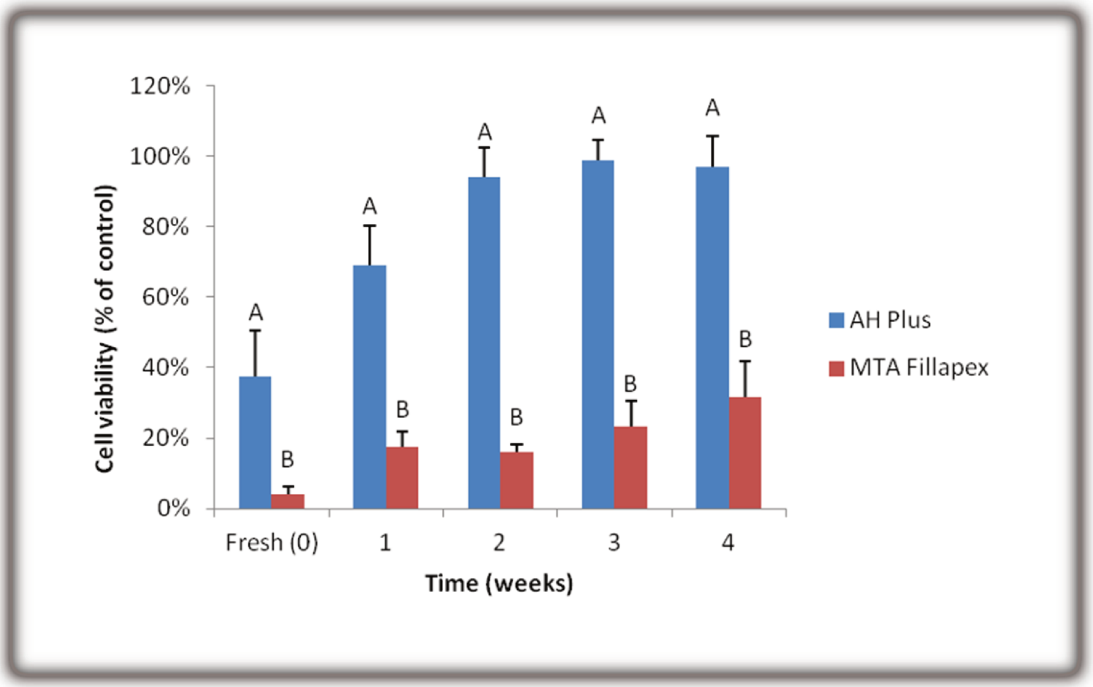

Figure 1. Cytotoxic effects following exposure to sealers in 3T3 fibroblast cells. Results are expressed as mean and standard deviation at different experimental periods. Different letters indicate statistically significant differences according ($P < .05$) in the same experimental time.

pH Analysis

The pH values at the different evaluation periods are shown in Table 2. MTA Fillapex presented an alkaline pH in all experimental times, with the maximum pH value at the 3-hour evaluation. AH Plus cement showed a slightly neutral pH. Differences between sealers were statistically significant at all evaluation periods ($P < .05$).

TABLE 2. Means and Standard Deviations of pH Values at the Different Periods

	3 hours	24 hours	48 hours	72 hours	168 hours
AH Plus	7.08 ± 0.21[a]	6.93 ± 0.08[a]	6.78 ± 0.13[a]	6.90 ± 0.13[a]	6.92 ± 0.06[a]
MTA Fillapex	9.68 ± 0.08[b]	9.34 ± 0.32[b]	8.25 ± 0.27[b]	8.02 ± 0.31[b]	7.76 ± 0.28[b]
Control	6.50	6.50	6.50	6.50	6.50

Values followed by different superscript letters indicate statistically significant differences according to ANOVA ($P < .05$) in comparison between sealers in the same experimental time.

Flow Analysis

During the flow test, no repetitions were required besides those standardized at the beginning of the experiment. According to the flow test, MTA Fillapex sealer showed a flow greater than 20 mm (31.09 ± 0.67 mm). AH Plus presented a flow significantly lower (25.80 ± 0.83 mm, $P < .05$) than that of MTA Fillapex.

DISCUSSION

The endodontic sealer properties include good sealing, visible radiopacity, easy handling, good resistance, dimensional stability, high flow, and low solubility (14). Thereby, this study evaluated some of the main properties that should be considered for a suitable endodontic sealer. It is important to point out that all materials should be tested in a laboratory before being considered for clinical use. However, these tests must attend international standards. The ISO is the world's largest international standards developer. Cytotoxicity evaluation was performed according to ISO 10993-5 specifications. Radiopacity and flow evaluations were performed according to ISO 6876/2001.

MTA Fillapex was created in an attempt to combine the physicochemical properties of a root canal sealer with the biological properties of MTA. The present study assessed the cytotoxicity, radiopacity, pH, and flow of this novel endodontic sealer. The cytotoxicity analysis, which uses a long-term methodologic strategy, was used to evaluate MTA Fillapex for the first time. According to the present results, MTA Fillapex showed severe cytotoxicity when cells were exposed to fresh elute of the sealer. This toxicity did not decrease over the tested time periods. Our findings are in agreement with previous studies that showed strongly affected cell viability with MTA Fillapex (14, 15). One possible explanation for these results is the presence of toxic components such as salicylate resin, diluting resin, and silica in MTA Fillapex composition. AH Plus exhibited mild cytotoxicity in fresh conditions and became noncytotoxic after 2 weeks, probably as a result of the diminishment in the leaching of toxic substances present in this formulation.

