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Cytotoxicity and Genotoxicity Analysis of two Endodontic cements in Human Fibroblast Culture *in Vitro*

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Abstract*— *The present in vitro study aims to evaluate cytotoxic and genotoxic potential of MTA Fillapex endodontic sealer and to compare it with AH Plus sealer. It was used human fibroblast cell lines FG11 and FG15 for this study. Cytotoxicity and genotoxicity was analysed in human gingival fibroblast submitted to growth condition with MTT test conditioned cells, respectively. Cells cultivated in DMEM means was used as command. Celular viability was mensured in 24, 48 and 72h. Results was analysed by the software Biostat 4.0 Shapiro-Wilk normality test was made but sample presented non-normal behavior. Descriptive analysis was made and its results was submitted to Kruskal-Wallis test (Dunn). All sealers and control groups presented MTT values lower in 24h period than 48 and 72h ($p<0.05$). The biggest cell viability was observed in AH Plus sealer and in control group related to MTA Fillapex in all experimental periods ($p\leq0.0002$). In terms of genotoxicity, the biggest value was mensuared in AH Plus sealer in the 24h period with significantly difference compared to MTA Fillapex and the control group ($p=0.0004$). It may be concluded that MTA Fillapex sealer showed higher cell cytotoxicity than two control groups and AH Plus sealer presented higher genotoxicity than other groups.

Keywords*— *Cytotoxicity, Genotoxocity, Fibroblasts.

I. INTRODUCTION

Seeking to seal the root canals of dental elements affected by some pathological condition of endodontic origin, the dental surgeon is the professional responsible for performing instrumentation protocols, disinfection by chemical processes and filling with appropriate materials. Of these, gutta-percha stands out as a medium that serves as the nucleus for filling the conduit (DONNERMEYER et al., 2018; ELYASSI; MOINZADEH; KLEVERLAAN, 2019).

However, it is significant to indicate that the establishment of optimal contact between the dentin wall and this central filling material should be achieved by using a low solubility sealing material (DONNERMEYER et al., 2018; ELYASSI; MOINZADEH; KLEVERLAAN, 2019). This compound should favor bacterial sealing and improve the resistance against mismatch of the entire three-dimensional obturation complex (DONNERMEYER et al., 2018).

Significant examples of these sealers are epoxy resin-based materials and associated amines in different pastes (SAYGILI et al., 2017). Of these, AH Plus cement (Dentsply, Konstanz, Germany) is the most investigated by several scientific methodologies, proving its effectiveness in dental sealing, besides having a positively influenced by other endodontic substances, such as EDTA and NaOCl. (DONNERMEYER et al., 2018).

Another standard with endodontic applications is Mineral Trioxide Aggregate (MTA), which is based on Portland cement associated with hydrophilic particles of elements such as calcium, silicon and bismuth oxide, making them suitable for dental use from their association. in aqueous vehicles (MOON et al., 2018). It is still known that MTA has good endodontic therapy properties, since it stimulates osteogenic and angiogenic cells (ALI et al., 2019). This material is commercially available through products such as ProRoot MTA (Dentsply Tulsa Dental, Tulsa, USA) and MTA Fillapex (Angelus, Londrina, PR, Brazil).

With this range of materials that assist in the root canal system obturation process, it is significant to indicate that, in addition to their excellent physical and chemical properties, they must have excellent biocompatibility as they remain in contact with periapical tissues for a long period of time. In this regard, it is known that the toxicity of the material could lead to a local inflammatory response, preventing periapical healing by inhibiting cell respiration

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metabolism, fibroblast proliferation and reducing the activity of the alkaline phosphatase enzyme involved in bone tissue neoformation. (SZCZURKO et al., 2017).

Therefore, with the relevance of endodontic cements associated with their risks to the quality of root canal sealing therapy, in view of their possible toxicity, this study aims to evaluate the cytotoxic and genotoxic potential of MTA Fillapex and AH Plus.

II. METHODOLOGY

This study was approved by the Research Ethics Committee of the São Leopoldo Mandic College of Campinas - São Paulo, with the Certificate of Presentation for Ethical Appraisal under the opinion of 63345516.0.0000.5374. Thus, the analyzes were performed in the laboratory of Cellular and Molecular Biology of this faculty.

Also at this study site, through its cell bank, the two human fibroblast culture cell lines (FG11 and FG15) were also obtained. These cells were previously isolated through the primary culture of human gums, removed from three different patients by explant technique.

Thus, in laminar flow hood, these cell cultures were maintained in Dulbecco Minimum Modification Essential (DMEM) medium (Nutricell®, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (Cultilab®, Campinas, SP, Brazil).) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Missouri, USA).

