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Cytotoxicity, cytoprotection and morphological analysis of MTA, MTA Repair HP and Biodentine

Citotoxicidade, citoproteção e análise morfológica do MTA, MTA Repair HP e Biodentine

Citotoxicidad, citoprotección y análisis morfológico de MTA, MTA Repair HP y Biodentine

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Abstract
The aim of the present study was to evaluate the *in vitro* cytotoxicity, cytoprotection and morphological changes by SEM technique of MTA, MTA-HP and Biodentine, on fibroblast 3T3 cell line. MTA, MTA-HP, and Biodentine were disposed into teflon sterile discs and incubated in culture media for 24 hours to obtain elutes. Fibroblast 3T3 cells were cultured with the respective elutes and control group with culture medium. Cytotoxicity and cytoprotection assays were determined by MTT method. The results were statistically processed by Mann-Whitney (α=0.05) and Kruskal-Wallis analysis. Cells cultured in coverslips and treated with the elutes were submitted to fixation and dehydration process to evaluate morphological alterations by SEM technique. In cytotoxicity assay, cells treated with MTA, MTA HP and Biodentine showed viability above 95%, like control cells. In cytoprotection to the 3T3 cells, materials promoted at the same magnitude ($p>0.05$), with improved cell growth and were considered statistically different from the obtained for cells only treated with peroxide solution (positive control) ($p=0.046$). Also, viability results of the tested root canal materials were close to that of the negative control (cells treated only with culture medium) ($p=0.05$). No morphologic cell changes of 3T3 cells in contact with the endodontic materials were revealed by SEM technique. The bioceramic materials has demonstrated high bioactivity and biocompatibility, as presented in cytoprotection and morphological trials.
Keywords: Cell culture techniques; Microscopy; *In vitro* Techniques.

Resumo
O objetivo do presente estudo foi avaliar a citotoxicidade *in vitro*, citoproteção e alterações morfológicas pela técnica de MEV do MTA, MTA-HP e Biodentine, na linhagem celular de fibroblastos 3T3. MTA, MTA-HP e Biodentine foram dispostos em discos estéreis de teflon e incubados em meio de cultura por 24 horas para obtenção dos eluatos. Células de fibroblastos 3T3 foram cultivadas com os respectivos eluatos e o grupo controle com meio de cultura. Os ensaios de citotoxicidade e citoproteção foram determinados pelo método MTT. Os resultados foram processados

estatisticamente pela análise de Mann-Whitney (α=0,05) e Kruskal-Wallis. As células cultivadas em lamínulas e tratadas com os eluatos foram submetidas ao processo de fixação e desidratação para avaliação das alterações morfológicas pela técnica de MEV. No ensaio de citotoxicidade, as células tratadas com MTA, MTA HP e Biodentine apresentaram viabilidade acima de 95%, assim como as células controle. Na citoproteção das células 3T3, os materiais promoveram na mesma magnitude (p>0,05), com melhor crescimento celular e foram considerados estatisticamente diferentes do obtido para células tratadas apenas com solução de peróxido (controle positivo) (p=0,046). Além disso, os resultados de viabilidade dos materiais endodônticos testados foram próximos aos do controle negativo (células tratadas apenas com meio de cultura) (p=0,05). Nenhuma alteração morfológica das células 3T3 em contato com os materiais endodônticos foi revelada pela técnica de MEV. Os materiais biocerâmicos demonstraram alta bioatividade e biocompatibilidade, conforme apresentado em ensaios de citoproteção e morfológicos.
Palavras-chave: Técnicas de cultura celular; Microscopia; Técnicas *in vitro*.