Radiopacity is an essential property of endodontic sealing materials. Among other physical, chemical, and biological properties, the ideal root canal sealing material should have a certain degree of radiopacity to be clearly visible on radiographs and enhance the radiopacity of the root filling materials (16). International standards require a minimal radiopacity equivalent to 3.00 mm Al, yet some commercially available products do not meet this requirement (17). In this study, the radiopacity of both root canal sealers were found to be in agreement with ISO 6876/2001 recommendations (11). The differences between radiopacities of the tested root canal sealers in the present study probably are caused by the presence of different radiopacifying agents in each material. In AH Plus, the radiopacifying agent is zirconium oxide, and in MTA Fillapex it is bismuth oxide.

The pH of MTA Fillapex was significantly higher up to the 7-day period. This result indicates that MTA Fillapex has a strong capacity of releasing hydroxyl ions. A high pH activates alkaline phosphatase, which is present in the tissues, is involved in the mineralization process, and requires a pH around 8.6 to 10.3 to operate (18). The high pH of this sealer may also neutralize the acids secreted by osteoclasts, and this may help prevent further destruction of mineralized tissue. One disadvantage of its alkaline pH is a possible high cytotoxicity, which could explain the results shown by this material. However, its initial cytotoxicity could also be considered as an advantage. The high pH usually has a destructive effect on bacterial cell membranes and protein structure, which seems interesting, especially knowing that microorganisms can remain in the ramifications of the root canal system after chemomechanical preparation and intracanal dressing. Having antimicrobial activity, the sealers can act against such microorganisms, reducing their numbers and providing a better chance of successful root canal treatment (19).

In regard to flow, both MTA Fillapex and AH Plus showed acceptable values according to ISO 6786/2001 recommendations (11) that state the minimal flow required for cements is 20 mm. Flow is the ability of a sealer cement to penetrate into the irregularities and accessory canals of the root canal system, and it is considered to be a very important property—the greater the flow, the greater the ability to penetrate into irregularities. Conversely, if the flow is excessive, the risk of material extravasation to the periapex is increased, which could damage periodontal tissues (20). MTA Fillapex showed significantly superior flow values compared with AH Plus ($P < .05$). The different composition of the tested sealers seems to be the main factor related to their flow

differences. The flow ability is also influenced by the size of the sealer particles—the smaller the particles, the greater the flow ability of the sealer. Because of this property, MTA Fillapex will probably penetrate easier into the ramifications and irregularities of root canal walls than AH Plus. It is important to emphasize that it was not possible to compare the results obtained with MTA Fillapex with previous results because this is one of the pioneer studies using this material. Despite the flow ability shown by MTA Fillapex, this sealer does not seem to display bond strength to root dentin (21). However, recent studies have shown acceptable push-out bond strength values for MTA Fillapex, which were similar to those observed in samples filled with AH Plus sealer (22, 23). These values are related to the degree of residual moisture on dentin surface for both materials (23).

In conclusion, the results of this study showed that the MTA-based endodontic sealer MTA Fillapex showed suitable physicochemical properties although further studies evaluating the biological properties must be conducted to confirm its use in endodontic therapy.

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CAPÍTULO 2

Long-term cytotoxic effects of contemporary root canal sealers

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ABSTRACT

Objectives: The aim of the present study was to investigate the effects of root canal sealers on the cytotoxicity during a period of 5 weeks in 3T3 fibroblasts.

Materials and Methods: Fibroblasts (3T3, $1x10^5$ cells per well) were incubated with elutes of fresh specimens from eight root canal sealers (AH Plus, Epiphany, Endomethasone N, EndoREZ, MTA Fillapex, Pulp Canal Sealer EWT, RoekoSeal and Sealapex) and with the elutes of the same specimens for 5 succeeding weeks after immersing in simulated body fluid. The cytotoxicity of all root canal sealers was determined using the MTT assay. Data were analyzed using ANOVA and Tukey's test.

Results: RoekoSeal was the only sealer that didn´t show any cytotoxic effects ($p < 0.05$). All the other tested sealers exhibited severe toxicity initially (week 0). MTA Fillapex remained moderately cytotoxic after the end of experimental period. Toxicity of the other tested sealers decreased gradually over time. The evaluated root canal sealers presented varying degrees of cytotoxicity mainly in fresh mode.

Conclusions: RoekoSeal had no cytotoxic effect both freshly mixed and in the other tested time points. MTA Fillapex was associated with significantly less cell viability when compared to the other tested root canal sealers.

Key-words: Cytotoxicity; cell culture; fibroblasts; root canal sealers

INTRODUCTION

A complete sealing of the root canal system after cleaning and shaping is critical for successful endodontic therapy[1]. In endodontic treatment, most root canals are filled with gutta-percha points in combination with a root canal sealer. Although endodontic sealers are designed to be used only within the root canal during endodontic therapy, sometimes they can extrude though the apical constriction[2,3]. Indeed, they are often placed in intimate contact with the periapical tissues for an extended period of time[2,4]. Thus, the biocompatibility of root canal sealers is an important factor in choosing the best material.

The sealers that have been currently used are based on zinc oxide-eugenol, calcium hydroxide, polydimethylsiloxane, silicone, epoxy resin and methacrylate resin. They exhibit a variable degree of cytotoxicity depending on the conditions under which testing was performed[5,6]. Most of these sealers exert some toxic effect when they are fresh or in short time of test[5-9]. However these intervals are probably inadequate to predict the biologic response of sealers that may remain in contact with periapical tissues for decades. Only a few studies have attempted to evaluate the longitudinal cytotoxicity effects of root canal sealers[7,8,10,11].

Whereas the cytotoxicity of conventional endodontic sealers has been well documented[6-8,10-12], little is known about the long term toxicity of newer endodontic sealers such as Epiphany, RoekoSeal and MTA Fillapex. Previous reports have shown variable results[4,13-15], so it seems prudent to compare new and old sealers by standardized cell culture methods in the same study. Therefore, the aim of this study was to assess the cytotoxicity of eight root canal sealers, namely AH Plus, Endomethasone N, EndoRez,

Epiphany, MTA Fillapex, Pulp Canal Sealer EWT, RoekoSeal and Sealapex over long time periods. The reason for this study was that long term tests provide a more extensive toxicity profile that would be useful in to determine the clinical performance of root canal sealers.