In addition, this cell set structure was kept in a greenhouse at 37 ° C in a humid atmosphere, changing the culture medium every 3 days, with the cell progression evaluated by inverted phase microscope. The suspension of these cells was obtained by trypsinization of 24 thermometric wells with trypsin, later inactivated by the culture itself.

From a material perspective, AH Plus endodontic cements and MTA Fillapex were manipulated at room temperature (25 ° C) following the instructions of their respective manufacturers: mixing equal amounts of paste A and paste B on a glass plate using a metal spatula.

Thus, obtaining a homogeneous consistency of the samples, they were inserted in silicone devices of 6 mm in diameter and 2 mm in height, allowing to be prey within 24 hours in an environment of 37 ° C with 100% humidity. . This set was further dried for 24 hours at room temperature and sterilized by 37.2 Gy gamma radiation before being added to the cell culture.

This structure was further divided so that three experimental groups were obtained, with four samples per group, so that one was the control, the second would be the association of DMEM solution with the MTA Fillapex cement and the last one would associate the DMEM solution with the one. AH Plus cement.

Thus, by assembling the experiment groups, the evaluation of cell proliferation was performed using the Trypan blue vital exclusion method at 24, 48 and 72 hours for each cement tested.

For this, after reaching subconfluence, the cells were removed from the plates by enzymatic action and the cell precipitate resulting from centrifugation by Eppendorf® centrifuge was suspended in 1 ml of medium. 10 µL was removed from the cell suspension and 10 µL Trypan Blue was added to it, and 1 µL of this solution was placed in a hemocytometer (Neubauer-Fisher Scientific chamber, Pittsburgh, PA, USA) and taken under a microscope. inverted phase for cell counting and observation.

Thus, the total number of cells present in each well at different times of analysis was obtained by the following mathematical equation:

$$\text{Total number of cells} = \frac{number\ of\ counted\ cells\ X\ initial\ volume\ X\ Dilution}{\text{Number of squares used for counting}}\ X\ 10^4$$

With this information, it was possible to perform cytotoxicity analyzes of endodontic cements also at 24, 48 and 72 hours after incubation. This process was performed by colorimetric assays with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT assay), a yellow substance which, when absorbed by cellular mitochondria is reduced to purple-colored formazan crystals by enzymatic action of living cells (SZCZURKO et al., 2017; ZAKERZADEH; ESNAASHARI; DADFAR, 2017).

The scientific literature indicates that this fast and accurate method of execution generates directly proportional results between the amount of purple crystals created with the number of viable cells. That is, mitochondrial activity of cells indicates their viability through optical analysis (ZAKERZADEH; ESNAASHARI; DADFAR, 2017). For this study, this quantification was performed by an ELX800 multiplier reader (Epoch biotek instruments, inc.) At 570 nm. Therefore, for this cytotoxicity assay, 110 cells per mm² were used in each well of 96-well thermometric plates incubated

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with the tested substances for 24.48 and 72 hours at 37 ° C. Immediately after, 10 µl of diluted MTT solution (5 mg / mL - SIGMA) was placed in DMEM culture medium without serum, added to the treated cultures and incubated for 4 hours at 37 ° C. After this incubation period, 100µl of 10% sodium dodecyl sulfate (SDS) solution and 0.01N hydrochloric acid were added and the experiment maintained 1 hour at 37 ° C.

From the perspective of genotoxicity analyzes, the cells were seeded on glass slide and placed on 35 mm discs at the bottom of the cell culture. These were incubated for 24 hours at 37ºC in a humid atmosphere containing 95% air and 5% carbon dioxide. Then the culture medium was replaced with diluted conditioned medium and incubated for 24h. After this period, the conditioned medium was discarded and the cells were washed twice with buffered saline.

With this, the cell culture was fixed with 1.5% formaldehyde solution at room temperature for 20 min. This content was discarded and replaced with 100% methanol solution (-20 ° C), keeping the cells at room temperature for 20 minutes so that the latter solution was discarded and the cells three times with PBS.

Thus, Hoechst's solution (Sigma, St Louis, MO, USA) was placed on the cells which were incubated for 15 minutes at room temperature. The glass slides were visualized and photographed by a fluorescence microscope. The percentage of micronuclei was determined by the number of micronucleus cells in 100 cells observed in five determined microscopic fields (at the four extreme points and in the center of the slide) at 400X magnification - Figure 1. All experimental groups were tested in triplicate.

