

# Cytotoxicity, Cell Adhesion, and Apoptotic Gene Expression in Periodontal Ligament Fibroblasts treated with Endodontic Sealers

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**Objective:** The present study assessed cytotoxicity, cell adhesion, and apoptotic gene expression in periodontal ligament fibroblasts (PLF) treated with 2 endodontic sealers.

**Methods:** PLF cells were obtained from nonerupted third molars and cultured. MTS and LIVE/DEAD assays were performed using different treatments and time periods. Cellular adhesion was evaluated using immunocytochemistry for integrin  $\beta 1$  and vinculin expression, and the gene expressions of nuclear factor  $\kappa B$  (NF- $\kappa B$ ), P53, and apoptotic protease-activating factor 1 (Apaf-1) were evaluated using PCR.

**Results:** Cell proliferation at 12, 24, and 48 h was statistically significant in the control and PLF groups receiving different treatments; PLF treated with culture medium containing non-hardened (NH) sealers showed a decrease in the number of cells. PLF treated with culture medium containing hardened (H) sealers also exhibited a decreased cell population. Integrin  $\beta 1$  and vinculin were expressed in both cell cultures treated with Acroseal (NH and H); however, the cell morphology changed and the cell population decreased. The gene expression of NF- $\kappa B$  and that of P53 were significantly different between the control group and the groups treated with the different sealers; mineral trioxide aggregate (MTA) (NH and H) inhibited Apaf-1, and PLF treated with Acroseal H exhibited increased Apaf-1 expression.

**Conclusion:** Both sealers showed a certain level of cytotoxicity. The gene expression of NF- $\kappa B$  and P53 in PLF treated with the sealers showed significant changes compared to that of the control group, and MTA inhibited Apaf-1. [*P R Health Sci J* 2019;38:237-243]

*Key words:* Cytobiocompatibility, Root-end filling material, Gene expression

Seal quality is essential for the clinical success of endodontic treatments (1). A widely accepted technique for endodontic filling is to use a sealant and thermoplastic core material, in conjunction (2). The use of a sealer is critical because it establishes a connection between the root dentin and core (3,4).

Sealers are designed to remain in the root canal and may come into contact with periradicular tissues; they access tissues surrounding the dental root through dentinal tubules, lateral canals, and apical foramen accessories (5). Therefore, using materials that are well tolerated by the affected tissues after endodontic therapy is critical, and cytotoxicity tests should be performed to detect the potentially harmful effects of toxic components in the oral tissues prior to their clinical use (6,7).

Apoptosis is a form of programmed cell death that is characterized by several morphological changes: those affecting the cell membrane, cell shrinkage, nuclear fragmentation, and chromatin condensation. Two pathways regulate apoptosis, the intrinsic and the extrinsic pathways. P53 regulates the intrinsic pathway, which controls apoptotic protease-activating factor 1

(Apaf-1); Apaf-1 is the main molecule of the complex known as an apoptosome (8). Resistance to apoptosis contributes to the development of adverse processes in the cell by the promotion of cell survival (9,10). The intrinsic apoptosis pathway plays an important role in the elimination of damaged cells that have suffered intracellular stress; nuclear factor  $\kappa B$  (NF- $\kappa B$ ) is a pleotropic transcription factor that inhibits apoptosis, and activated NF- $\kappa B$  translocates to the nucleus and binds to the transcription factors of apoptotic genes (11).

Acroseal exhibits the chemistry of an advanced epoxy matrix, and it contains calcium hydroxide, which creates a high pH in the root canal during final sealing (12); it is eugenol-free,

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and it is compatible with most sealers. MTA-Fillapex is an endodontic sealer based on mineral trioxide aggregate (MTA) with the advantages of an MTA product and seals the upper conduit (13). MTA is more stable than calcium hydroxide, which stability ensures a constant release of calcium ions into the tissues and the maintenance of a high pH, which produces antibacterial effects (14).

The present study assessed the cytotoxicity, cell adhesion, and apoptotic gene expression in periodontal ligament fibroblasts (PLF) treated with 2 endodontic sealers.

