Cytotoxicity Evaluation of Root Canal Sealers Using an *In Vitro* Experimental Model with Roots

[1]Dental School, UANERP - Universidade de Ribeirão Preto, Ribeirão Preto, SP, Brazil
[2]Araraquara Dental School, UNESP - Universidade Estadual Paulista, Araraquara, SP, Brazil
[3]Presidente Prudente School of Dentistry, UNOESTE - Universidade do Oeste Paulista, Presidente Prudente, SP, Brazil

Ligia Teixeira[1-3], Fernanda Gonçalves Basso[1], Josimeri Hebling[2], Carlos Alberto de Souza Costa[2], Graziela Garrido Mori[3], Yara Teresinha Corrêa Silva-Sousa[1], Camila Fávero de Oliveira[1]

Correspondence: Profa. Dra. Camila Fávero de Oliveira, Avenida Costábile Romano, 2201, 14096-900, Ribeirão Preto, SP, Brasil. Tel: +55-16-98119-8746. e-mail: camilafaveroo@gmail.com

The aim of the present study was to evaluate the cytotoxicity of root canal sealers under conditions closely resembling a clinical reality. A primary human fibroblast cell line was seeded in 24-well acrylic plates with Dulbecco's modified Eagle's medium supplemented with 10% serum fetal bovine (SFB) and incubated for 24 h. Root canals from premolars were filled and individually attached to nylon devices to be stabilized in the wells with the already seeded cells. Specimens were divided into groups as follows: Control: gutta-percha cones (GPC); AH Plus+GPC; Sealapex+GPC; MTA Fillapex+GPC and Endofill+GPC. After 24 and 48 h, cell viability and morphology were evaluated by MTT assay and scanning electron microscopy (SEM), respectively. Statistical analysis was performed by Mann-Whitney test, complemented by Kruskal Wallis test (p<0.05). Only Endofill presented cytotoxicity after 24 h. MTA Fillapex and Endofill reduced the production of succinic desidrogenase after 48 h. AH Plus was non-toxic at any time point. SEM showed that the AH Plus and MTA Fillapex groups presented fibroblasts with morphology close to the control group, while the Endofill group presented few cells with thin extensions cells. The present study showed that good results were present in AH Plus and Sealapex, but not the Endofill group after 48 h. The method used enabled evaluation of the cytotoxicity of the studied sealers that diffused through the root apex.

Key Words: endodontics, root canal filling, cytotoxicity.

Introduction

The aim of root canal filling is to promote the hermetic sealing of root canals, neutralize the remaining microbiota and stimulate mineralization of the pulp stump without causing toxic effects on the cells of the periapical region (1). Therefore, the biological properties of root canal sealers and the degree of cytotoxicity or stimulation they may have on different cells, including fibroblasts, osteoblasts and cementoblasts must be evaluated (2,3).

Studies have evaluated the biological effects of sealers based on zinc-oxide-eugenol (ZOE), glass ionomer, epoxy resin, silicones, calcium silicate, methacrylate resin and sealers containing calcium hydroxide in their composition (1). The *in vitro* cytotoxicity of different types of sealers has been evaluated by direct exposure of different cell lines to sealers or their components solubilized in liquid media (4-6).

Studies published in the literature report variations regarding the tested sealers, cell type, time of cell exposure to extracts, extract dilutions and specimen sizes. Most researchers follow the International Organization for Standardization (ISO) recommendations to assess the effects of biocompatibility and cytotoxicity of the endodontic cements (4-7). Camps et al. (8) challenged such

methodologies because the ISO standardization advocates that the area of sealer exposure to the culture media should be 0.5 to 6.0 cm² of sealer per 1 mL of culture medium for such tests, which does not present clinical significance because this is not the actual apical foramen diameter area of exposure. According to those authors, the results of current ISO tests exceed the actual toxicity of sealers relative to clinical reality. Thus, the methods used for this purpose, which are still recommended by the ISO, apparently overestimate the negative effects of the tested materials (9).

The literature presents few studies that evaluate the cytotoxic effect of root canal sealers in an *in vivo* situation and also in a reproducible model that comes close to the real response to the area of exposure of the apical foramen diameter without using animal models (6,8-10). The present study aimed to evaluate the cytotoxicity of the root canal sealers AH Plus, Sealapex, MTA Fillapex and Endofill using a new methodological approach in fibroblasts culture in which the clinical situation is simulated.

