**In vitro** toxicogenomic activity of an MTA/salicylate-based endodontic sealer

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**A R T I C L E   I N F O**

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**A B S T R A C T**

This study was designed to investigate whether mineral trioxide aggregate (MTA) Fillapex®, an MTA/salicylate-based endodontic sealer, exerts cytotoxic and toxicogenomic effects on human gingival fibroblasts (HGFs). HGFs were exposed *in vitro* to MTA Fillapex® at concentrations of 5%, 10%, 20%, and 40% for 24 h. Cytotoxicity, cell survival (5 days), cell cycle kinetics (flow cytometry), genotoxicity (comet assay), and gene (TP53, BAX, and BCL2) expression profiles were evaluated using reverse-transcriptase quantitative polymerase chain reaction. MTA Fillapex® was cytotoxic to HGFs at the two highest concentrations (20% and 40%), and cell survival decreased after 5 days treatment only with 40% concentration. After MTA Fillapex® treatment, there was an increase in the expression of apoptosis-related genes BAX, BCL2, and TP53, but no increase in DNA damage. Cement also induced changes in cell cycle kinetics, apoptosis, and necrosis rates. The data show the ability of MTA Fillapex® endodontic sealer to induce cellular and genetic alterations in HGFs. Our findings suggest that this compound should be used with caution to avoid health-related risks to the buccal tissue.

1. Introduction

Endodontic treatment, an important practice in the dental clinic, aims to remove damaged or infected tooth pulp, clean and shape the root canal system, and completely fill the space to prevent apical and coronal penetration of liquids and microorganisms. For therapeutic effectiveness, it is imperative to use an adequate sealant component that does not cause or allow future injuries to patients. To determine the best range and application for each clinical case, it is important to know the characteristics and qualities of endodontic sealers. The essential characteristics of the filling cements are dimensional stability, setting and working time, low solubility, biocompatibility, release of Ca²⁺ ions, radiopacity, antimicrobial activity, and adhesion to root dentin [1].

Endodontic sealers are categorized according to the setting reaction and composition [2]. At present, several cements are composed of a mineral trioxide aggregate (MTA), which comprises fine hydrophilic particles of tricalcium silicate, tricalcium aluminum oxide, tricalcium oxide, calcium sulfate dihydrate (gypsum), and others mineral oxides [3, 4]. MTA has excellent biological activity and favorably reacts with tissues, especially as it does not elicit a severe inflammatory response [5–7]. Furthermore, studies have shown that MTA induces osteoblast differentiation and stimulates dental pulp cell mineralization [8–10]. Nonetheless, MTA also presents some disadvantages such as difficulty in manipulation, slow setting time, and high cost [11].

MTA Fillapex®, an MTA-based endodontic sealer, was developed in an attempt to address the limitations of MTA. This cement combines the biological properties of MTA with components such as salicylate resin, natural resin, calcium tungstate, nanoparticulate silica, and pigments [12]. Studies have shown that MTA Fillapex® is a biocompatible material with physicochemical properties suitable for an endodontic sealer [13–15]. However, the presence of toxic elements such as salicylate and silica has raised concerns regarding its biocompatibility [16]. Previous studies have shown that MTA Fillapex® can mediate cytotoxic [11, 12, 17] and genotoxic effects [18, 19].

Although an ideal sealer should integrate a hermetic seal with therapeutic effects, it is of utmost importance that it exhibits acceptable biocompatibility, nontoxicity, non-mutagenicity, and non-carcinogenicity [20]. Considering the wide applicability of MTA Fillapex® for endodontic treatments and that it can remain in close contact with periapical regions for a long time, it is essential to clarify its toxicogenetic potential by understanding the underlying mechanism of action and its main targets. Thus, the present study aims to investigate whether MTA Fillapex® induces primary DNA damage in human gingival fibroblasts (HGFs) and modulates the expression of three

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2. Material and methods

2.1. Cell line and endodontic sealer

HGFs were kindly provided by Prof. Carlos Alberto de Souza Costa, Department of Pathology, Faculty of Dentistry, UNESP, Araraquara, SP, Brazil, with approval of the Brazilian Ethics Committee (CAAE 85676318.0.0000.5411). Cells were cultured as monolayers using minimum essential medium (MEM; Gibco, NY, USA) supplemented with 10% fetal calf serum (Cultilab, Brazil) and 1% antibiotic/antimycotic minimum essential medium (MEM; Gibco, NY, USA) supplemented with (10,000 units/mL penicillin, 10,000 mg/mL streptomycin, and 25 mg/ mL amphotericin B-Gibco), and incubated at 37 °C in a humid atmosphere containing 5% CO₂ for 24 h. This study was carried out using cells from the sixth passage, and the supplemented culture medium was changed every 3 days. The experiments were carried out in triplicates and always with exponentially growing cells.