Resumen
El objetivo del presente estudio fue evaluar la citotoxicidad, citoprotección y cambios morfológicos *in vitro* por técnica SEM de MTA, MTA-HP y Biodentine, en la líneo celular de fibroblastos 3T3. MTA, MTA-HP y Biodentine se colocaron en discos estériles de teflón y se incubaron en medios de cultivo durante 24 horas para obtener eluidos. Las células de fibroblastos 3T3 se cultivaron con los eluidos respectivos y el grupo de control con medio de cultivo. Los ensayos de citotoxicidad y citoprotección se determinaron mediante el método MTT. Los resultados fueron procesados estadísticamente por Mann-Whitney (α=0,05) y análisis de Kruskal-Wallis. Las células cultivadas en cubreobjetos y tratadas con los eluidos fueron sometidas a proceso de fijación y deshidratación para evaluar alteraciones morfológicas por técnica SEM. En el ensayo de citotoxicidad, las células tratadas con MTA, MTA HP y Biodentine mostraron una viabilidad superior al 95 %, al igual que las células de control. En la citoprotección a las células 3T3, los materiales promovieron en la misma magnitud (p>0.05), con crecimiento celular mejorado y se consideraron estadísticamente diferentes a los obtenidos para las células tratadas únicamente con solución de peróxido (control positivo) (p=0.046). Además, los resultados de viabilidad de los materiales del conducto radicular probados fueron similares a los del control negativo (células tratadas solo con medio de cultivo) (p = 0,05). La técnica SEM no reveló cambios celulares morfológicos de las células 3T3 en contacto con los materiales endodónticos. Los materiales biocerámicos han demostrado una alta bioactividad y biocompatibilidad, tal como se presenta en los ensayos de citoprotección y morfológicos.
Palabras clave: Técnicas de cultivo celular; Microscopía; Técnicas *in vitro.*

1. Introduction

Bioceramics are biocompatible materials specifically designed for the repair and reconstruction of diseased or damaged parts of the body in medicine and dentistry (Baino et al., 2015). They contain alumina, zirconia, bioactive glass, resin layers, hydroxyapatite, resorbable calcium phosphate and radiotherapeutic glass particles (Best et al., 2008; Jefferies, 2014). Bioceramic endodontic materials are used to seal root canal communications with the periodontal ligament, being nonsensitive to moisture and blood contamination and they are dimensionally stable and expand slightly after hardening, becoming rigid and insoluble (Jefferies, 2014; Zhang et al., 2009; Gandolfi et al., 2009; Nekoofar et al., 2010; Zhang et al., 2010). When the bioceramic materials come in contact with the tissue fluids, they release calcium hydroxide, which interacts with the phosphates to form the hydroxyapatite, having a tissue-inducing capacity (Richardson, 2008; Debelian & Trope, 2016). These materials are indicated for pulp coating, pulpotomy, repair of perforations and resorptions, retrobturation and obturation of immature teeth with open apices (Tran et al., 2012).

MTA (aggregated mineral trioxide) is an original material present in all bioceramics as a chemical property, that is, when the value is maintained, it is biocompatible and bioactive. It consists of components of Portland cement (mainly di-tricalcium silicate) and radiopacifiers as bismuth oxide, varying in proportion according to the trademark (Parirokh & Torabinejad, 2010). It appeared commercially as the ProRoot MTA (Tulsa Dental Products, Tulsa, OK) and subsequently, MTA gray and white - Angelus (Angelus Dental Products Industry S/A, Londrina, PR, Brazil) was introduced (Camilleri, 2008).

Despite its advantages in clinical application, the MTA presents some disadvantages, such as the time of setting, difficulty of handling and removal. Besides that, both MTA, gray and white, stain dentin due to the quantity of heavy metals in

their composition or to the inclusion of blood pigment during its setting and the discoloration of the marginal gingiva related to these materials have been already reported (Bortoluzzi et al., 2007). An interaction of Bismuth Oxide with the collagen present in the dental tissue is one of the main causes of discoloration (Marciano et al., 2014).

MTA Repair HP had as a main modification to substitute bismuth oxide for calcium tungsten radiopacifier in the powder formula, avoiding a dental discoloration. The liquid supplied for mixing with the cement powder consists of water and a plasticizing agent (Palczewska-Komsa et al., 2021). According to the manufacturer, this material has high-plasticity and improved physical properties, as compared with White MTA (Angelus® homepage, 2015).

Another bioceramic material, considered as second generation is Biodentine (BD, Septodont, Saint Maur de Fossés, France), which has a similar property to the MTA with its clinical indications (Bozeman et al., 2006; Parirokh & Torabinejad, 2010). Biodentine powder is mainly composed of tricalcium silicate, calcium carbonate (filling material) and zirconium oxide (radiopacifier). While the liquid used to blend with the powder for calcium chloride (used to speed up the setting and a water-soluble polymer (water-reducing agent / super-plastification) (Laurent et al., 2008). As its advantages over MTA is that it has a shorter hardening time (approximately 10-12 minutes) and a resistance to dentin compression (Grech et al., 2013; Chang et al., 2014; Corral Nuñez et al., 2014; Mori et al., 2014). Its capacity to induce an apposition of reactional tertiary and restorative dentin, via stimulus of the odontoblastic activity, was confirmed with positive influence without repair by cell-part difference and biomineralization in in vitro studies after the direct pulpal coating (Laurent et al., 2012; Cushley et al., 2021).

