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BASIC RESEARCH:

Biocompatibility of Bioactive Sealers Bio-C Sealer *vs* MTA Repair HP in Human Fibroblasts

Biocompatibilidad de selladores bioactivos Bio-C Sealer *vs* MTA Repair HP en fibroblastos humanos

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ABSTRACT: Bioactive cements based on tricalcium silicate have been introducedto the market for use in dentistry, with a variety of clinical applications. These cements are in contact with vital tissues such as dental pulp or periodontium in cases of unintentional extrusion; thus, it is important to know the genotoxicity and cytoxicity of these materials. The objective of this study was to evaluate the cytotoxicity and genotoxicity of bioactive sealers, Bio-C® Sealer and MTA Repair HP®, in human fibroblasts. Discs of bioactive sealers Bio-C® Sealer, and MTA Repair HP®, were prepared and set for 24h under sterile conditions. The discs were placed in culture medium at 2.5mg/mL inside a SRT6D roller mixer (Stuart, UK) at 60rpm for 24h. The eluates obtained were incubated for 24h with previously activated and cultured ATCC cell line fibroblasts at 80% confluence. The cytotoxicity was evaluated by Alamar Blue® and LIVE/DEAD assays, as well as the analysis of the Tunel and Mitotracker assays to evaluate genotoxicity using the confocal laser-scanning microscope. In the Alamar Blue® assay, the Bio-C® Sealer presented a cell proliferation of 87%, while the MTA Repair HP® Sealer was 72%. A statistically significant difference was found between the MTA Repair HP® Sealant and the negative control (p=<0.001). Regarding the genotoxicity tests, in the Tunel assay, both materials stain the nucleus of the fibroblast cells exposed to the eluates, while in the Mitotracker assay, the MTA Repair HP® Sealer showed greater mitochondrial function than the Bio-C® Sealer. Calcium silicate-based sealers, Bio-C® Sealer and MTA Repair HP®, are not cytotoxic and have low genotoxicity.

KEYWORDS: Bio-C® Sealer; MTA HP® repair; Cytotoxic; Genotoxicity; Cements; Tricalcium silicate.

RESUMEN: Los cementos bioactivos a base de silicato tricálcico se introdujeron en el mercado para uso en odontología, con una variedad de aplicaciones clínicas. Estos cementos pueden estar en contacto con tejidos como la pulpa dental o el periodonto, en caso de extrusión no intencionada. Por lo tanto, es importante conocer la genotoxicidad y la citoxicidad de estos materiales. El objetivo de este estudio fue evaluar la citotoxicidad y genotoxicidad de los selladores bioactivos Bio-C® Sealer y MTA Repair HP® en fibroblastos humanos. Se prepararon discos de selladores bioactivos Bio-C® Sealer y MTA Repair HP® y se colocaron durante 24h en condiciones de esterilidad. Los discos se colocaron en medio de cultivo a 2,5mg/mL dentro de un mezclador de rodillos SRT6D (Stuart, Reino Unido) a 60rpm durante 24h. Los eluidos obtenidos se incubaron durante 24h con fibroblastos de la línea celular ATCC previamente activados y cultivados al 80% de confluencia. La citotoxicidad se evaluó mediante ensayos Alamar Blue® y LIVE/DEAD; así como el análisis de los ensayos Tunnel y Mitotracker para evaluar la genotoxicidad, utilizando el microscopio confocal láser de barrido. En el ensayo Alamar Blue®, el Sellador Bio-C® presentó una proliferación celular del 87%, mientras que el sellador MTA Repair HP® fue del 72%. Se encontró una diferencia estadísticamente significativa entre el sellador MTA Repair HP® con respecto al control negativo (p=<0.001). En cuanto a las pruebas de genotoxicidad, en el ensayo Tunel, ambos materiales tiñen el núcleo de las células fibroblásticas expuestas a los eluidos, mientras que el ensayo Mitotracker, el sellador MTA Repair HP®, mostró una mayor función mitocondrial que el Bio-C® Sealer. Los selladores a base de silicato de calcio, Bio-C® Sealer y MTA Repair HP® no son citotóxicos y tienen una baja genotoxicidad.

PALABRAS CLAVE: Sellador Bio-C® Sealer; Reparación MTA HP®; Citotóxico; Genotoxicidad; Cementos; Silicato tricálcico.