Material and Methods

One hundred and twenty single-rooted mandibular

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premolars were instrumented using the ProTaper rotary system (Dentsply Maillefer Instruments S.A., Ballaigues, Switzerland). This procedure was performed with intermittent irrigation with 2 mL 2.5% NaOCl solution (Rio Química Indústria Farmacêutica, Ltda, São José do Rio Preto, SP, Brazil) between each file up to the finishing file F4. After instrumentation, the apical foramen diameter of all specimens was standardized using a manual #30 K-file (Dentsply Maillefer). The teeth were cross-sectioned at the cementoenamel junction using a diamond blade (11-4254, 4"x 0,012"/ series 15LC, Diamond Wafering blade; Buehler Ltd., Lake Bluf, IL, USA) coupled to a serial-sectioning device (ISOMET 1000, Buehler). The root lengths were standardized at 18 mm. The root canals were initially filled with a 17% ethylenediaminetetraacetic acid (EDTA) solution (Biodinâmica, Ibiporã, PR, Brazil), washed with phosphate-buffered saline (PBS, pH 7.4) and dried using sterile absorbent paper cones (Dentsply-Herpo, Petrópolis, RJ, Brazil).

The roots were individually adjusted to the circular nylon devices (5.53 mm high and 17.85 mm total diameter) developed for this study in order to maintain the filled roots in partial contact with the culture medium at a distance of 1 mm from the cultured cells. The sets of roots/devices were individually sterilized in ethylene oxide (Acecil Central de Esterilização Comércio e Indústria Ltda, Campinas, SP, Brazil) (Fig. 1).

The roots of the sterile sets were divided in groups according to the sealing materials (n=12): Control Group: only gutta-percha cone (Dentsply Ltda, Petrópolis, RJ,

Brazil); Group AH Plus sealer (Dentsply DeTrey, Konstanz, Germany) and gutta-percha cones; Group Sealapex sealer (Kerr, Romulus, MI, USA) and gutta-percha cones; Group MTA Fillapex sealer (Angelus, Curitiba, PR, Brazil) and gutta-percha cones; Group Endofill sealer (Dentsply Ltda) and gutta-percha cones.

A single, previously calibrated operator, under aseptic conditions and under a laminar flow, performed all the procedures from filling the root to the cell analysis. The filled roots/devices sets were individually placed on top of each well so that the root apex would remain immersed in DMEM at a standardized distance from the bottom of the well where the cultured fibroblasts were attached.

Cell Culture

A primary culture of human gingival fibroblasts was cultured and re-cultured until reaching a sufficient number of cells to perform the experiment. This cell line was selected because in a real situation the products contact this type of cell. Subsequently the fibroblasts (30,000 cells/well) were cultured in sterile 24-well acrylic plates. Dulbecco's modified Eagle's medium (DMEM; Sigma- Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (SFB Culti-Lab, Campinas, SP, Brazil) was used for cell culturing. Cells were incubated (Isotemp Fisher Scientific, Pittsburgh, PA, USA) for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell Viability Analysis (MTT Assay)