## Methods

The third molars used in the study were donated by patients who were seen at the Clinic of Oral and Maxillofacial Surgery. This study was approved by the Institutional Ethics Committee (CEI-FE-052-015), and the patients signed an informed consent.

### Preparation of culture medium with Acroseal and MTA-Fillapex

The biomaterials Acroseal (Septodont, Saint-Maur-des-Fossés Cedex, France) and MTA-Fillapex (Angelus, Londrina, Paraná, Brazil) were prepared according to the manufacturers' instructions. The culture medium was prepared with both sealers set in 2 ways: hardened (H), for 24 h, and non-hardened (NH). The sealers were mixed with supplemented medium (5.0 mg/mL), agitated overnight, and stored at 4°C for later use.

### Cell culture

The primary PLF cell culture and subcultures were performed as previously reported by Escobar-García et al. (15).

### Cell proliferation assay (MTS)

Cells were grown to confluence (80 to 90%) and then were detached using trypsin, and 50,000 cells were plated in 100  $\mu$ L of culture medium in 96-well microplates. The cells were incubated for 24 h at 37°C in 5% CO<sub>2</sub> and 95% humidity. Fibroblasts treated with different sealers were evaluated, as follows: i) Acroseal H (A/H) and NH (A/NH) and ii) MTA-Fillapex H (M/H) and NH (M/NH), each with its respective control. These groups were evaluated at 12, 24, and 48 h. The culture medium initially contained no sealer, to allow for the adaptation of the cells to the well for 24 h. A 100  $\mu$ L portion of the medium was extracted and the 2 sealants were added to it; the non-sealant-containing portion of culture medium that remained acted as control. A working solution was prepared at the end of the evaluation periods, immediately prior to use (CellTiter 96<sup>®</sup> Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corporation, Madison, WI, USA), and 20  $\mu$ L was added to each well, directly onto the cells, for 4 h at 37°C, 5% CO<sub>2</sub>, and 95% humidity. The assay was read on a microplate reader (Multiskan<sup>™</sup> FC, Thermo Fisher Scientific, Vantaa, Finland) at 490 nm.

### LIVE/DEAD<sup>®</sup> test

Two- or 3-day cell cultures were performed in 24-well culture boxes. The cell culture were incubated with different biomaterials (Acroseal and MTA-Fillapex, H and NH) for 24 h at 37°C in 5% CO<sub>2</sub> and 95% humidity. Cells were washed with PBS. Live cells were distinguished by the presence of intracellular esterase activity following the addition of calcein and evidenced by a fluorescent green color. Ethidium homodimer-1 enters cells with damaged membranes and binds to nucleic acids to produce a red fluorescence in dead cells. The LIVE/DEAD<sup>®</sup> working solution was prepared according to the manufacturer's directions (LIVE/DEAD<sup>®</sup> Viability/Cytotoxicity Kit, Life Technologies, Carlsbad, CA, USA). This solution (100  $\mu$ L) was added directly to the cell culture and incubated for 45 min at room temperature. The cells were washed with PBS and observed using a confocal laser-scanning microscope (CLSM) (DMI4000B; Leica Microsystems, Wetzlar, Germany). The sealers were compared with negative and positive controls.

### Immunocytochemical assay

The expression of integrin  $\beta$ 1 and that of vinculin were evaluated in the following treatment groups: a) fibroblasts treated with A/H; b) fibroblasts treated with A/NH; c) fibroblasts treated with M/H; d) fibroblasts treated with M/NH, and e) control groups at 24 h. Culture medium was added without sealer to allow for the adhesion of the cells, and the samples were incubated for 24 h at 37°C, 5% CO<sub>2</sub>, and 95% humidity. The culture medium was replaced with culture medium prepared with the sealer and cultured for 24 h. The samples were fixed with 10% neutral formalin and blocked in PBS with 1% albumin and 0.025% Tween, prior to contact with the primary antibody (integrin  $\beta$ 1 mouse monoclonal IgG 2a and vinculin mouse monoclonal IgG1). This incubation was performed overnight. The samples were washed with PBS and placed in contact with the secondary antibody (normal mouse IgG Alexa Fluor 488). Finally, the samples were observed under the CLSM.