INTRODUCTION

The main objectives of endodontic treatment are the shaping and cleaning of all pulp spaces, as well as their complete obturation using an inert filling material (1-3). Root canal sealing is important for the long-term success of endodontic treatment; therefore, all materials used in pulp therapy must have suitable physical and chemical properties to achieve a three-dimensional seal (1, 4, 5). It becomes important to know the toxicity of the new sealers since the compounds that constitute them can damage the surrounding tissues (6). Currently, new materials known as "bioactive cements" have been developed for use in dentistry based on tricalcium silicate with a variety of clinical applications, ranging from vital pulp therapy to filling of the root canal system; they are used in apical surgeries, apexification, internal resorption, and the repair of perforations (7-10). In these applications, the cements are in contact with tissues such as the dental pulp or the periodontium in cases of unintentional extrusion (6, 11, 12).

Calcium silicate-based sealants have low cytotoxicity, induce a mild or moderate inflammatory reaction, and are premixed with zirconium oxide and monobasic calcium phosphate (13). However, during the setting process, where the ceramic reacts with moisture from tissue fluids and then becomes stable under moist conditions. The biological properties of calcium silicates depend on the formation of calcium hydroxide

as a byproduct of this hydration reaction (14, 15). Within these calcium silicate-based sealers, premixes have been developed that have adequate physicochemical and biological properties, such as EndoSequence BC and TotalFill BC Sealer, which include calcium silicates, zirconium oxide, and phosphate monobasic calcium within their formulas, conferring biocompatibility, dimensional stability, and high radiopacity. Regarding zirconium oxide, it is used as a radiopacifier that is based on calcium silicate and that stimulates the formation of fibroblasts and the formation of collagen in the subcutaneous tissue of rats (13). Similarly, new calcium silicate-based endodontic sealers have come onto the market, such as Bio-C® Sealer, which is made of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, zirconia oxide, silicon oxide, polyethylene glycol, and iron oxide (10, 13, 16). The objective of this study was to evaluate the cytotoxicity and genotoxicity of bioactive sealers, Bio-C® Sealer and MTA Repair HP®, in human fibroblasts. The hypothesis was that Bio-C® Sealer presents greater biocompatibility with respect to MTA Repair HP®.

MATERIALS AND METHODS

An experimental *in vitro* study was carried out with cultures of the ATCC cell line (PCS-201-010) of human dermal fibroblasts neonatal (hDFN) with a confluence of ≥80%. The genotoxicity and toxicity of the calcium silicate-based sealers to be evaluated were MTA Repair HP (Ángelus, Londrina, PR, Brazil) and Bio-C Sealer (Angelus, PR, Brazil). For the preparation of the MTA Repair HP® and Bio-C® Sealer bioactive sealants, it was carried out according to the manufacturer´s instructions under sterile conditions in a laminar flow hood, where they were allowed to set for 24h. The set bioactive sealant discs were placed in falcon tubes in contact with culture medium in a ratio 2.5mg/mL and placed in the SRT6D Roller mixer (Stuart, UK) at 60rpm for 24h ; after, it was centrifuged at 3000rpm for 10min to obtain an eluate. These experiments were performed according to ISO-10993-5:2009 standard for the biological evaluation of medical devices, Part 5: cytotoxicity tests *in vitro* (17).

FIBROBLAST CULTURE

hDFN from the ATCC cell line were activated. For this, a 37°C water bath was used. 1mL of low-glucose DMEM medium, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic, was added, and centrifuged at 1000rpm for 10min. The cell pellet obtained was separated from the supernatant by direct decantation and resuspended in 4mL of culture medium in 25-cc culture bottles to be incubated at 37°C under 95% humidity with 5% CO_2, with medium replacements every 48h until obtaining confluence ≥ 80%.

IN VITRO CYTOTOXICITY AND GENOTOXICITY ASSAY

LIVE/DEAD® CELL VIABILITY

For cell viability, the LIVE/DEAD® (LIVE/DEAD-Life Technologies, LIVE/DEAD® viability/cytotoxicity kit, Life Technologies, catalog # L-3224) assay was performed. The working solution was prepared according to the manufacturer's instructions. The culture medium was removed and washed three times with PBS, and 150µL of the working solution was added directly to each of the wells; the samples were incubated for a period of 30min in dark conditions. Finally, the samples were washed with PBS twice before evaluation in the confocal laser-scanning microscope at 495nm (CLSM) (DMI400B, Leica Microsystems, Wetzlar, Germany). For cell viability, the controls were defined as follows: 1) Negative control: culture medium; 2) Positive control: culture medium + H_2O_2.