The MTA Fillapex® Root Canal Sealer (Angeluss Industria de Productos Odontologicos S/A, PR, Brazil, lot 46454) is a paste material presented in automated double tubes (A-18 g base paste: salicylate resin + calcium tungstate + fumed silica; B-12 g catalyst paste: fumed silica + titanium dioxide + 13.2% mineral trioxide aggregate + base resin). Before cell treatment, the cement was weighed (0.1 g base paste + 0.1 g catalyst paste), mixed on a sterilized glass plate using a metal spatula, according to the manufacturer’s instructions, and placed into a 9 cm² cell-culture dish. Then, 1.6 mL supplemented culture medium was added to obtain 6 cm²/mL ratio (cement sample/volume of culture medium) [21]. The dish was incubated at 37 °C and 5% CO₂ for 6 h. Later, the culture medium was collected and filtered through a 0.22 μm pore size filter. From this stock solution, four serially diluted test solutions were obtained as follows: 5%, 10%, 20%, and 40%.

2.2. Cytotoxicity and cell survival

Cytotoxicity and cell survival (5 days) were evaluated using the Cell Titter 96 AQueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA), based on the colorimetric method of reduction of the MTS tetrazolium salt by metabolically active cells. For cytotoxicity (1 × 10⁴ cells) and 5-day cell survival (3 × 10⁵ tests), HGFs were seeded in a 96-well plate and maintained at 37 °C and 5% CO₂ for 24 h for adhesion and stabilization. The cells were then treated with four different concentrations of MTA Fillapex®/medium solution for 24 h. Immediately after treatment, the cement/medium solution was removed, and the cells were incubated with Cell Titter reagent for 2 h for cytotoxicity evaluation. Alternatively, the cells were incubated in supplemented culture medium at 37 °C for 5 days. Cell suspension absorbance was read on a spectrophotometer (490 nm). The obtained value was proportional to the number of viable cells.

2.3. Cell cycle and cell death

Cultured HGFs (3 × 10⁵ cells) were treated with different concentrations of cement/medium for 24 h. Cell cycle (percentage of cells in G0/G1, S, and G2/M phases) and apoptosis rates were evaluated by flow cytometry (GUAVA EASYCYTE; Merck Millipore, Darmstadt, Germany) using propidium iodide (Sigma-Aldrich, Darmstadt, Germany). Cell death was investigated using the Guava Nexin reagent (Merck Millipore, Darmstadt, Germany), according to the manufacturer’s instructions. Cell status was defined as the percentage of viable cells (Annexin (-) 7-AAD (-)), initial apoptosis (Annexin (+) 7-AAD (-)), late apoptosis (Annexin (-) 7-AAD (+)), and necrosis (Annexin (+) 7-AAD (+)). The percentage of each population was calculated using the GUAVA Cytosoft software version 4.2.1.