Materials and Methods

Sample and extract preparation

Eight root canal sealers were evaluated: AH Plus, Endomethasone N, EndoRez, Epiphany, MTA Fillapex, Pulp Canal Sealer EWT, RoekoSeal and Sealapex. The tested materials, product names, manufacturers and components are listed 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 in diameter and 2 mm in height. Excess material was removed with a sterile scalpel. After one hour sealers were carefully removed from Teflon blocks. Cytotoxicity of the sealers was assessed immediately after mixing and for 5 succeeding weeks (weeks 1-5). The extraction was made eluting the sealers in cell culture medium using the surface area-to-volume ratio of approximately 150 mm^2/mL between the surface of the samples and the volume of medium (16). The extraction vials were agitated for 24 h in a water bath at 37°C. Between tests, the specimens were aseptically removed and rinsed twice with sterile simulated body fluid (SBF) as previously reported[11,12]. Control samples containing only culture medium were treated similarly. Undiluted extracts were used for the testing.

Table 1. Composition of materials and their manufactures

Root Canal Sealer	Components	
AH Plus, Dentsply Germany	**Paste A:** Epoxy Resins, Calcium Tungstate, Zirconium Oxide, Silica, Iron Oxide Pigments, Aerosil	**Paste B:** Adamantane amine, N,N-Dibenzyl-5-oxanonane, TCD-Diamine, Calcium Tungstate, Zirconium Oxide, Aerosil
Epiphany, Pentron USA	BisGMA, UDMA, Hydrophilic Methacrylates	
Endomethasone N, Septodont France	**Powder:** Hydrocortisone Acetate, Thymol Iodide, Barium Sulphate, Zinc Oxide, Magnesium Stearate	**Liquid:** Eugenol
EndoREZ, Ultradent USA	30% UDMA, Zinc Oxide, Barium Sulphate, Resins, Pigments	
MTA Fillapex, Angelus Brazil	Salicylate Resin, Diluting Resin, Natural Resin, Bismuth Trioxide, Nanoparticulated Silica, MTA, Pigments	
Pulp Canal Sealer EWT, SybronEndo USA	**Powder:** Silver Powder, Zinc Oxide, Thymol Iodide, Dimeric Acid Resin	**Liquid:** Clove Oil, Canada Balsam
RoekoSeal, Coltene Germany	Polymethylsiloxane, Silicone Oil, Paraffin-base Oil, Hexachloroplatinic Acid, Zirconium Dioxide	
Sealapex, SybronEndo USA	**Paste A:** Isobutyl Salicylate Resin, Silicon Dioxide, Bismuth Trioxide, Titanium Dioxide Pigment	**Paste B:** N-ethyl Toluene Solfanamide Resin, Silicon Dioxide, Zinc Oxide, Calcium Oxide

Cytotoxicity Assay

Balb/c 3T3 cells (American Tissue Type Collection; ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS) (Sigma Chemical Co, St Louis, MO), 100 µg/mL of streptomycin, 100 mg/mL of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO2. Confluent cells were detached with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) for 5 minutes, and aliquots of separated cells were subcultured. Cells were seeded in 24-well plates ($1x10^5$ cell/well). After overnight attachment, cells were exposed to the extracts of the different tested sealers (500 µl/well). Cytotoxicity testing was repeated immediately after mixing (fresh), and then after 1, 2, 3, 4 and 5 weeks to study temporal trends in cytotoxicity of the sealers.

Cell viability was determined each week (1-5 weeks) by the MTT assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). After the removal of culture medium from each well, the cells were gently washed with 1.0 mL of phosphate-buffered saline. The wash was replaced with an MTT-succinate solution (1mg/mL; Sigma-Aldrich, St Louis, MO) for 4 hours. After aspiration of the solution, the cell monolayers were rinsed with double-distilled water. Then the water was completely removed. Formazan crystals produced within the cells by succinate dehydrogenase (SDH) reduction of MTT were dissolved using destaining solution (isopropanol–10%NP40-0.4N HCl). Aliquots (100µl) 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 (Hitachi, Tokyo, Japan).

The formazan content of each well was computed as a percentage of the control group (untreated cells). Cytotoxicity responses were rated as severe (30%), moderate (30%-60%), slight (60%-90%) or noncytotoxic (>90%)[17].

Data and statistical analysis

All assays were repeated three times to ensure reproducibility. Toxicity of the endodontic sealers was assessed by measuring cell viability that had been determined by the SDH activity (MTT assay). The data were analyzed by one-way analysis of variance (ANOVA), and follow-up comparison between the groups was made using Tukey multiple comparison test (at 95% confidence interval level, a = 0.05). Data were analyzed using the statistical software SPSS® (SPSS, Inc., Chicago, IL, USA).

Results

The results of the MTT assay over the entire time periods are listed in Table 2 and collectively represented in Figure 1. RoekoSeal was the only sealer that didn´t show any cytotoxic effects in any time points and was not significantly different from the control ($p<0.05$). The other sealers appeared to show some toxic effects when they were evaluated in fresh conditions. After one week, Endomethasone N and Pulp Canal Sealer EWT became noncytotoxic with no significant difference from the control group or RoekoSeal ($p<0.05$). AH Plus fresh was moderately cytotoxic, was mildly cytotoxic after one week and become noncytotoxic after two weeks. Sealapex fresh was mildly cytotoxic and became noncytotoxic after three weeks. EndoREZ and Epiphany exhibited significant increases in the SDH activities over time ($p<0.05$, table 1) and after 5 weeks both became noncytotoxic.