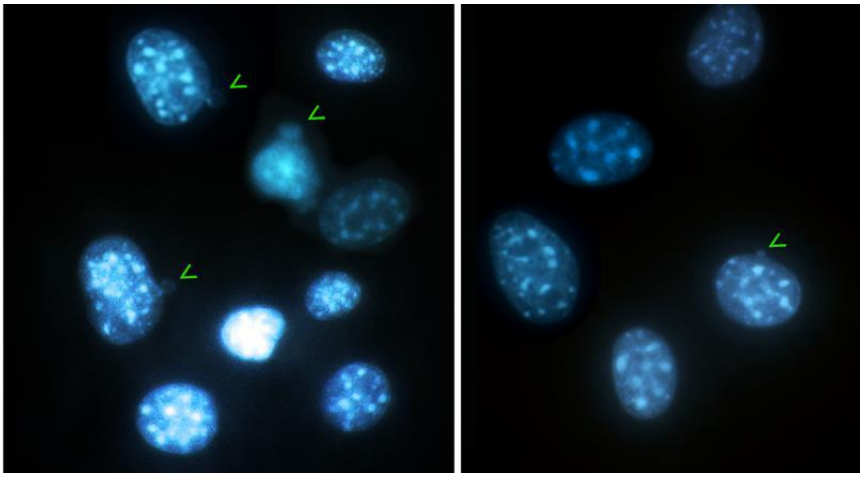

Fig.1: micronucleus formation

Thus, the results obtained were stored and analyzed in the Biostat 4.0 Program. In this system, the Shapiro Wilk normality test was performed, obtaining a non-normal distribution sample. Therefore, the descriptive analysis was performed and the results submitted to the Kruskal-Wallis (Dunn) test with a significance level of 5% ($p < 0.05$).

III. RESULTS

In the comparative scope between the evaluated cements and the control group in the first 24 hours of analysis, it is indicated that there was no significant difference in cell proliferation values ($p = 0.1930$), as can be observed by the medians of cell development in each analysis. Performed as shown in graph 1.

However, this same graph indicates that after 48 hours of experiments, there was greater proliferation in the control group, with a statistical difference presented in relation to the MTA Fillapex ($p = 0.0058$). This relationship with the control group remained active after 72 hours, but at this time, there were statistically significant differences from this group to the two cements studied ($p = 0.0140$).