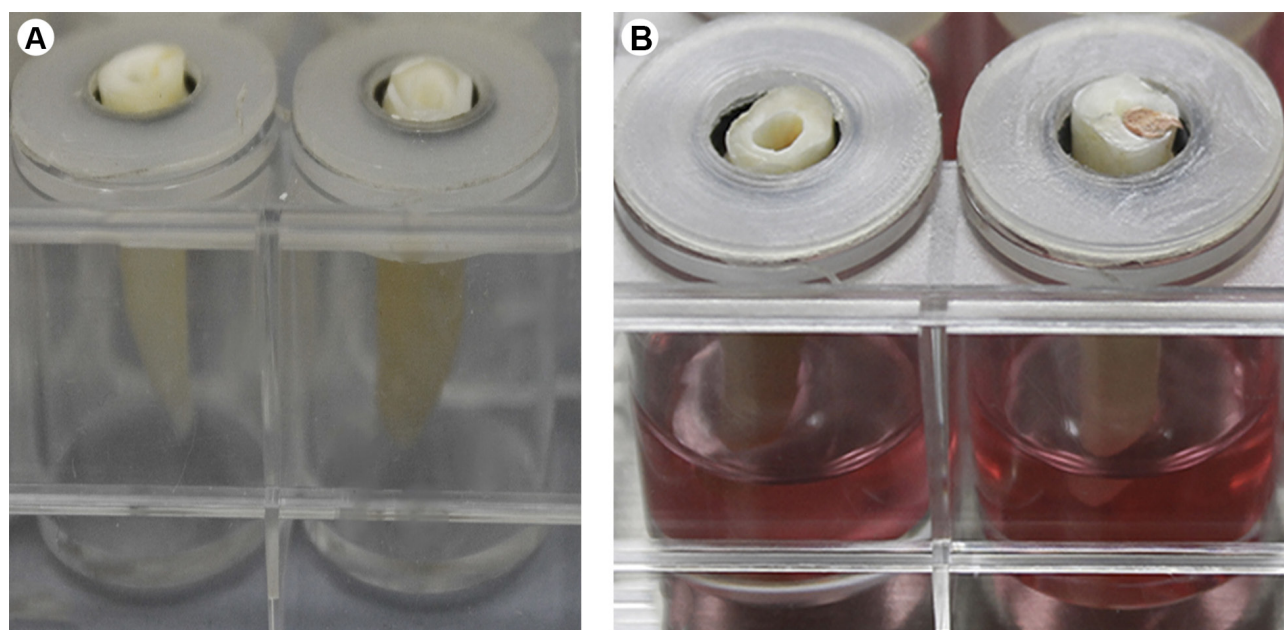Eight specimens from each experimental and control

Figure 1. The device specially designed for this experiment. A: Circular nylon devices with the root. B: Filled roots in partial contact with the culture medium at a standardized distance (1 mm) from the cultured cells in the 24-well plates.

groups were used for analysis of fibroblast cell metabolism after 24 and 48 h contact of the filled roots with the culture medium. Cell metabolic activity was evaluated by succinic dehydrogenase (SDH) production, which is a measure of the mitochondrial activity of the cells. The methyltetrazolium (MTT) assay was used for this purpose.

The devices with the filled roots were carefully removed from the top of the wells, and the culture medium was aspirated and replaced by 900 µL of culture medium (DMEM) solution without SFB and 100 µL MTT solution (5 mg/mL sterile PBS). The cells were incubated at 37 ℃ for 4 h. This step is important because the absence of SFB allows to analyze the cell metabolism at this moment and it has no influence on the cell proliferation during this 4 h period. Thereafter, the culture medium (DMEM) with the MTT solution were aspirated and replaced by 700 µL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the violet formazan crystals resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. Three 100 µL aliquots of each well were transferred to a 96-well plate (Costar Corp., Cambridge, MA, USA). Cell viability was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Multiskan, Ascent 354; Labsystems CE, Les Ulis, France). The values obtained from the three aliquots were averaged to provide a single value and the inhibitory effect of the different groups on cell mitochondrial activity was calculated and expressed as median.

Statistical Analysis

The absorbance was expressed in numerical values recorded and statistically analyzed to determine the effect of the mitochondrial cell activity. A test was performed to assess the degree of normality of the sample and the Kruskal-Wallis test complemented by the Mann-Whitney test for pairwise comparisons were applied to all collected data. A significance level of 5% was set for all analyses.

Cell Morphology Analysis Under Scanning Electron Microscopy (SEM)

Four specimens of each experimental and control group were used to analyze the cell morphology by SEM at 24 and 48 h after the contact of the filled roots with the culture medium. The roots were carefully removed from the wells of 24-well plates. Then, the fibroblast cells that remained attached to the glass coverslips placed at the bottom of each well before preparing the cells, were fixed in 1 mL of buffered 2.5% glutaraldehyde for 24 h and post-fixed with 1% osmium tetroxide for 1 h.

The cells were dehydrated in a series of increasing ethanol concentrations (30, 50, 70, 95 and 100%) and immersed in 1,1,1,3,3,3-hexamethyldisilazane (HMDS; Acros Organics, Springfield, NJ, USA) for 90 min and maintained in a desiccator for 24 h. The glass discs with the fibroblast surface upward were mounted on metallic stubs, sputter-coated with gold and the morphology of the surface-adhered fibroblast cells was examined with a scanning electron microscope (JMS-T33A; JEOL Ltd, Tokyo, Japan).