BLUE ALAMAR® TEST

For the Alamar Blue assay, 10,000 cells per sample determined in a Neubauer chamber were cultured for 24h in a 96-well plate. They were cultured for 24h in an atmosphere with 95% humidity and 5% CO_2 at 37°C. At the end of this period, the culture medium was replaced by the eluates previously obtained and incubated for 24h. At the end of the period, 10µL of the Alamar Blue solution was incorporated directly into each of the wells and incubated for. After the incubation period, it was read in a microplate reader (Thermo Scientific FC Multiskan Vabtaa Finland) at a wavelength of 570nm. Preparation of control groups proceeded as follows: 1) Negative control: culture medium + reagent; 2) Positive control: cells treated with H_2O_2 + reagent.

TEST TUNNEL

Cells were cultured on circular coverslips within a 24-well plate for 24h in an atmosphere of 95% humidity and 5% CO_2 at 37°C. At the end of this period, the medium was removed, and the cells were exposed to the eluates for 24h under the same conditions. The cells that grew on the glass coverslips were fixed with 300µL of 4% formaldehyde in PBS for 25min at 4°C. After that, they were washed with PBS for 5min. Triton X-100 0.2% solution in PBS was added for 5min for cell permeabilization. Subsequently, they were washed with PBS for 5min and 100µL of equilibration buffer was added for 10min at room temperature. The medium was removed, and 10µL of a mixture containing 9.8% nucleotides, 1.96% rTdT enzyme, and 88.2% equilibration buffer was added and allowed to incubate for 60min. At the end of the period, a 2X SSC solution was added with a 1:10 concentration in deionized water. They were washed with PBS and stained for 15min with a solution of propidium iodide diluted in PBS in a 1:1000 ratio. Finally, the samples were washed with PBS and analyzed under a 520nm CLSM. For the TUNEL assay, controls were defined as follows: 1) Negative control: without the rTdT enzyme; 2) Positive control: 100µL of DNase 1 was added during cell permeabilization.

MITOTRACKER® ASSAY

A MitoTracker® stock solution was prepared at a concentration of 100nM in DMSO. Subsequently, from the stock solution, the working solution was prepared in PBS to obtain a concentration of 20nM. The culture medium was removed from both the control and treated cells. 200µL of the working solution was added to each of the wells and incubated for 30min, protecting them from light. At the end of this period, the working solution was removed, and the glass coverslips were washed with PBS. Finally, the samples were analyzed in a CLSM. For the MitoTracker® assay, the controls were defined as follows: 1) Negative control: culture medium; 2) Positive control: culture medium + Cisplatin.

STATISTIC ANALYSIS

The data were analyzed using SigmaPlot software version 11.0 (Systat Software Inc.). The Shapiro-Wilk test was used to determine the normality of the data from the Alamar Blue Test. For the analysis of cell viability, an ANOVA was performed in one direction with a statistical significance of $p<0.05$.

RESULTS

CYTOTOXICITY TESTS

LIVE/DEAD ASSAY

Micrographs from the LIVE/DEAD assay are presented in Figure 1. In the negative control (Figure 1.A), viable cells with an intact plasma membrane showing intense green fluorescence produced by calcein AM are observed. In the positive control

(Figure 1.B), cells stained red due to the penetration of ethidium homodimer-1, are seen, indicating loss of plasma membrane integrity (dead cells). Figure 1.C shows hDFN treated with the Bio-C® Sealer eluates, where viable cells and some cells with a membrane permeable to ethidium homodimer-1 that bind to DNA are observed, while in the hDFN treated with MTA Repair HP (Figure 1.D) a greater number of red-stained nuclei are observed due to the permeability of ethidium homodimer-1, evidencing the presence of non-viable cells, as well as stained intact membranes of green.