Conversely, MTA Fillapex remained severely and mildly cytotoxic over the entire experimental period. At the end of the fifth week, this sealer exhibited a toxicity level that was significantly more severe ($p<0.05$) than the other tested sealers, which had become noncytotoxic.

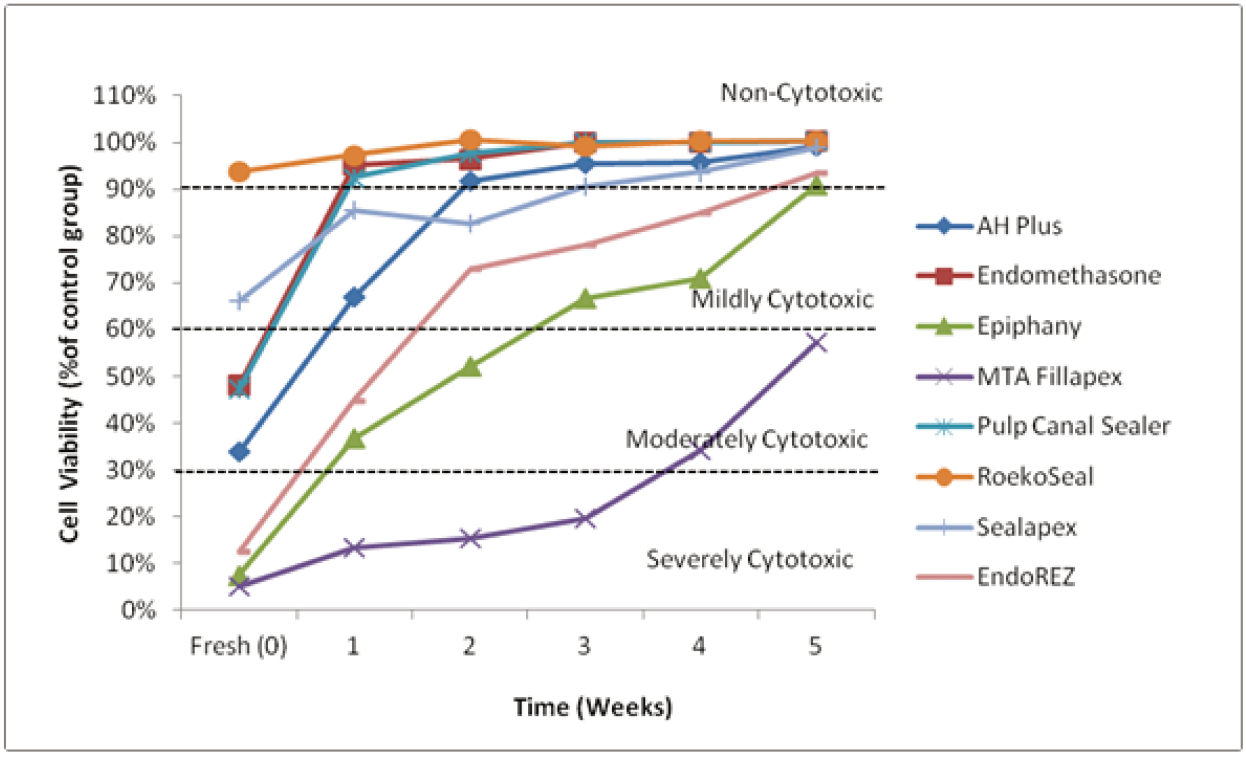

Figure 1 – A line chart depicting the changes in cell viability with time after the eight endodontic sealers were repeatedly immersed in a simulated body fluid. Values are expressed as percentages relative to the control group and classified as severe (<30%), moderate (<60%), slight (60%-90%) or non-cytotoxic (>90%).

TABLE 2. Succinate Dehydrogenase Activities Exhibited by 3T3 Cells in the Presence of Different Root Canal Sealers

Materials	Fresh	Week 1	Week 2	Week 3	Week 4	Week 5
Control	$100^{A,1}$	$100^{A,1}$	$100^{A,1}$	$100^{A,1}$	$100^{A,1}$	$100^{A,1}$
AH Plus	$33.9^{D,3}$(±4.6)	$67.0^{C,2}$(±5.3)	$91.7^{AB,1}$(±5.3)	$95.4^{A,1}$(±8.2)	$95.8^{A,1}$(±6.0)	$99.1^{A,1}$(±6.3)
Endomethasone N	$48.2^{C,2}$(±3.8)	$95.1^{AB,1}$(±6.4)	$96.4^{A,1}$(±6.3)	$100.4^{A,1}$(±15.4)	$100.0^{A,1}$(±9.3)	$100.3^{A,1}$(±7.6)
EndoREZ	$12.4^{E,4}$(±5.7)	$44.8^{D,3}$(±7.9)	$73.0^{C,2}$(±12.3)	$78.1^{B,2}$(±13.6)	$84.9^{AB,1}$(±10.9)	$93.5^{A,1}$(±13.2)
Epiphany	$7.5^{E,5}$(±4.4)	$36.8^{D,4}$(±2.5)	$52.1^{D,3}$(±8.7)	$66.7^{B,2}$(±12.4)	$70.8^{B,2}$(±9.6)	$91.3^{A,1}$(±12.6)
MTA Fillapex	$5.0^{E,4}$(±3.4)	$13.4^{E,3}$(±4.7)	$15.3^{E,3}$(±1.0)	$19.7^{C,3}$(±4.3)	$34.0^{C,2}$(±5.9)	$57.3^{B,1}$(±13.4)
Pulp Canal Sealer EWT	$47.3^{C,2}$(±3.7)	$92.4^{AB,1}$(±11.3)	$97.7^{A,1}$(±12.2)	$100.5^{A,1}$(±12.9)	$100.1^{A,1}$(±13.6)	$100.3^{A,1}$(±13.4)
RoekoSeal	$93.7^{A,1}$(±10.1)	$97.3^{A,1}$(±7.0)	$100.7^{A,1}$(±4.0)	$99.1^{A,1}$(±5.5)	$100.4^{A,1}$(±12.0)	$100.2^{A,1}$(±11.9)
Sealapex	$66.0^{B,3}$(±9.7)	$85.4^{B,2}$(±3.5)	$82.7^{BC,2}$(±3.4)	$90.5^{AB,1}$(±4.2)	$93.6^{A,1}$(±11.8)	$98.9^{A,1}$(±9.8)

The data are normalized against the control group. Values represent means (standard deviations) and are expressed as relative percentages of the SDH activities of the control group (100%). For each column, data with different letter superscripts denote significant difference ($p<0.05$). For each row, data with different numerical superscripts denote significant difference ($p<0.05$).