Results

Statistically significant (p<0.05) differences between the analyzed periods were identified in all the groups. The 48 h period resulted in a lower cell metabolism compared with the 24 h period (Table 1 – rows). Statistically significant differences (p<0.05) were also observed when the evaluation focused on the different sealers at the same assessment period. The control, AH Plus, Sealapex and MTA Fillapex groups were not different from each other (p>0.05) at the 24 h time point. However, the Endofill group showed reduced SDH production (p<0.05; Table I - column).

AH Plus and Sealapex were statistically similar to the control (p>0.05) at the 48 h time point. However, they showed a higher expression of SDH than the other groups (p<0.05). The MTA Fillapex group was statistically similar to the control group. However, the MTA Fillapex group expressed lower levels of SDH than the other two sealers mentioned above, although the SDH expression in this group was higher than in the Endofill group. The group with the most reduced expression of this enzyme at that time point was the Endofill group (Table 1 – column).

The SEM evaluation (Figs. 2 and 3) showed a normal morphology of the control group after both periods. The fibroblasts presented normal extensions, some of them thin and short with a higher cytoplasm and they covered all the

*In vitro experimental model for root canal sealers*

Table 1. Succinic dehydrogenase (SDH) enzyme production by the fibroblasts, detected by the MTT assay, after 24 and 48 h contact with the dissolved sealers of filled roots

Root canal sealers	Time	
	24 h	48 h
Control (gutta-percha)	0.451 (0.430 – 0.566) [a,A]	0.327 (0.305 – 0.387) [b,AB]
AH Plus	0.523 (0.480 – 0.619) [a,A]	0.378 (0.306 – 0.402) [b,A]
Sealapex	0.517 (0.496 – 0.566) [a,A]	0.354 (0.319 – 0.373) [b,A]
MTA Fillapex	0.436 (0.331 – 0.524) [a,A]	0.279 (0.253 – 0.318) [b,B]
Endofill	0.209 (0.135 – 0.335) [a,B]	0.020 (0.008 – 0.032) [b,C]

a,A Values are medians (percentile 25-percentile 75), n=8. Groups identified by same lowercase letters in rows and uppercase letters in columns do not differ significantly (Mann-Whitney, p>0.05).

glass. For AH Plus the glass was similar to the control with a homogenous distribution and the morphology was close to