### Gene expression

The cell cultures were incubated for 48 h with the different treatments, under the conditions described previously. The cells were detached from the culture box using 0.025% trypsin-EDTA solution and collected in a tube for subsequent RNA extraction.

### RNA isolation and cDNA synthesis

RNA was isolated by using TRI Reagent (Sigma-Aldrich BioSciences, St. Louis, MO, USA), according to Martínez-Herrera et al. (16). cDNA synthesis was performed with 1  $\mu$ g of RNA using reverse transcription long-range reverse transcriptase (QIAGEN GmbH, D-40724 Hilden, Germany). The resulting cDNA was stored at -80°C for later use.

### PCR amplification

The PCR was performed in a total volume of 25  $\mu$ L with 300 ng cDNA. The sequences of the primers and the annealing

temperatures are described in Table 1. The PCR products were subjected to electrophoresis on 1% agarose gels at 90 volts for 1 h. The agarose gels were analyzed using Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were quantified following densitometry analysis, and the relative expression of each gene was compared with the control group. GAPDH was used as a housekeeping gene.

**Table 1.** Primer sequences, T<sub>m</sub>, and product sizes of genes amplified

Gen	T <sub>m</sub> °C	Sequences	Fragment size (pb)
GAPDH	65.2	Fw 5'CCATCAATGACCCCTTCATTGACC3'	435
		Rv 5'TGGTCATGAGTCCTCCACGAT3'	
P53	59.1	Fw 5'TTGCACTTGGGACAGCCAAGT3'	491
		Rv 5'CAGGCACAACATGCACCTCAAAG3'	
Apaf-1	55.7	Fw 5' ATGAGGCTCTAGACGAAGCCATGT3'	490
		Rv 5' TGAATCAGATGAGCAGGCCTACA3'	
NF-κB	57.8	Fw 5'CCTTGCACTGGCAGTGATCACTA3'	294
		Rv 5'ACTTCTGCTCCTGAGCATTGACG3'	

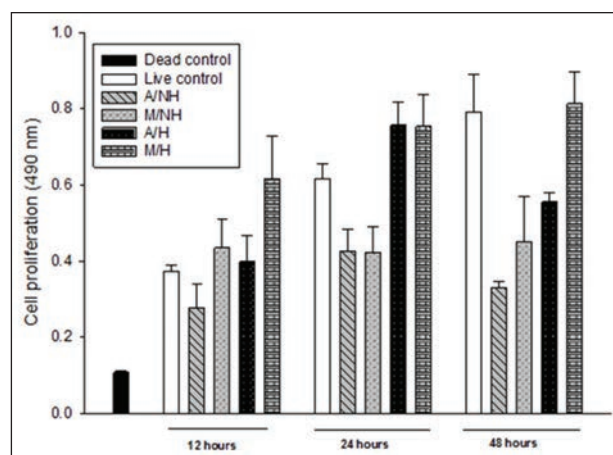
### Statistical analysis

Results are expressed as means and standard deviations. Means were compared by using one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons employing SigmaPlot ver. 11.0 (Systat Software, Inc., San Jose, CA, USA) statistical software. The significance level was set at 0.05 ( $p < 0.05$ ) in a 2-tailed test. All the tests were performed in triplicate.