Discussion

Root canal sealers should be biocompatible since they may come in intimate contact with the periapical tissues for an extended period of time. The direct contact and the degradation of sealers over time could induce cytotoxic damage to cells and tissues and adversely affect the outcome of the root canal treatment[2-4,18]. This study was designed to determine the longitudinal citoxicity behavior of eight contemporary endodontic sealers on fibroblasts. Although cytotoxicity testing of freshly mixed sealers is relevant as they are placed into the root canal system in a freshly mixed and incompletely polymerized stage, 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. It could be confirmed as all tested sealers showed different degrees of toxicity reduction after repeated testing at extended time periods.

This long term evaluation is also better than previous strategies that assessed cytotoxicity for the shorter term, because it enables the establishment of distinct toxicity profiles that are characteristic of each sealer. RoekoSeal was the only sealer that did not exhibited citotoxic effects both freshly mixed and in the other tested time points. RoekoSeal is a sealer based in silicone, which is described as a biocompatible material[19]. The findings of the present study are in agreement with many previous studies, that have shown that RoekoSeal was only little or non-cytotoxic even in fresh conditions[4,8,13,14]. The relatively severe and mildly cytotoxicity responses exhibited by the other sealers in fresh conditions, confirm the results reported in previous studies[4,7,9,13,14,19-21]. This could be attributed to the

release of small amounts of toxic substances present in the sealers. Probably as a result of the diminishment in the leaching of these toxic substances, the cytotoxicty of the tested sealers decreased in the aged specimens. Previous reports with different methodologies showed similar citotoxic reduction after 5 weeks[11,12,19].

MTA Fillapex was created in an attempt to combine the physicochemical properties of an endodontic sealer with the excellent biological properties of MTA. According to the present results, MTA Fillapex showed a severe citotoxicity when cells were exposed to fresh elute of the sealer. This toxicity did not decrease over time. A longer period may then be required before the sealer can be rendered non-cytotoxic. The findings of the present study are in agreement with a previous study which has shown strongly affected cell viability by MTA Fillapex, using several methodologies[15,22]. The results suggest the correlations between the components, such as Salicylate Resin, Diluting Resin and Silica with the citotoxic effects.

Although the relevance of in vitro toxicity tests to clinical conditions has been frequently questioned, it appears that biological risks of endodontic sealers is relatively high as the components of various root canal sealers may induce potential tissue toxicity, leading to apical periodontal tissue damage and inflammatory responses[6,23]. This is unfortunate knowing that these materials might be placed in close contact with periapical tissues for long periods of time. Even in cases that the sealer does not reach directly the periapical region, there is always the possibility of elutable substances or degradation products from root canal fillings leaching through the dentinal tubules, lateral and accessory canals or apical foramina[24]. However, according to the results of this study, after

5 weeks, endodontic sealers exhibited non-citotoxic effects to 3T3 fibroblast cells reflecting no long-term risk for adverse effects. So, despite the transitory irritability that endodontic sealers may cause to periapical tissues, endodontists should evaluate the advantages and disadvantages of sealer extrusion since the remaining areas not sealed in the apical region may serve as microorganism niches, initiating or perpetuating an endodontic failure[25].

Conclusion

The evaluated root canal sealers presented varying degrees of cytotoxicity mainly in fresh mode. RoekoSeal had no cytotoxic effect both freshly mixed and in the other tested time points. MTA Fillapex was associated with significantly less cell viability when compared to the other tested root canal sealers.

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CAPÍTULO 3

A multiparametric assay to compare the long-term cytotoxicity of AH Plus and MTA Fillapex

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Running title: Long-term cytotoxicity of AH Plus and MTA Fillapex

Key-Words: AH Plus, cytotoxicity, cell culture, MTA Fillapex

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ABSTRACT

Aim: To verify, through a multiparametric *in vitro* assay, the long term cytotoxic effects of the MTA Fillapex and to compare with AH Plus.

Methodology: 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 succeeding weeks. A multiparametric cell viability assay was performed, evaluating mitochondrial activity, membrane integrity and cell density. The results were analysed by one-way analysis of variance, complemented with the Tukey post-test ($P < .05$).

Results: No significant difference was found among the materials when fresh mixed ($p>0.05$). After one week AH Plus become noncytotoxic, on all three parameters evaluated. 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 in all tested time points ($p<0.01$), except in fresh conditions ($p>0.05$).

Conclusions: Both materials showed high cytotoxic levels for human primary cells when freshly mixed. After one week AH Plus become noncytotoxic and MTA Fillapex remained cytotoxic over the entire experimental period, as shown by three different cell viability tests.

Key Words: AH Plus, cytotoxicity, cell culture, MTA Fillapex

INTRODUCTION

During the last decade, mineral trioxide aggregate (MTA), a calcium silicate–based material, has gained popularity in endodontics as a root-end filling and pulp-capping material, for the repair of root canal perforations, and for apexification (Torabinejad *et al*. 1993, Main *et al*. 2004, Felippe *et al*. 2006, Nair *et al*. 2008). MTA is a biocompatible, noncytotoxic, nonmutagenic, and neither genotoxic nor carcinogenic material (Braz *et al*. 2006, Ribeiro *et al*. 2006, Washington *et al*. 2011, Silva *et al*. 2012a). In addition, MTA has shown antibacterial (Eldeniz *et al*. 2006) and excellent sealing properties (Torabinejad & Parirokh 2010). However, despite its favorable characteristics, MTA does not exhibit the physical properties needed to be used as an endodontic sealer because of its working time, setting time, and difficult handling (Roberts e*t al*. 2008).