2.4. Comet assay

In brief, approximately 6 × 10⁴ HGF/well were cultured in 24-well plates for 24 h. Cells were treated with the cement/culture medium solution at concentrations of 5%, 10%, and 20% for 24 h. As a positive control, cells were treated with 100 μM peroxide (Merk KGaA, Darmstadt, Germany). The experiments were performed in triplicates and protected from light. Only cultures with cell viability (trypan blue test) greater than 70% were considered for this assay [22]. The alkaline comet assay was performed according to the methodology described by Singh et al. [23] and Tice et al. [22]. Cells from each well were transferred to microtubes and centrifuged at 70 × g for 3 min. After centrifugation, the supernatant was removed, and 100 μL of 0.5% low melting agarose (37 °C) was added. The agarose/cell suspension was placed onto a slide previously covered with a 1.5% normal melting point agarose (PBS) solution (1 ×) for 5 min and then in a horizontal electrophoresis chamber where it remained in an alkaline buffer (10 N sodium hydroxide [NaOH], 200 mM EDTA, pH 13) at 4 °C for 20 min. Electrophoresis was conducted at 1.25 V/cm and 300 mA for 20 min. Subsequently, the slides were placed in a neutralization solution (0.4 M Tris-HCl, pH 7.5) for 15 min and fixed in absolute ethanol. SYBR Gold (1:10,000, Invitrogen) was used to stain the nucleoids. A total of 50 nucleoids/slide (two slides per treatment) were analyzed under 400 × magnification using a fluorescence microscope coupled to an image analysis system (Comet Assay IV, Perceptive Instruments, UK). The tail intensity (% DNA in the comet’s tail) was used to measure DNA damage.

2.5. Gene expression by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR)

Relative expression of BCL2 (Hs 00608023 m1), BAX (Hs 00414514 m1), and TP53 (Hs 99999147 m1) genes was evaluated using RT-qPCR. Total RNA was isolated using the Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA, USA), and cDNA was synthesized using the High-Capacity kit (Applied Biosystems, Foster City, California, USA). PCR was performed in an automatic thermocycler (ABI Prism 7500 Fast Sequence Detection System, Applied Biosystems) using the TaqMan® system (Applied Biosystems, Branchburg, New Jersey, USA), which is composed of a universal PCR Master Mix and specific probes for each gene marked with the fluorochrome FAM. The analysis was performed using the comparative cycle threshold method (ΔΔCT) [24]. The beta-actin gene was used as a control.

2.6. Statistical analysis

Cytotoxicity and cell survival data were analyzed using analysis of variance (ANOVA) and Tukey’s test. A negative binomial distribution, was used for the comet assay results. Cell cycle and cell death data were analyzed using a binomial distribution, followed by comparison with the Wald test. For gene expression, p-values were calculated based on the Student’s t-test of the replicate 2-ΔΔCT values for each gene in the control and experimental groups. Only genes with a fold-change > 2 were considered differentially expressed. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. Cytotoxicity and cell survival

The cytotoxicity test showed a significant (p < 0.05) decrease in the viability of cells treated with 20% and 40% MTA Fillapex®/medium.
3.4. Gene expression

was not recommended for this assay [22].

The number of cells in the late apoptosis and necrosis stages was significantly higher after treatment with the highest concentration (40%) of cement solution (Fig. 1b).

3.2. Cell cycle and cell death

We observed changes in the kinetics of HGF cycle after exposure to 40% MTA Fillapex® (p < 0.05). The percentage of cells in the G1 and S phases decreased and that in the G2 phase increased (Fig. 2). Flow cytometry analyses revealed a significant decrease in the percentage of apoptotic and necrotic cells strongly associated with MTA Fillapex® treatment increased the percentage of cells at the initial apoptosis stage. The number of cells in the late apoptosis and necrosis stages was higher after treatment with the highest concentration (40%) of cement solution (Fig. 3).

3.3. Genotoxicity

We observed no significant increase (p < 0.05) in primary DNA damage in HGFs exposed to the cement solution (Fig. 4). The 40% MTA Fillapex® solution was not tested for genotoxicity because the percentage of viable cells at this concentration was lower than 70%, which was not recommended for this assay[22].

3.4. Gene expression

Transcriptome analysis showed a significant increase (p < 0.05; fold change > 2) in the expression of BAX, BCL2, and TP53 genes in HGFs treated with 20% MTA Fillapex® (Fig. 5). The ratio of BAX/BCL2 (pro-apoptotic and anti-apoptotic genes, respectively) was less than 1 in cells treated with 20% test sample (relative expression for BAX and BCL2, 8.384 and 11.099, respectively; BAX/BCL2 = 0.760).