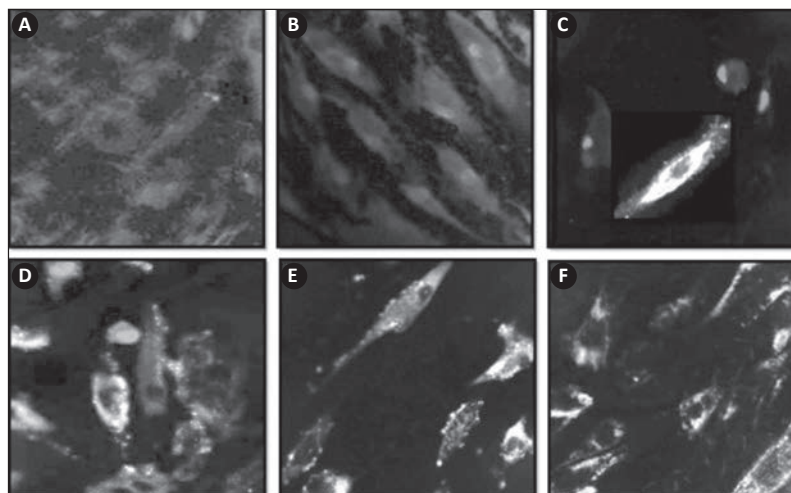
## Results

Cell proliferation at 12, 24, and 48 h was statistically significant in the control and PLF groups treated with enriched medium ( $p < 0.05$ ). In PLF treated with A/NH, a significant reduction in cell proliferation at the evaluated times was observed. Similar results were obtained in the proliferation assay of PLF treated with M/NH, but cell proliferation was higher than that of the control group at 12 h, only. PLF cultured with medium supplemented with H sealers exhibited better cell proliferation at 24 h, which revealed that A/H at 12 h had slightly higher growth than did the control cells. Cell proliferation decreased at 48 h and reached only 0.7 of the proliferation observed in the control group, while the proliferation of M/H cells exceeded the results obtained in the control group at 3 tested times (12, 24, and 48 h, with the results 1.6, 1.2, and 1.0, respectively) (Fig. 1).

Calcein produced an intense green fluorescence in living cells due to the presence of esterase activity and plasma membrane integrity (Fig. 2A). Ethidium homodimer-1 dyes inside the cell nucleus exhibited a bright red color on binding to nucleic acids in dead cells with damaged membranes (Fig. 2B). PLF treated with culture medium containing A/NH and M/NH demonstrated a decrease in cell number and mortality rate—approximately 75% and 14%, respectively (Figs. 2C and 2D). PLF treated with A/H and M/H exhibited a normal cell population, with 100% of the cells found to be alive (Figs. 2E and 2F). Some alterations in cellular morphology were observed in cells treated with the different sealers, primarily M/NH. Integrin  $\beta 1$  was expressed in focal contacts throughout the cytoplasm and cell membrane (Fig. 3A). Cells treated with A/NH exhibited a changed morphology, a decrease in cell population, and minimal integrin  $\beta 1$  expression (Fig. 3B). Integrin  $\beta 1$  (focal contacts) expression was observed in cells treated with M/NH, and no significant changes in cell morphology were observed (Fig. 3C). Cells treated with A/H exhibited a significantly decreased population, with an intact morphology (Fig. 3D). The expression of integrin  $\beta 1$  in M/H was similar to that in the control group (Fig. 3E).

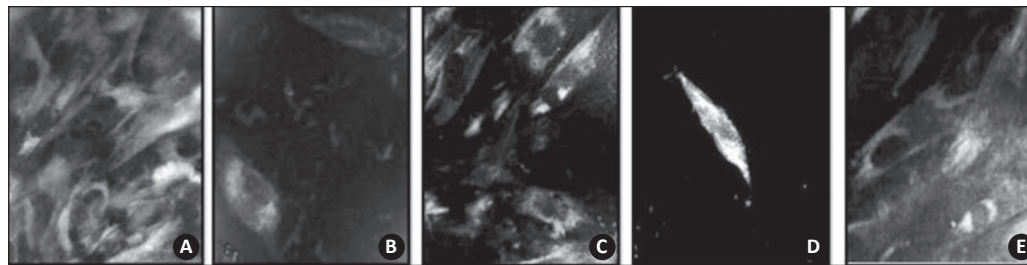


**Figure 1.** Cell proliferation.



**Figure 2.** LIFE/DEATH<sup>®</sup> assay. A) Live control, B) Dead control, C) Acroseal non-hardened, D) MTA-Fillapex non-hardened, E) Acroseal hardened, and F) MTA-Fillapex hardened.





**Figure 3.** Integrin  $\beta 1$  expression in PLF. A) Control, B) Acroseal non-hardened, C) MTA-Fillapex non-hardened, D) Acroseal hardened, and E) MTA-Fillapex hardened.