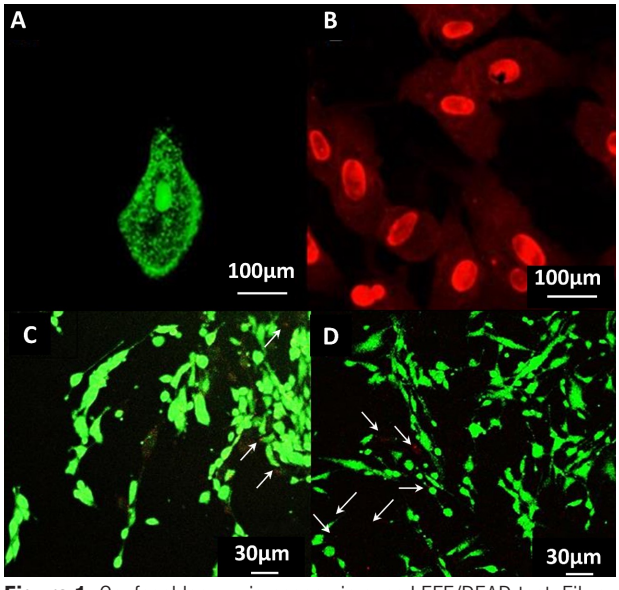

Figure 1. Confocal laser microscope images LEFE/DEAD test. Fibroblasts evaluated at 24h in contact with the eluates; A) Negative control, B) Positive control, C) Bio-C® Sealer, and D) MTA Repair HP.

ALAMAR BLUE TEST

Figure 2 shows the results of the Alamar Blue® assay. The Bio-C® Sealer group had less cell shrinkage than the MTA Repair HP group. However, a statistically significant difference was only found in the MTA Repair HP group compared with the negative control group (life) (p=<0.001). However, despite the evident decrease in cell proliferation with the eluates and treatment with MTA Repair HP, the cell concentration was not reduced by more than 30%.

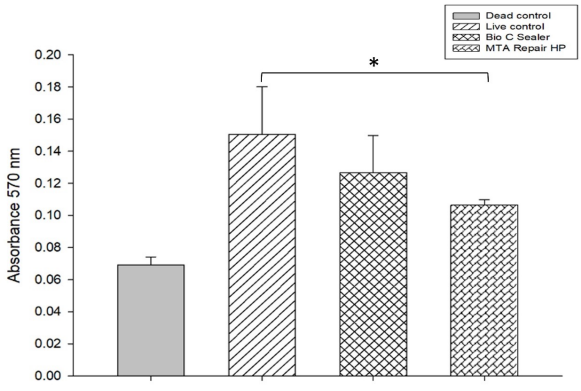

Figure 2. Alamar Blue® test. The effect of the cytotoxicity of the eluates of Bio-C® Sealer and MTA Repair HP® after 24h in contact with fibroblasts. * Statistically significant difference.

GENOTOCICITY TESTS

TUNNEL ASSAY

In relation to the Tunnel assay, the micrographs are seen in Figure 3. In the negative control, nuclear staining is seen in a few cells, which was generated by the incorporation of fluorescein-12-dUTP into the DNA breaks, characteristic of apoptosis. In the positive control, the increase in staining of the nucleus is notorious since damage to the genetic material was generated with the treatment of the Cisplatin cells. In relation to the treatments with Bio-C® Sealer (Figure 3.C), cells stained by fluorescein-12-dUTP are observed in less quantity than those observed in the hDFN treated with MTA Repair HP (Figure 3.D). show

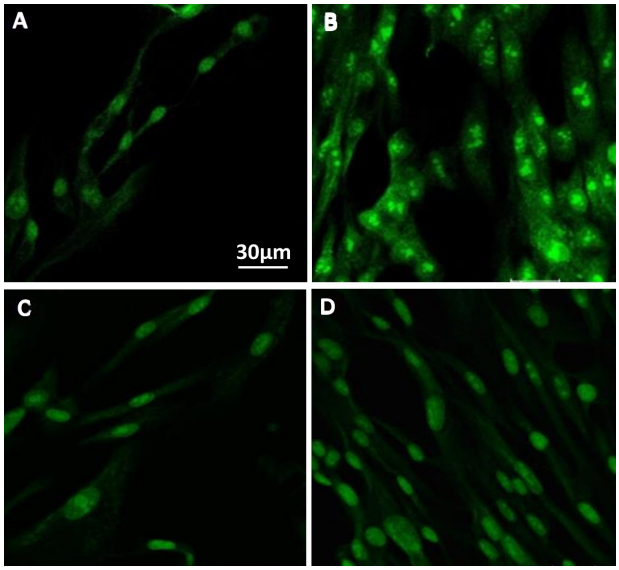

Figure 3. TUNEL® assay. Fluorescence microscope image (40X). Fibroblasts evaluated at 24h in contact; A) Negative control, B) Positive control, C) Bio-C® Sealer and D) MTA Repair HP.