the same group. Some mitosis was noticed for both periods. The Sealapex showed similar aspects after 24 h, however
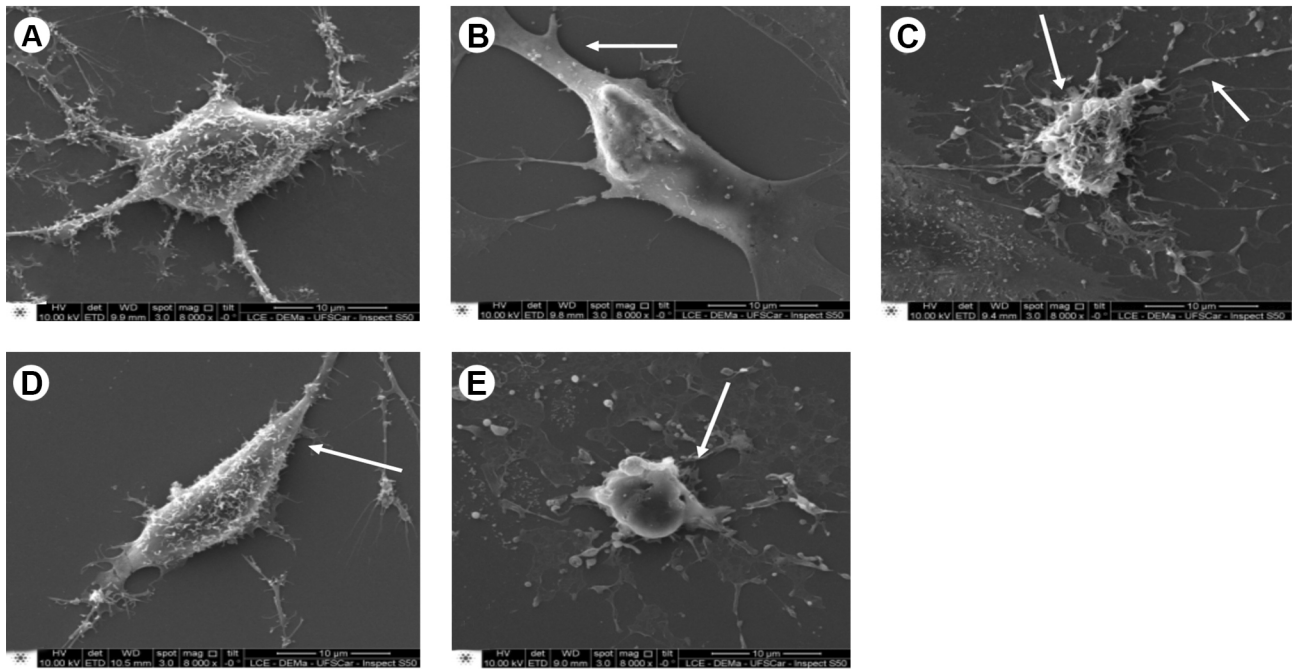
Figure 2. Micrographs recorded after 48 h for each specimens (8000×). A (Control group): fibroblasts organized in large number on the glass surface. B (AH Plus group): the cells were distributed homogeneously on the glass substrate. C (Sealapex group): a large group of high-density fibroblasts with normal morphology was also present. D (MTA Fillapex group): note the presence of cell-free areas at both periods, cells with rounder morphology. E (Endofill group): fewer cells covered the glass substrate.
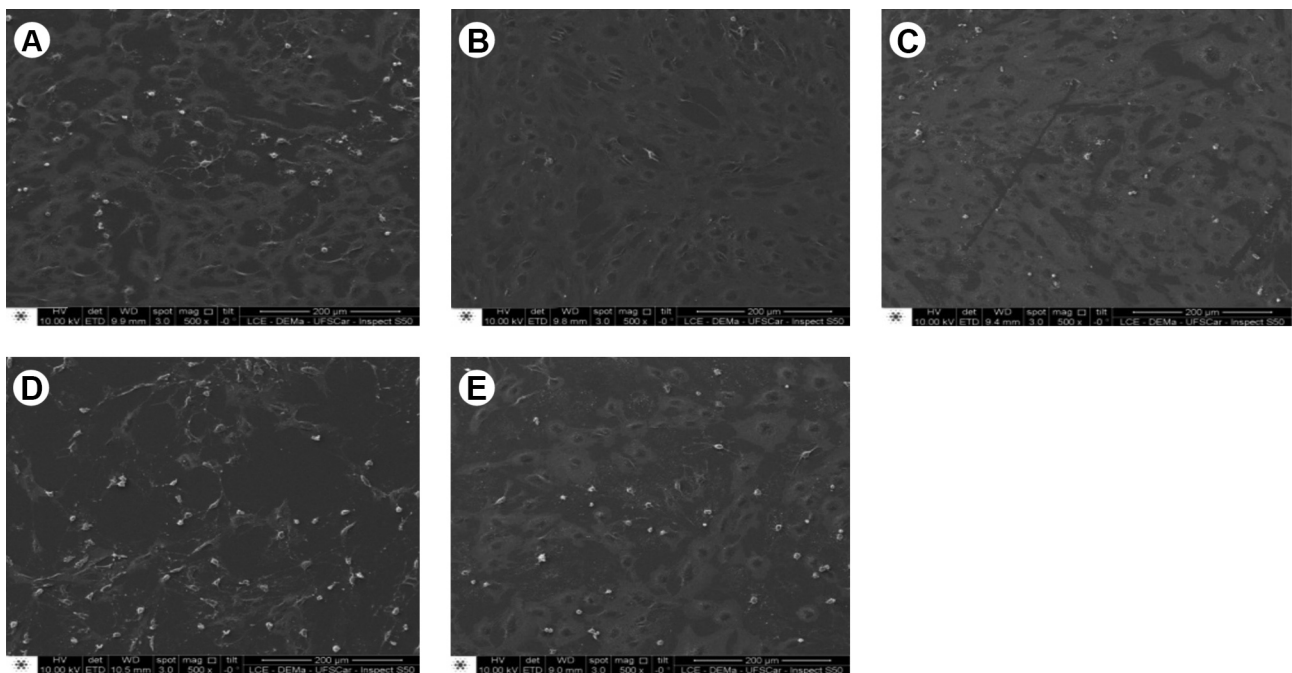