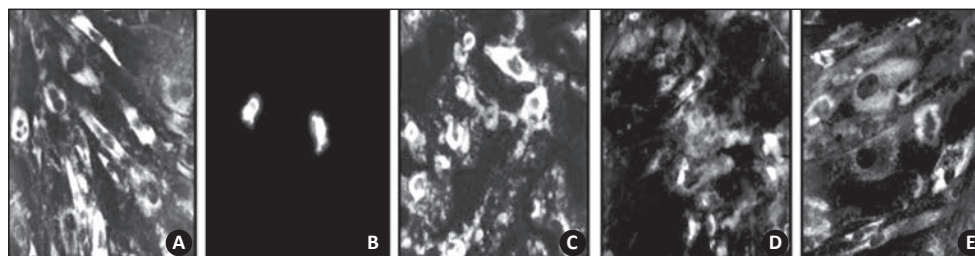
The expression of vinculin was present along the focal contacts (Fig. 4A). In PLF treated with A/NH, a change was observed in the morphology, including the presence of rounded cells and a marked decrease in cell proliferation; however, vinculin expression was observed in the focal contacts (Fig. 4B). In fibroblasts treated with M/NH, major changes in cell morphology were observed and vinculin expression was observed in the focal contacts. PLF cultured with A/H (Fig. 4C) exhibited an increased expression of vinculin and increased cell proliferation compared to cells treated with A/NH (Fig. 4D), and PLF treated with M/H exhibited cell numbers, morphology, and vinculin expression similar to those of the control group, respectively (Fig. 4E).

The expression of NF- $\kappa$ B was modified between the different treatments. A/H was expressed 1.1 times more than it was in the control (Fig. 5). M/NH produced major modifications in the expression of NF- $\kappa$ B, which was expressed 0.7 times less than it was in the control. PLF treated with M/H and A/NH exhibited decreased expression of NF- $\kappa$ B and expressed 0.9 times less than did the control (Fig. 5A). P53 gene expression was also modified in PLF with the different treatments, and a statistically significant difference was found between the control group and the different treatments ( $p < 0.05$ ). A/H expression was increased to 1.1 times that of the control. A/NH, M/H, and

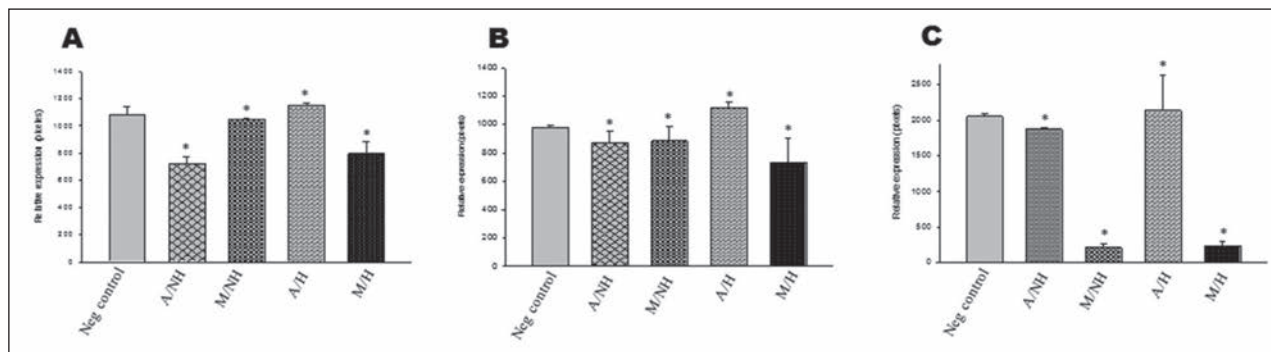
M/NH decreased the expression of this gene, which was only expressed 0.7 times that of the control for A/NH and M/H and 0.9 times that of the control for M/NH (Fig. 5B). The different treatments inhibited expression of the Apaf-1 gene. M/NH and M/H modified Apaf-1 gene expression to a level that was 0.1 times lower than that of the control group ( $p < 0.05$ ). A/NH inhibited the expression of Apaf-1 by only 8.6%, and PLF cultured with A/H exhibited increased Apaf-1 expression (4%), relative to that of the control group (Fig. 5C).