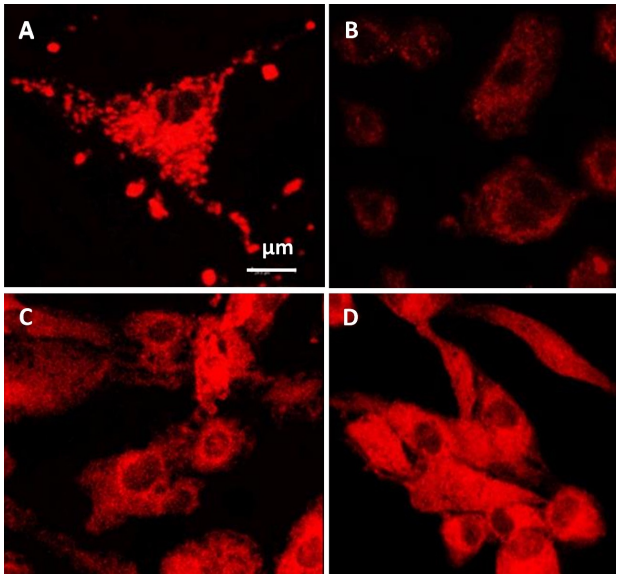

Figure 4. MitoTracker® assay. Fluorescence microscope image (40X). Fibroblasts evaluated at 24h in contact; A) Negative control, B) Positive control, C) Bio-C® Sealer and D) MTA Repair HP®.

MITOTRACKER DEEP RED FM TEST

Cells with active mitochondria express fluorescent red pigmentation. Untreated cells (negative control) show the highest number of mitochondria (Figure 4.A), while in the positive control (cells treated with Cisplatin), a lower intensity is shown in the characteristic red color of the mitochondria. In relation to the hDFN treated with the eluates of the bioactive sealers, it is observed that the Bio-C® Sealer group (Figure 4.C) does not affect the mitochondrial state, and they do not alter the number of mitochondria in the cells since an important number of stained organelles per cell are observed, although it was observed slightly decreased in relation to the hDFN treated with MTA Repair HP®, where each one of the cells shows numerous mitochondrial organelles stained with an intense red color (Figure 4.D).

DISCUSSION

Bio-C® Sealer and MTA Repair HP®, based on tricalcium silicate, have a variety of clinical applications, ranging from vital pulp therapy to filling the root canal system, and they may come into contact with pulp tissue and periradicular tissues (14, 17, 18). So, the present study aimed to evaluate and compare the cytotoxicity and genotoxicity of hDFN treated with different sealers. The classic assay to evaluate the possible cytotoxic effects of the materials is the MTT assay after 24h of cell exposure, following the ISO 10993 guidelines (17). However, in this study, the cytocompatibility of the different materials was evaluated using the Alamar Blue® assay, which is based on cell processing and contains resazurin and a mix of stabilizing salts to minimize spontaneous background reduction in the absence of

cells (19, 20). Resazurin assays are based on the reduction by living cells of the oxidized blue dye to a pink fluorescent resorufin product and the reduction of resazurin, which is accomplished by reductase or diaphorase enzymes from the mitochondria and cytosol (20, 21). Besides, resazurin is water-soluble, sensitive, and non-cytotoxic, making it a robust, easy-to-perform, and relatively inexpensive assay (20).

Regarding the results of the Alamar Blue® assay obtained, both eluates did not generate a greater than 30% decrease in cell viability; so, according to the ISO 10993 guidelines, both materials are not cytotoxic (17). However, the MTA Repair HP® group had a statistically significant difference with respect to the control group at 24h.

These results agree with those obtained by Celso *et al*., where the MTA Repair HP® group showed the lowest cell viability at 24h. However, Celso *et al*. evaluated cell viability using the MTT assay, and in this type of assay, cell viability may be associated with cells in the apoptotic process, where cells are detected as viable even though they have reduced metabolic activity, and/or a necrotic process, where the cells are found to be non-viable (22). The differences in the results obtained in this report respect other studies can be explained by: (i) the type of cells used, when the proliferation capacity is variable; for example, the proliferation rate of cancer cells is faster than normal cells (23); (ii) the number of passages of the cell culture used, this is the case of the use of primary cultures where it is recommended to work with cells from a quarter or a seventh pass since the sensitivity of the test can vary with the number of passes according to ISO 10993-5; (iii) the treatment time, since some evaluate at 24, 48 or 72h; (iv) the use of fresh or set materials; since fresh materials can release toxic compounds and (v) the concentration of the material to be evaluated according to the recommendation of the ISO 10993-5 standard (20, 22, 24).