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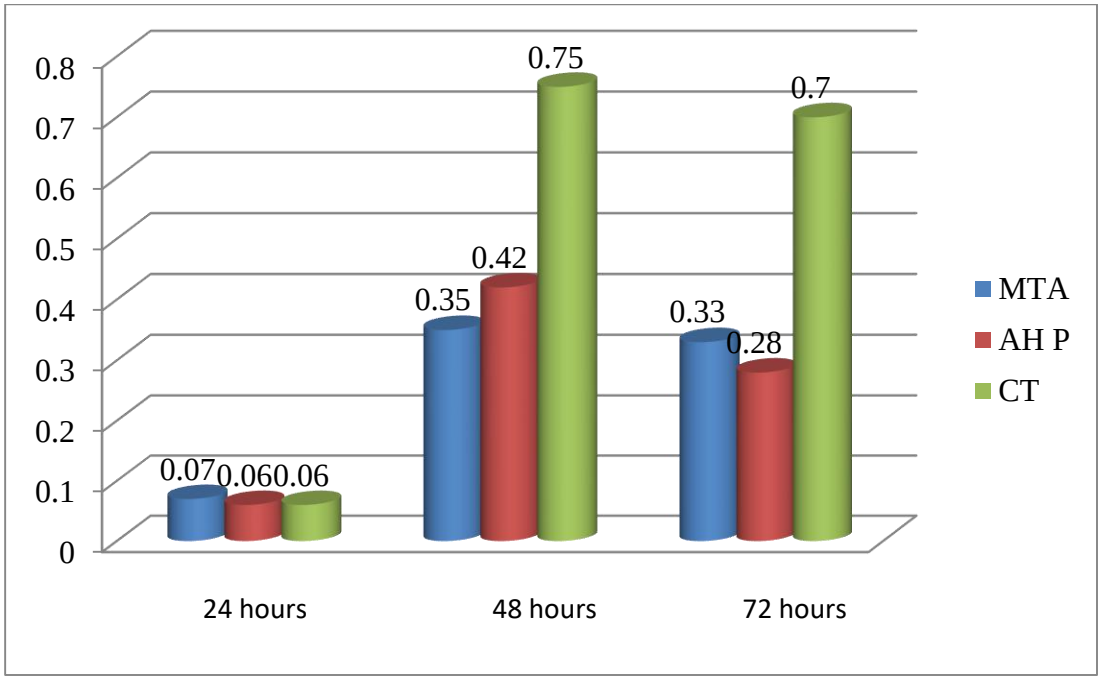
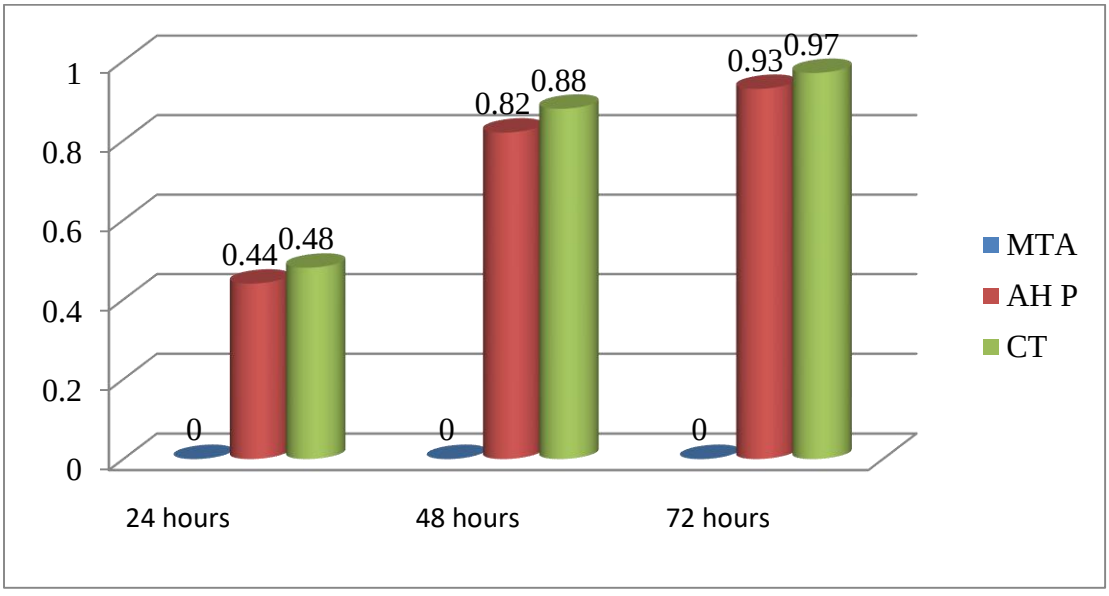
Graph 1: Median cell proliferation of MTA Fillapex, AH Plus and the control group in the same experimental period (number of viable cells x104)



From the perspective of cell viability, it is indicated that the highest values were related to AH Plus cement and the control group in all experimental periods ($p < 0.0002$), as can be shown in graph 2.

Graph 2: Median cell viability of MTA Fillapex, AH Plus and the control group in the same experimental period.



Contrary to this last analysis, Graph 3 indicates that the highest value in relation to genotoxicity analyzes were present in AH Plus cement within 24 hours, with significant difference compared to the control group and MTA Fillapex ($p = 0.0004$).

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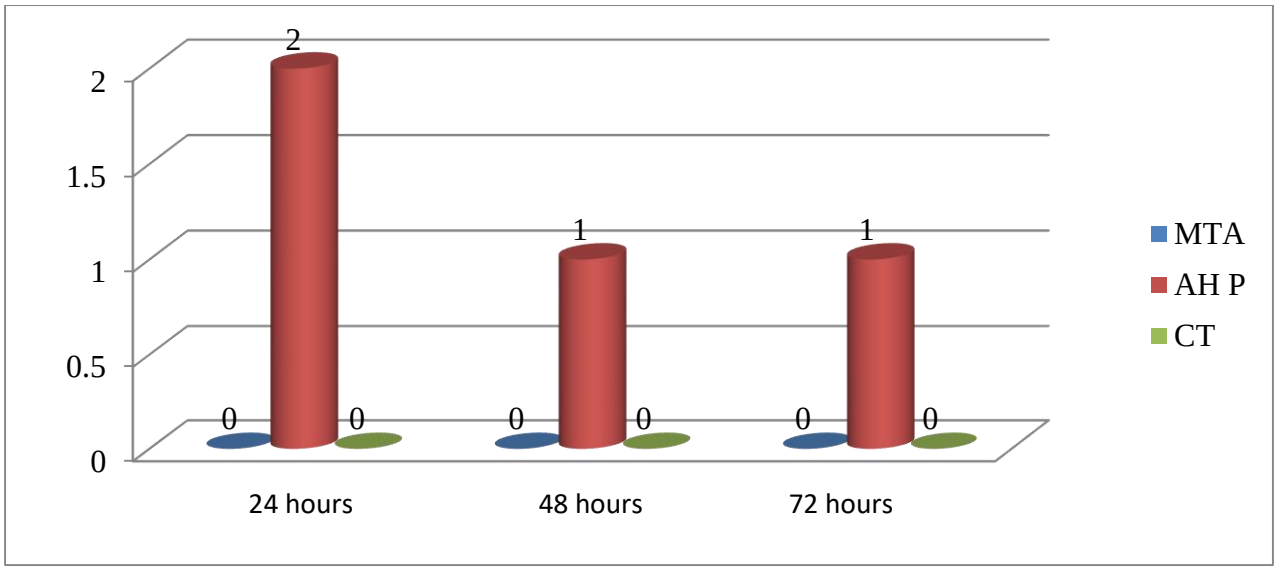
Graph 3: Median genotoxicity of MTA Fillapex, AH Plus and control group in the different experimental periods.