### Discussion

Although endodontic sealers are designed to remain in the root canal, they may be extruded into contact with periapical tissues; if such materials are not biocompatible, then their eluates or degradation products may damage cells and periapical tissues or interfere with wound repair (17–19). The present study evaluated the biocompatibility of 2 sealers via a technique that simultaneously permitted the evaluation of cell viability/cytotoxicity in vivo (20). The possible biological mechanisms involved in these processes were also evaluated (21). PLF were selected because these cells are the main constituents of connective tissue and are the predominant cell type in the periodontal ligament (22). In the clinical environment, fresh



**Figure 4.** Vinculin expression in PLF. A) Control, B) Acroseal non-hardened, C) MTA-Fillapex non-hardened, D) Acroseal hardened, and E) MTA-Fillapex hardened.



**Figure 5.** Relative expression. A) NF- $\kappa$ B relative expression, B) P53 relative expression, C) Apaf-1 relative expression.

sealers are set in place, and the setting reaction is completed after placement. Sealers release soluble components that may be dissolved by tissue fluids, which allows for the assessment of a possible dose–response effect similar to the effects that occur *in vivo* (23). Studies show that the biocompatibility of different sealers varies when these substances are fresh and that the effects of these variations are reduced over time (24–27).

Sealers based on calcium hydroxide are touted for their ability to form hard tissue, but this tissue dissolves over time and compromises the endodontic sealing (27). Acroseal is a combination of calcium hydroxide and epoxy resin, and the manufacturer states that it exhibits lower solubility than other sealers do (28). MTA-Fillapex in a hardened state exhibited the highest rates of cell proliferation, which remained constant during the evaluation periods. NH cell proliferation in this state remained constant at 24 and 48 h. Calcium silicate is one of the main components of this sealer, and it reacts with water to form a calcium silicate hydrate gel, which contributes to biocompatibility. However, these results are not consistent with those of some studies, which reported that this sealer exhibited high levels of cytotoxicity (6,29,30). The Acroseal sealer exhibited higher levels of cell proliferation compared to those of the positive control at 24 h in the A/H and A/NH, and a statistically significant difference was found compared to the positive control at 12 and 48 h in the uncured settings at 24 and 48 h. These results are consistent with those of a study performed under similar conditions (31).

Cell adhesion takes place through receptors that bind specific adhesion integrins to matrix proteins; these interactions are essential to numerous processes, such as differentiation and wound repair. The lack of an appropriate matrix or the separation of anchor-dependent cells from the substrate induces a special form of apoptosis denominated “anoikis” (32), which plays a major role in the adhesion of fibroblasts, and integrin  $\beta 1$  ensures fibronectin binding (33). Vinculin plays an important role in regulating aspects of assembly, rotation, focal adhesion, and the transmission of forces by these cell structures (34). In this study, integrin  $\beta 1$  and vinculin expressions were observed in PLF treated with MTA-Fillapex and A/H and A/NH, and significant alterations in morphology and decreased cell populations were observed in samples treated with Acroseal. Samples treated with MTA-Fillapex expressed integrin  $\beta 1$  on the periphery of the cell membrane and expressed vinculin in the cytoplasm.

To understand the molecular mechanisms involved in the cytotoxicities of the 2 sealers, a semiquantitative analysis of gene expression was performed using PCR. NF- $\kappa$ B is a transcriptional regulator that is activated by different stimuli; inappropriate NF- $\kappa$ B activation is associated with inflammatory diseases, and persistent its inhibition results in retarded cell growth (35). NF- $\kappa$ B expression was slightly increased in cells treated with A/H, which suggests a response to the cytotoxic components present in this biomaterial. The cell group treated with MTA-Fillapex demonstrated a slight inhibition of expression, which may not be favorable because the cells were not responding to stimuli