4. Discussion

The clinical use of root canal sealers relies on their physicochemical and biological characteristics. Therefore, the biological properties and possible cytotoxic effects of endodontic sealers on different cells, including fibroblasts, osteoblasts, and cementoblasts, need to be thoroughly investigated. In the present study, the cytotoxicity and toxicogenomic potentials of MTA Fillapex®, an MTA/salicylate-based endodontic sealer, were investigated in HGFs, one of the key target cells. MTA Fillapex® cytotoxicity, detected by different methodologies, and the increase in the percentage of apoptotic and necrotic cells strongly indicate the ability of this compound to cause cell injury at the two highest concentrations tested (20% and 40%). Previous studies have demonstrated that freshly prepared MTA Fillapex® exhibits high cytotoxicity in human gingival, periodontal fibroblasts, and dental pulp stem cells after setting time, probably owing to its high pH and individual components (salicylate resin, diluent resin, silica, and titanium dioxide) [25–28]. In addition, a study with fibroblasts of the periodontal ligament revealed necrosis as the main cause of cell death following treatment with MTA Fillapex®, and predicted the presence of salicylate and silica as the cause of this activity [28]. Cell death due to necrosis is thought to occur owing to the involvement of organelle membranes, allowing the release of proteolytic enzymes from the lysosomes to the cytosol, triggering cell destruction and possibly causing inflammatory reactions [29,30].

Genotoxicity tests are critical for assessing the risks of potentially toxic materials that come into contact with human cells. The MTA Fillapex® endodontic sealer tested in the present study had no genotoxic effect on HGFs. Using a modified micronucleus test without cytokinesis-blocking, no mutagenicity of the cement was also detected in human fibroblast cell lines FG11 and FG15 [31]. Previously, neither regular nor white MTAs were found to be genotoxic in Chinese hamster ovary (CHO) cells or in vitro human peripheral lymphocytes obtained from 10 healthy volunteers. [32,33]. However, literature reports significant genotoxic and mutagenic activities of MTA Fillapex® to cultured cells [12,18,19,26]. Darrag & Fayyad [18] have compared the genotoxic potential of two MTA-based sealers (MTA Fillapex and MTA Plus) and a RealSeal self-etch (SE) sealer using the comet assay. The authors show that RealSeal SE followed by MTA Fillapex induced the most DNA damage in Hamster kidney fibroblast cell culture [18]. Similarly, the mutagenicity
The modulation in the expression of genes is another important genetic modification reported in cells treated with MTA Fillapex®. The sealer (20%) induced simultaneous overexpression of apoptosis-related genes BCL2, BAX, and TP53, consistent with a BAX/BCL2 ratio < 1.00. Previous studies have reported that cells with BAX/BCL2 ratios < 1 are more resistant to a given apoptotic stimulus than those with higher BAX/BCL2 ratios [34,35]. BAX and BCL2 are the major members of the B cell lymphoma 2 (BCL2) family and play a key role in promoting or inhibiting the intrinsic apoptotic pathway triggered by mitochondrial dysfunction [36]. While BAX promotes cell death through permeabilization of the mitochondrial outer membrane in response to different cellular stresses, BCL2 prevents apoptosis by inhibiting the activity of BAX [37]. Considering the overexpression of TP53 in response to cellular stress caused by MTA Fillapex®, it is important to note that the simultaneous elevation in TP53 and BAX mRNA expression may indicate the possible change in the integrity of the genomic DNA associated with malignant transformation. TP53 can directly activate BAX, and the TP53-mediated regulation in the ratio of BAX/BCL2 protein levels may influence the fate of a cell in response to stress [38,39]. Interestingly, although the cell death rate increased in the flow cytometry analysis, no changes were detected in the expression of the three genes following exposure of cells to the highest concentration (40%) of MTA Fillapex®. One possible explanation could be the activation of different molecular pathways underlying cell death. Instead of promoting or inhibiting intrinsic or BCL2-regulated apoptosis, cell death may be triggered by other molecular programs [40].

5. Conclusion

The results demonstrate the clear in vitro cytotoxic potential of MTA Fillapex® in HGFs. The toxicogenomic approach provided a more sensitive measurement, allowing identification of the ability of the sealer to modulate gene expression. Therefore, our data suggest that MTA Fillapex® should be used with caution to avoid health risks to the buccal tissue.
Fig. 5. Relative expression (RQ) of BAX, BCL2, and TP53 genes in human gingival fibroblasts treated with MTA Fillapex®. C.: negative control. *p < 0.05.

CRediT authorship contribution statement

The authors declare that they contributed equally to the conduction of the study and the writing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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