Guimaraes *et al*. reported that MTA Repair HP® showed the highest values of calcium release at 24h and 72h compared to Angelus white MTA, as well as a higher solubility that could be due to the plasticizer contained in the MTA Repair HP® liquid mixture (24). As described in other studies, the release of calcium, which is related to the formation of soluble calcium salts and calcium hydroxide during the hydration and setting reactions of the material, could be related to the cytotoxicity of the material (24, 25). In addition, the different additives that are part of the sealers play an important role in the biocompatibility of endodontic sealers; such is the case of radiopacifiers, which influence the release of calcium ions and the alkaline pH of the environment, stimulating the recruitment of inflammatory cells for the production of cytokines necessary for the recovery or proper functioning of the tissue in contact with the sealants. Of the sealants evaluated, Bio-C® Sealer contains ZrO_2 (zirconium oxide) as a radiopacifier, and MTA Repair HP® contains calcium tungstate as a radiopacifier, which could explain the reason for Bio-C®'s greater biocompatibility. Li *et al*. reported that the addition of ZrO_2 does not affect the mechanical properties and decreases the initial release of Ca^{2+} ions, so they concluded that the presence of ZrO_2 improved some properties based on its indication as dentin replacement material and/or pulp repair (13, 24, 26). In the present study, damage to cell membranes was evaluated using the Life and Death assay; the results showed that after 24h of treatment, both Bio C Sealer® and MTA Repair HP® cause minimal damage to the cell membrane, manifested in the presence of some cells with red-stained nuclei (white arrows). These results agree with the data obtained in the Alamar Blue® Assay, where MTA Repair HP® has less biocompatibility with respect to the living

control group, while the group of cells treated with Bio-C® Sealer behaved in the same way as that of the cell viability test, with greater biocompatibility than MTA Repair HP®.

The quantity and volume of active mitochondria in the cells treated with the evaluated materials was analyzed using the MitoTracker Deep Red® assay, where it was observed that both the cells of the control group and those treated with the different sealants present an intense red color, which means active mitochondrial content, which translates into better cellular respiration (measured by the oxygen consumption rate (OCR)), better respiratory reserve capacity, and greater energy production (27). All of this indicates the biocompatibility of the sealants evaluated in this research. Bioactive materials used in root canal repair must have little or no capacity to cause DNA damage; therefore, it was of interest to evaluate the genotoxicity of the sealers to determine the presence or absence of fibroblasts with DNA damage or in an apoptotic state by means of the TUNEL assay, in which the nuclei of apoptotic or DNA-damaged cells are stained with an intense green color due to the incorporation of fluorescein-12-dUTP into the nucleus. Confirming the results obtained in the cell viability assays (MTS and LIVE/DEAD), the materials show similar results to the life control group, with the number of cells with fluorescent green-stained nuclei being lower in the Bio-C® Sealer group in comparison with MTA Repair HP®. These results agree with the data found in the scientific literature, where they conclude that, in addition to Bio-C® Sealer and MTA Repair HP® being the most studied silicate-based sealants from 2005 to 2020 (28, 29), only a few studies showed slight genotoxicity in mammalian cells. Dos Santos et al. report that MTA Angelus is the most tested sealant and showed an absence of genotoxicity with the comet assayin L5178Y cells, CHO-K1 cells, hPB lymphocytes, and 3T3-L1 cells; likewise, this sealant does not show genotoxicity evaluated with the micronucleus test performed on V79 cells and bone marrow cells from swiss mice (30). With these results, it would be interesting to perform these same techniques with periodontal ligament cells, which are the type of cells that are in contact with the different sealants used in the endodontic field.

CONCLUSION

Calcium silicate-based sealers, Bio-C® Sealer and MTA Repair HP®, are not cytotoxic. MTA Repair HP® Sealer showed greater active mitochondrial function than Bio-C® Sealer, and both materials presented low genotoxicity.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization and design: V.M.M.G. and D.M.E.G.
Literature review: J.M.L., D.M.E.G. and M.G.S.
Methodology and validation: A.M.G.A., A.P.G., D.M.E.G. and M.G.S.
Formal analysis: A.P.G., D.M.E.G. and M.G.S.
Investigation and data collection: J.M.L, D.M.E.G. and A.P.G.
Resources: A.P.G. and V.M.M.G.
Data analysis and interpretation: A.P.G., D.M.E.G. and M.G.S.
Writing-original draft preparation: V.M.M.G.
Writing-review & editing: A.P.G., D.M.E.G. and M.G.S.
Supervision: D.M.E.G. and M.G.S.
Project administration: D.M.E.G.
Funding acquisition: A.P.G. and V.M.M.G.

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