Another important aspect of biomaterials is the outermost chemical surface, as this is the layer that will be in close contact with vital tissue. Several reactions occur at the interface between cells and the biomaterial (Tomás-Catalá et al., 2018).

The goal of the present study was to compare conventional MTA, MTA Repair HP and Biodentine by evaluation of the cytotoxicity, cytoprotection and morphological alterations in fibroblast 3T3 cell line.

2. Methodology

Preparation of endodontic materials eluates

Calcium silicate-based endodontic materials MTA, MTA Repair HP and Biodentine were prepared according to manufacturer's instructions. Their complete compositions are described in Table 1. Biodentine capsules were vibrated at 4.000 rpm for 30 seconds. Six samples of each material were distributed into discs (5.5 mm in diameter and 3.0 mm in thickness) of sterile teflon molds (Applied Plastics Technology, Inc, Bristol, RI) and disposed in 6-well tissue culture plate. The endodontic materials were covered with 3 mL of cell culture media RPMI (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, Paisley, UK), and antibiotics - 100 U mL^{-1} penicillin/ 100 µg mL^{-1} streptomycin (Sigma, St Louis, MO, USA) and set for incubation at 37ºC/ 48 hours. After this period, eluates were sterilized by filtering with 0,22 µm pore filter membrane (Corning, NY, EUA). Samples containing only cell culture media were used as the control.

Cell culture

Fibroblast cells (3T3, American Type Culture Collection) were cultured in RPMI media supplemented with 10% FBS and 100 U mL^{-1} penicillin/ 100 µg mL^{-1} streptomycin. Cells were kept in a humidified atmosphere at 37°C, 5% CO_2. Cell culture viability was determined by trypan blue method. Cultures with more than 95% of viable cells were considered able to be seeded in 96-well plates at the density of 3 x 10^3 cells/well. After starvation, cells were treated in sixtuplicates with the sealer eluates and incubated at 37ºC/ 24 hours, before performing the cytotoxicity assay (Molecular Probes, Eugene, OR).

Cytotoxicity assay

Cytotoxicity was accessed by MTT method (Stockert et al., 2018; Bertin et al., 2019). MTT salt was dissolved in sterile phosphate buffer solution pH 7,4 (0,5 mg mL^{-1}). Eluates of each root canal sealer and the media of control group were substituted for MTT solution (100 µL). Cells were set for incubation at 37ºC for three hours. After incubation, MTT solution was completely removed and 100 µL of dimethyl sulfoxide was added in each well for dissolution of the formazan crystals. The 96-well plate was homogenized for five minutes in a plate shaker and absorbance was measured at 570 nm in an ELISA plate reader (Labsystem, Helsinki, Finland).

Cytoprotection assay

Cells were seeded in a 96-well plate (1 x 10^3 cells/well). After starvation, cells were treated with eluates of each root canal sealer, except the control groups: negative control (cells only treated with culture media) and positive control group (cells that will be treated only with peroxide solution). After incubation for 24 hours, the media was removed, except for the negative control group, and replaced by a solution of H_2O_2 600 µM in PBS (concentration of hydrogen peroxide that promoted a 50% reduction in cell viability). Cells were incubated at 37ºC for 2 hours, after this period, cell viability was assessed by MTT method (Maluf et al., 2018; Garcia et al., 2021).

Morphological analysis by Scanning Electron Microscopy (SEM) technique

Cells were seeded at 6 x 10^4 cells per well in a 24-well plate that had been previously prepared with a 13 mm coverslip placed on the bottom. Then, cells were treated with the eluates obtained from endodontic materials and incubated at 37ºC for 24 hours. After incubation, cells that remained adhered to the glass substrate were fixed in 1 mL of 2.5% buffered glutaraldehyde at 37ºC for 24 hours. Fixed cells were submitted to dehydration process with 1 mL of the respective hydroethanolic solutions 30, 50, 70, 95 and 100% for one hour each one. Then, cells were maintained in colloidal silica for 24 hours followed by sputter-coating with gold in a metallizer (Shimadzu, Kyoto, Japan). Subsequently, they were taken to the samples of the scanning electron microscope (Shimadzu, Kyoto, Japan) to evaluate the morphology of attached cells (Burattini & Falcieri, 2013; Pupo et al., 2017).