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 through expansion during setting. A recent study showed suitable radiopacity, pH and flow of MTA Fillapex (Silva *et al*. 2012b). However, in regard to it cytototixicity and biocompatibility, controversial results have been presented (Gomes-Filho *et* al. 2011, Salles *et* al. 2012, Silva *et al.* 2012b, Tavares *et al*. 2012, Zmener *et al*. 2012). 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 tissues for an extended period of time (Ricucci & Langland 1998, Lodiene *et al*. 2008). Due to

these controversial results, more studies about MTA Fillapex cytotoxic behavior are required to be evaluated before its clinical indication.

Thus, the aim of this study was to assess, through a multiparametric *in vitro* assay, the long term cytotoxic effects of the MTA Fillapex on primary human osteoblasts (hOB) cell line. AH Plus (Dentsply, Germany) was employed as the reference material for comparison. The null hypothesis is that there are no differences between the tested endodontic sealers in any tested time point.

MATERIALS AND METHODS

Sample and extract preparation

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

Table 1. Composition of the materials and their manufactures

Root Canal Sealer	Components	
AH Plus, Dentsply Germany	**Paste A:** Epoxy Resins, Calcium Tungstate, Zirconium Oxide, Silica, Iron Oxide Pigments, Aerosil	**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	

The sealers were mixed according to the manufacturers' instructions. Discs of each sealer were fabricated under aseptic conditions in sterile cylindrical Teflon blocks with 5

mm in diameter and 2 mm in height. Excess flash material was removed with a sterile scalpel. Cytotoxicity of the sealers was assessed immediately after mixing and for up to 4 weeks. The extraction was made in cell culture medium using the surface area-to-volume ratio of approximately 50 mm^2/mL between the surface of the samples and the volume of medium (International Organization for Standardization, 2009), for 24 h at 37°C. Between tests, the specimens were aseptically removed and rinsed twice with PBS. Control samples containing only culture medium were treated similarly. Undiluted extracts were used for the testing.

Culture of Osteoblastic Cells

Human osteoblasts on second passage (hOB) from the Rio de Janeiro Cell Culture Bank were subcultured for 24h at 37°C on 96-well culture plates ($1 \cdot 10^4$ cells per well). After 24 h, the medium was removed gently from each well and replaced by 200 μL of one of the selected test media (AH Plus and MTA Fillapex extracts) in triplicates and was incubated for a further 24 h. Medium not exposed to filling materials was used as a control of culture conditions. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cytotoxicity assay

After 24 h of cell exposure to each extract medium, cytotoxicity was evaluated with a commercial kit (In Cytotox, Xenometrix, Allschwil, Switzerland) which allows the use of three different tests of cell survival and integrity on 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-coloured soluble compounds of formazan, measured by their absorbance at 480 nm using a microplate UV/Vis spectrophotometer (PowerWave MS2; BioTek Instruments, Rio de Janeiro, RJ, Brazil). NR is a survival/ viability test based on the ability of living cells to incorporate NR dye on their lysosomes, where it accumulates on membrane-intact 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 (De-Deus *et al*. 2010, Scelza *et al*. 2012, Silva *et al*. 2012c).

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 methods employed. No significant difference was found among the materials in this time point ($p>0.05$). After one week AH Plus become noncytotoxic, on all three parameters evaluated. 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 in all tested time points ($p<0.01$), except in fresh conditions ($p>0.05$), in all evaluated parameters.

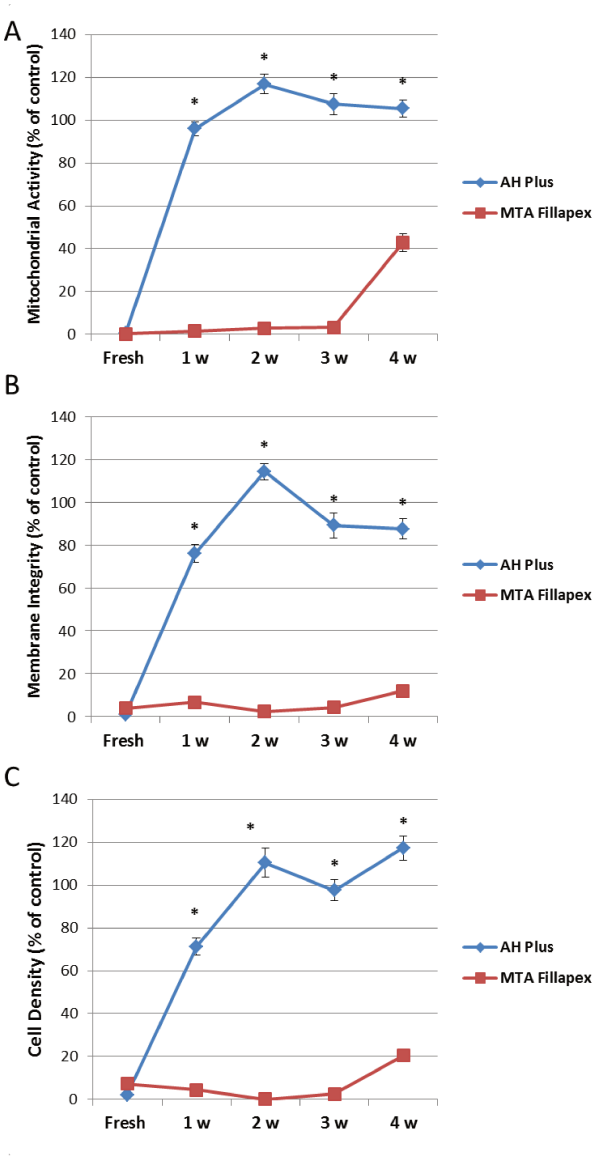

Fig 1. Multiparametric Long-Term cytotoxicity assay. Cytotoxic effects of endodontic sealers on hOB by (a) XTT, (B) NR and (C) crystal violet tests, expressed as percentage of control group. (*) indicates significant difference between the groups (P<0.05).