Figure 3. Micrographs recorded after 48 h for each specimens (500×) A (Control group): fibroblasts with discrete fusiform morphology adhered to the glass substrate. B (AH Plus group): cells had large cytoplasm volumes and numerous processes originating from their membranes. C (Sealapex group): cells with large and irregular cytoplasmic membrane volumes from which numerous cytoplasmic processes originated. D (MTA Fillapex group): cells were fragmented, narrower and had fewer processes. E (Endofill group): cells show altered morphology with a rounder pattern.

for 48 h the cells were circular, with irregular extensions and reduced cytoplasm. MTA Fillapex presented a glass covered with fibroblast close to the control group, with normal morphology, but at 48 h some cells presented broken and narrow extensions. The Endofill was the worst group because the glass presented some cell-free areas and the ones that still stuck were rounded and with morphological changes like contraction of the cytoplasm.

Discussion

An injury to the periapical cells may occur during the root canal filling step by leakage of the filling material through the foramen or by diffusion of the filling material (11) through the apical foramen, accessory/lateral canals and dentinal tubules. Therefore, filling material components diffusion may trigger local toxic effects to cells. However, they seem to be less intense than those observed in laboratorial studies in which cultured cells are exposed to the materials, following protocols recommended by the ISO standards (6,8,10,12). Different studies (8-10) evaluated the cytotoxicity of root canal sealers using a method they termed "root dipping". In this test, root canals filled with different sealers were immersed in culture medium to obtain the "extracts", which were applied on L929 fibroblasts in culture. Exacerbation of the results was observed by the authors when comparing their results with those obtained according to the ISO standards.

On the other hand, the present study evaluated not the direct action of the cytotoxic effect from root canal sealers on the medium, but filled roots in partial contact with the culture medium at a standardized distance (1 mm). This methodology is closer to the clinical situations, because it does not use just the biomaterial in contact with the cells, but also the properties of the sealers, like diffusion, degradation and flow during the reaction period. The present study showed that good results were presented by AH Plus and Sealapex, followed by MTA Fillapex and then the Endofill groups after 48 h.

The results of this study show that there were no statistically significant differences between the sealer AH Plus and the control group, in the model tested at the 24 and 48 h time points. The SEM analyses agree with this information because the cell morphology was very close to the control group. Similar data were previously reported by Al-Hiyasat et al. (7) and Karapinar-Kazandag et al. (6), who evaluated the same sealer, albeit the specimens with sealer were immersed directly in culture medium. However, other authors who observed toxicity with this sealer in the early setting period (13-17) evaluated the effect of the products released by the specimens directly into the culture medium, without its diffusing effect. This characteristic was ascribed to the release of formaldehyde and the degradation of its

amines in the formulation to accelerate the epoxy sealer polymerization (6,14-18). However, Bin et al. (14) claimed that the AH Plus manufacturer reports that the new formulations no longer release formaldehyde, which may explain the low toxic effects observed in the present study.

The Sealapex was not significantly different from the control group in the present study after 24 and 48 h. These results corroborate previous findings (4,16,19,20). According to Schafer et al. (21) and Desai et al.(22), Sealapex shows significant mass loss when subjected to the solubility tests recommended by ISO 6876 (2001), with high solubility in tissue fluids; this high solubility suggests increased solute degradation in the culture medium. On the other hand, Sealapex shows good responses of tissue repair after its use (2,20,22). Its action may be attributed to the dissolution of calcium and hydroxyl ions, which increase the pH of the periapical region, favoring repair, exerting antimicrobial effects, promoting the degradation and inactivation of lipopolysaccharides of the bacterial plasma membrane (17). Besides, it induces biological sealing with calcified tissue deposition in the apical foramen and associated with favorable repair processes, it induces the formation of mineralized tissue and acts to control inflammatory root resorption (2,19,20,22,23). However, some morphological changes are noticeable just in some cells after 48 h, but this is discreet, like the loss of some extensions and also a more circular morphology.