caused by treatment, and decreased NF- $\kappa$ B expression prevents the expression of genes that lead to the repair or proliferation of the activated cell. The P53 gene encodes a protein that responds to cellular stress and regulates target gene expression that induces cell-cycle arrest, apoptosis, senescence, DNA repair, and changes in metabolism (36). The present results showed a decrease in the expression of this gene in cells treated with A/H, M/NH, and M/H, because the induction of high levels of the expression of P53 leads to apoptosis, and decreased P53 expression promotes cell-cycle arrest, which may be beneficial. However, the inhibition of this gene is related to cell mutations (37). Apaf-1 is a gene that encodes a cytoplasmic protein that initiates apoptosis. Cells treated with A/H exhibited behaviors similar to those of the control group, which suggests that cells treated with this biomaterial appropriately performed the cell cycle. Cells treated with MTA-Fillapex showed inhibition of the Apaf-1 gene compared to what occurred with the control group, and apoptosis is necessary for the regeneration of the cell population. Therefore, these changes may produce genetic damage that prevents the cell from performing the process of apoptosis (38); however, further studies are needed to corroborate this hypothesis. Similarly, the expression of NF- $\kappa$ B and P53 in PLF exposed to the different treatments showed a discrete but significant change; however, the expression of Apaf-1 in the PLF was markedly inhibited in cells treated with M/NH and M/H, which suggests that MTA-Fillapex did not allow the recruitment of this gene to form the apoptotic body along with other molecules.

Materials in the clinical setting are introduced into the root canal immediately after mixing. However, these materials may exert toxic effects after setting via the release of harmful components (39). Biocompatibility should be an important factor that influences the choice of sealer for endodontic treatment. The toxic potential of materials was evaluated during hardening and after setting, as it has been demonstrated that both of the evaluated sealers may produce harmful effects and induce cellular apoptosis and necrosis (40). All test models using set sealer samples have some limitations because the surrounding tissue will be exposed to unset material *in vivo*. Furthermore, it was shown that sealers were significantly more toxic immediately after mixing than in their set forms (41).

## Conclusions

Both sealers showed a certain level of cytotoxicity. The gene expression of NF- $\kappa$ B and P53 in PLF with sealers exhibited significant changes compared to that of the control group, and MTA inhibited Apaf-1.

## Resumen

Objetivo: El presente estudio evaluó la citotoxicidad, la adhesión celular y la expresión de genes apoptóticos en fibroblastos de ligamento periodontal (FLP) tratados con dos

selladores endodónticos. Métodos: FLP se obtuvieron a partir de terceros molares no erupcionados y se cultivaron, ensayos de MTS y LIVE/DEAD se llevaron a cabo con diferentes tratamientos en diferentes períodos de tiempo. La adhesión celular se evaluó por inmunocitoquímica con la expresión de integrina  $\beta 1$  y vinculina, y la expresión génica de NF- $\kappa$ -B, P53 y Apaf-1 (por sus siglas en inglés) mediante PCR. Resultados: La proliferación celular a 12, 24 y 48 h fue estadísticamente significativa entre el grupo control y los FLP tratados; los FLP tratados con medio de cultivo con selladores no endurecidos (NH, por sus siglas en inglés) mostraron una disminución en el número de células. Los FLP tratados con medio de cultivo y selladores endurecidos (H, por su sigla en inglés) exhibieron una disminución de la población celular. Integrina  $\beta 1$  y vinculina se expresaron en cultivos celulares tratados con Acroseal (NH y H) con cambios en la morfología y disminución de la población celular. La expresión génica de NF- $\kappa$ -B y P53 tuvo diferencias estadísticamente significativas entre el control y los grupos tratados; la expresión de Apaf-1 disminuyó por MTA (NH y H) y en los FLP tratados con Acroseal H aumentó la expresión de Apaf-1. Conclusiones: Ambos selladores mostraron un cierto nivel de citotoxicidad. La expresión génica de NF- $\kappa$ -B y la P53 de los FLP tratados con los selladores mostraron cambios significativos en relación con el control, Apaf-1 fue inhibido por MTA.

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