DISCUSSION

In the present study, the cytotoxicity of MTA Fillapex and AH Plus were tested, employing some *in vitro* methodological strategies that differs from most previous works on these materials. First of all, three different parameters were evaluated on the same samples using a multiparametric assay that evaluates: (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 (De-Deus *et al*. 2012, Scelza *et al*. 2012, Silva *et al*. 2012c).

A long-term cytotoxicity evaluation was also used in the present study. Although cytotoxicity testing of freshly mixed sealers is relevant as they are placed into the root canal system in a freshly mixed and incompletely polymerized stage, 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. It could be confirmed because the tested sealers showed different degrees of toxicity reduction after repeated testing at extended time 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 profiles that are characteristic of each sealer (Lodiene *et al*. 2008).

Several 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 (Koh *et al.* 1998). However, biocompatibility assessments through primary cell culture are appealing, because the biomaterials will interact with such kind of cells after *in vivo* implantation (Rosa & Beloti 2003). Also the employment of human primary cells of a relevant type on the study of endodontic materials, has been pointed out previously (Huang & Chang 2002), in the light of several expected differences in the responses of immortalized cells. For this, 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 the clinical setting (Zhu *et al*. 2000). It is also reported that hOB cells provides a useful tool, able to help to predict the effects of biomaterials on regenerative capability of periapical tissues (De-Deus *et al*. 2012).

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 have also observed severe cytotoxic effect of AH Plus and MTA Fillapex immediately after mixing and in the first days after mixed (Bin *et al.* 2012, Salles *et al*. 2012, Scelza *et al*. 2012, Silva *et al.* 2012b). The null hypothesis of the present study was rejected. After one week and in the succeeding weeks AH Plus became non-citotoxic, however MTA Fillapex toxicity did not decrease over time. Therefore, MTA Fillapex does not have the claimed biological advantages over other available products. As far as we know, this is the first attempt to evaluate this condition

with MTA Fillapex. The results suggest the correlations among the components present in MTA Fillapex formulation, such as Salicylate Resin, Diluting Resin and Silica with the citotoxic effects. Salycilate 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 (Mahdi *et al*. 2006). Arsenic, a heavy metal that may be found as a contaminant in MTA (Bramante *et al*. 2008) could be also related to MTA Fillapex cytotoxicity. Arsenic reacts with protein thiols, and exposure to high concentrations of this element may induce genotoxicity (Salles *et al*. 2012). Another possible explanation may related to the physic-chemical properties of MTA Fillapex. Borges e*t al*. 2012, demonstrated a higher solubility of MTA Fillapex when compared to AH Plus. This higher solubility can account for a greater amount of sealer particles release during the elution in DMEM, resulting in a higher exposition of MTA Fillapex to the cell culture. The high pH of MTA Fillapex (Silva e*t al*., 2012b) can also be related to an increase in the cytotoxicity values. Although the high pH can be related to cytotoxic effects, it activates alkaline phosphatase stimulating the mineralization process. The high pH may also neutralize acids secreted by osteoclasts inhibiting tissues demineralization (Eriksen 2010).

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 (Gomes-Filho *et al.* 2011). Contrary to this study, two recent studies showed intense and extensive inflammation in response to MTA Fillapex implanted in rat subcutaneous tissues (Tavares *et* al., 2012; Zmener *et al.*

2012). The conflicting results among the studies are probably related to details in the experimental procedures. In another study, MTA Fillapex clearly showed 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 (Salles *et al.* 2012).

Well-know and extensively used ISO standards cytotoxicity testes 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 has been suggested to test cytotoxicity of endodontic materials (Susini *et al*., 2006; De-Deus *et al*., 2009). This kind of model displays some advantages over assessments performed with isolate sealers, because more realistic material amounts are used, and the interaction between sealer and the surrounding dentin is taken into consideration as well (De-Deus *et al*. 2009). Thus, further studies are needed for a better understating of the cytotoxic effects of MTA Fillapex, and if this cytotoxicity may be related to some impairment of endodontic treatment.

CONCLUSION

AH Plus and MTA Fillapex had similar level of cytotoxicity in fresh conditions. AH Plus become non-cytotoxic after one week and MTA Fillapex remained cytotoxic after 4 weeks.

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CONSIDERAÇÕES GERAIS

O MTA Fillapex foi criado numa tentativa de combinar as propriedades físico-químicas de um cimento obturador endodôntico com as propriedades biológicas do MTA. No presente estudo foi possível verificar que a propriedades físico-químicas testadas no capítulo 1 foram apropriadas, baseadas nas normas ISO 6876/2001. No entanto, no que diz respeito as propriedades biológicas, o MTA Fillapex apresentou resultados de elevada citotoxicidade nos diversos ensaios realizados (Capítulo 1, 2 e 3).

Apesar da óbvia citotoxicidade do MTA Fillapex, a literatura endodôntica ainda é escassa no que diz respeito ao que os efeitos citotóxicos de um cimento endodôntico, podem gerar de prejuízo ao tratamento endodôntico. Como perspectivas futuras, mais estudos serão necessários para caracterizar novos determinantes biológicos, relacionados com a elevada citotoxicidade do MTA Fillapex, bem como, identificar se os mecanismos de citotoxicidade do MTA Fillapex são capazes de comprometer o prognóstico do tratamento endodôntico.