IV. DISCUSSION

The recognition of the need to use root canal sealers is a fact recognized in the relevant literature (VICTORIA-ESCANDELL et al., 2017). However, it is known that these materials can be expelled to the dental periapex through their communication with root canal system, delaying the healing of these areas (SZCZURKO et al., 2017; VICTORIA-ESCANDELL et al., 2017).

With this knowledge, it becomes evident the need for studies that analyze the biocompatibility of these sealers through methodologies that establish how their cytotoxic and genotoxic behavior is established, thus observing the feasibility of their use (SZCZURKO et al., 2017; VICTORIA-ESCANDELL et al., 2017).

Thus, it is necessary to use in vitro cell culture to perform these analyzes on biological compatibility of materials. Therefore, the relevance of the use of human fibroblasts, which have the ability to simulate a tissue response in vivo (SCELZA et al., 2018) is observed.

In addition, the timing of these assessments becomes significant, as in clinical practice endodontic cements are inserted into the root canal immediately after manipulation, at which time they present a higher degree of cytotoxicity (ELDENIZ et al., 2007). However, evaluations at other periods after manipulation become relevant for evaluating changes in possible toxic behavior.

Thus, with the results of this research, it can be stated that MTA Fillapex was the most cytotoxic cement of this evaluation, contradicting results obtained in other cell groups from other analyzes that infer that, even after 14 days of tissue exposure, MTA would have no cytotoxicity effect on human bone marrow mesenchymal stem cells (ALI et al., 2019).

The perspective generated by this work is in consensus with other analyzes carried out by the relevant literature, which infer that these findings possibly occur due to the presence of a higher amount of resins in the composition of MTA Fillapex cement in relation to the amount of MTA (ASSMAN, 2013).).

To this is added further analyzes which, aiming at observing the cytotoxicity of five endodontic cements (AH Plus, Endomethasone E, EndoSequence BC, MTA Fillapex and Pulp Canal Sealer EWT) using a three-dimensional cell culture model, observed that all proposed cements tested exhibited cytotoxic effects. However, MTA Fillapex was much more cytotoxic than other endodontic cements tested using the methodology employed by the authors (SILVA et al., 2016).

It is still significant to indicate that the results of this research still propose that, in the AH Plus resin cement optics, there was a good pattern of cell viability, showing no significant difference when compared to the control group and cytotoxicity between all experimental periods evaluated. Corroborating these analyzes, other studies testing the cytotoxicity of the MTA Fillapex and AH Plus cements identified that the former was more cytotoxic in all evaluation periods (SILVA et al., 2013).

However, it should be noted that these results contradict other studies, which point out the cytotoxicity of AH Plus possibly associated with its formaldehyde release

capacity, pointing out cytotoxicity moments through a seven-day evaluation using the MTT assay methodology (SAYGILI et al. ., 2017).

From the perspective of genotoxicity, the highest value obtained was concentrated in AH Plus cement within 24 hours, with significant difference compared to MTA Fillapex and the control group. This is a relevant milestone in endodontic therapy, as the eventual contact of genotoxic cement with periapical tissues may lead to damage to the DNA structure of connective tissue cells, delaying or preventing the repair process (CANDEIRO) et al., 2015).

V. CONCLUSION

From the results obtained in the present study, it can be stated that MTA Fillapex cement presented higher cytotoxicity potential on human fibroblast cell lines in all experimental periods. However, it is indicated that the AH Plus cement presented higher degree of genotoxicity from the applied methodology.

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