In the present study, the MTA Fillapex showed no statistically significant difference with the control group after 24 h. These low toxic effects of MTA Fillapex may be explained by the fact that MTA cements inactivate enzymes of the cell plasma membranes, induce formation of mineralized tissue, react with fibronectin expressed in tissues and stimulate alkaline phosphatase expression by the treated cells (14,20). Therefore, all these positive biological properties of MTA cements may determine the excellent performance of MTA Fillapex when applied to contact with connective tissues. Nevertheless, after 48 h, MTA Fillapex differs from AH Plus and Sealapex, showing an SDH production decrease. As it was corroborated by the SEM results in the same period, it is possible to notice some free areas or some cells with circular morphology. Probably, this effect is related to the addition of components to MTA Fillapex, such as resin composites like the salicylate (14) or other pigments, which may have increased the toxic effect, because in contact with cells, these soluble chemicals may lead to cell modification (24).

The Endofill was the sealer with lowest expression of the SDH enzyme in the present study, both in the 24 and 48 h periods. These results may also be associated with its high solubility and therefore with its increased degradation of products in the culture medium. Schafer et al. (21)

reported that ZOE-based sealers are associated with a loss of mass in aqueous media, that is, they have high solubility. Regarding the morphological changes of fibroblasts in the present study, the Endofill showed round-shaped fibroblasts with few processes and large acellular areas. Previous studies attributed the toxicity of ZOE-based cements to the eugenol and zinc ions released by the material in a wet environment (9,25). Therefore, one may suggest that the zinc and eugenol components released in the culture medium from the highly soluble cement Endofill may be responsible for higher cytotoxicity caused by this sealer to the cultured cells when compared to the other cements tested in the present investigation. The more intense toxic effect caused by Endofill was also confirmed by the evaluation of the morphology of the cells exposed to this sealer. To the other sealers, the cell's morphology was close to the control groups and no significant differences were observed in the analysis, corroborating the MTT results.

The methodology using instrumented and filled roots individually adjusted to the circular nylon devices and immersed at a standardized distance in culture medium with cells, was effective to evaluate the toxic effects of root canal sealers on the cellular metabolism of a fibroblast cell line. This methodology could also be used to new sealers and also to test the irrigant protocols.

By the proposed methodology it was possible to notice that AH Plus and Sealapex were similar to the control group in both evaluation periods; MTA Fillapex differed discretely from the control just after 48 h and the Endofill was the worst group in both periods, presenting some important modifications in the fibroblast cells. So it is possible to assume that this methodology brings results closer to the clinical situations, but this *in vitro* study should not be directly extrapolated, but it may be also used to test other materials and techniques.

Resumo

O objetivo do presente estudo foi avaliar a citotoxicidade dos cimentos dos canais radiculares em condições próximas à realidade clinica. Uma linhagem primária de fibroblastos humanos foi semeada em placas acrílicas de 24-poços com meio de cultura Dulbecco's modified Eagle's medium suplementado com 10% de soro fetal bovino e incubados por 24 h. Os canais radiculares de pré-molares foram obturados e individualmente adaptados aos dispositivos de nylon para serem estabilizados nos poços com as células já semeadas. Amostras foram dividas de acordo com os grupos: Controle: cones de gutta-percha (CGP); AH Plus+CGP; Sealapex+CGP; MTA Fillapex+CGP e Endofill+CGP. Após 24 e 48 h, a viabilidade e a morfologia celular foram avaliadas pelo ensaio de MTT e microscopia eletrônica de varredura (MEV), respectivamente. Análises estatísticas foram realizadas pelo teste de Mann-Whitney, complementadas por Kruskal Wallis (p<0,05). Apenas o Endofill apresentou citotoxicidade após 24 h. MTA Fillapex e Endofill reduziram a produção da enzima desidrogenase succinica após 48 h. AH Plus não apresentou toxicidade em nenhum momento. MEV mostrou que os grupos AH Plus e o MTA Fillapex apresentaram fibroblastos com morfologia próxima ao grupo controle, enquanto que o grupo do Endofill apresentou poucas células com finos prolongamentos. O presente estudo demonstrou que resultados satisfatórios foram apresentados nos grupos AH Plus e Sealapex, mas não para o Endofill após 48 h. O método utilizado permitiu avaliar a citotoxicidade dos cimentos que se difundem pelo ápice radicular.

Acknowledgements

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