CONCLUSÃO

Dentro da metodologia empregada e de acordo com os resultados apresentados pode-se concluir que embora o MTA Fillapex tenha apresentado propriedades físico-químicas testadas (radiopacidade, pH e escoamento) adequadas para a utilização na terapia endodôntica, o mesmo apresentou elevada citotoxicidade nas diversas condições testadas.

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ANEXO

Confirmação de Publicação do Artigo (Capítulo 1)

ARTICLE IN PRESS

Evaluation of Cytotoxicity and Physicochemical Properties of Calcium Silicate-based Endodontic Sealer MTA Fillapex

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Abstract

Introduction: The aim of the study was to evaluate the cytotoxicity, radiopacity, pH, and flow of a calcium silicate–based and an epoxy resin–based endodontic sealer, MTA Fillapex (Angelus, Londrina, PR, Brazil) and AH Plus (Dentsply, Konstanz, Germany), respectively. **Methods:** Cytotoxicity, radiopacity, and flow evaluation were performed following ISO requirements. The pH level was measured at periods of 3, 24, 72, and 168 hours. Cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay to check the Balb/c 3T3 cells viability at 1- to 4-week periods. Data were statistically analyzed by analysis of variance and the Tukey test with a significance level of 5%. **Results:** In all tested periods, MTA Fillapex was more cytotoxic than AH Plus ($P < .05$). Although AH Plus presented higher radiopacity than MTA Fillapex ($P < .05$), both sealers showed minimum required values. MTA Fillapex presented alkaline pH in all experimental times, whereas AH Plus cement showed a slightly neutral pH and a flow significantly lower than that of MTA Fillapex ($P < .05$). **Conclusions:** Although MTA Fillapex was more cytotoxic than AH Plus, it showed suitable physicochemical properties for an endodontic sealer. *(J Endod 2012; ■:1–4)*

Key Words

AH Plus, cytotoxicity, MTA Fillapex, physicochemical properties, root canal sealer

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The characteristics and physicochemical properties of endodontic sealers are fundamental to allow hermetic sealing, which with an adequate coronal restoration will avoid bacterial leakage (1, 2). Mineral trioxide aggregate (MTA) is a material consisting of tricalcium oxide and other mineral oxides such as tricalcium silicate and silicate oxide. The pH of the material has been determined as 12.5 when set, which is comparable with that of calcium hydroxide (3–6). MTA biocompatibility, low cytotoxicity, antimicrobial properties (3, 7), low microleakage (4, 8), and its ability to set in the presence of blood or moisture are among the material's additional advantages. Because of these properties, it has been used for several dental applications such as root-end filling, root repair materials, and pulp capping (3–5). However, despite its favorable characteristics, MTA does not exhibit the physical properties needed to be used as an endodontic sealer because of its working time, setting time, and difficult handling (9).

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 through expansion during setting. However, up to now, there are limited independent publications about the physicochemical and biological properties of MTA Fillapex and its possible use in clinical practice. Thus, the aim of the present study was to evaluate the cytotoxicity, radiopacity, pH, and flow of MTA Fillapex sealer and to compare them with those of AH Plus (Dentsply, Konstanz, Germany).

Materials and Methods

Cytotoxicity evaluation was performed according to ISO 10993-5 specifications (10). Radiopacity and flow evaluations were performed according to ISO 6876/2001 specifications (11). The pH level was measured at 3, 24, 72, and 168 hours. AH Plus cement was mixed according to the manufacturer's instructions for all the tests. MTA Fillapex is premixed, so further manipulations were unnecessary. The composition of the evaluated sealers is shown in Table 1.

Cytotoxicity

Discs of each sealer were fabricated under aseptic conditions in sterile cylindric Teflon blocks with a 5-mm diameter and a 2-mm height. Excess flash material was removed with a sterile scalpel. Cytotoxicity of the sealers was assessed immediately after mixing and during 4 succeeding weeks. The extraction was made in cell culture medium using a surface area to volume ratio of approximately 150 mm^2/mL between the surface of the samples and the volume of the medium (8). The extraction vials were agitated for 24 hours in a water bath at 37°C. Control samples containing only culture medium were treated similarly. Undiluted extracts were used for the testing.

Balb/c 3T3 cells (American Tissue Type Collection, Manassas, VA) were cultured in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma Chemical Co, St Louis, MO), 100 μg/mL streptomycin, and 100 mg/mL penicillin at 37°C in a humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and

Confirmação de Aceite do Artigo (Capítulo 2)

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Assunto: **[JAOS 2304] Editorial Review of Article - Decision**

Dear Authors,

I am delighted to inform you that the revised version of your manuscript "JAOS 2304 - Long-term Cytotoxic effects of Contemporary Root Canal Sealers" has been accepted for publication in the Journal of Applied Oral Science.

We will do our best to send the galley proofs for your approval in a timely fashion.

Thank you for choosing our journal to publish your work.

Please, do not hesitate to contact me for any further clarification or assistance.

Yours sincerely,

_____

Carlos F. Santos, DDS, MSc, PhD, Professor
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Confirmação de Envio do Artigo para Publicação (Capítulo 3)

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Assunto: **International Endodontic Journal – Manuscript ID IEJ-13-00024**

Dear Dr. Zaia,

Your manuscript entitled "A multiparametric assay to compare the long-term cytotoxicity of AH Plus and MTA Fillapex" has been successfully submitted online to the International Endodontic Journal.

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Please mention the above manuscript ID in all future correspondence or when calling the Editorial Office for questions. If there are any changes in your postal or e-mail address, please log in to ScholarOne Manuscripts at http://mc.manuscriptcentral.com/iej and edit your user information as appropriate.

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Thank you for submitting your manuscript to the International Endodontic Journal.

Kind regards

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APÊNDICE

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