3. Results

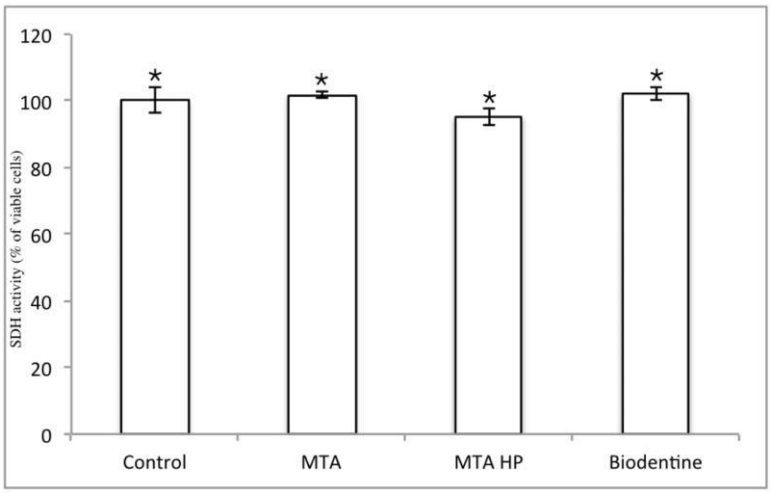
Statistical analysis

Data from the MTT assays for cytotoxicity and cytoprotection were expressed as percent viability over the negative control (culture media, 100%). Statistical analyzes were performed using SPSS software and the results were identified using non-parametric Kruskal-Wallis and Mann-Whitney tests ($\alpha=0.05$). A P value < .05 was considered significant.

Cytotoxicity of endodontic materials on fibroblast cells

The cellular viability of treatments performed with bioceramic materials was evaluated by the MTT method to obtain cytotoxicity. Figure 1 reports these results.

Figure 1: Viability of the 3T3 cell exposed to different root canal materials by the MTT method. * - p <0.05.



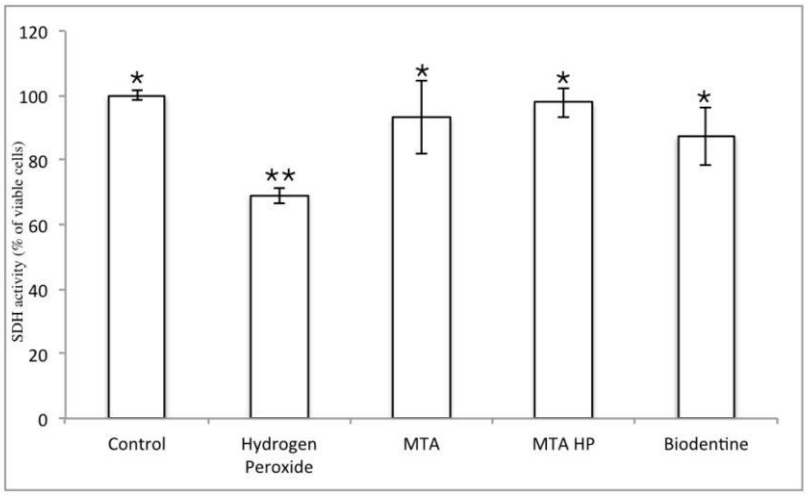
Source: Authors.

Treatments with the tested endodontic materials provide no variable responses on the metabolism of fibroblast 3T3 cells. Figure 1 shows the maintenance of cell metabolism observed in the control group. Kruskal-Wallis and Mann-Whitney analysis showed no statistically significant difference (*P<0.05*).

Cytoprotection assay

The regenerative capacity of the bioceramic materials evaluated was determined by the cytoprotection assay. Figure 2 shows a positive effect for all materials tested on the fibroblast cell protection capacity.

Figure 2: Cell response to H_2O_2 damage after treatment with endodontic materials. Hydrogen peroxide (Positive control group) and Bioceramic materials: MTA, MTA HP and Biodentine.



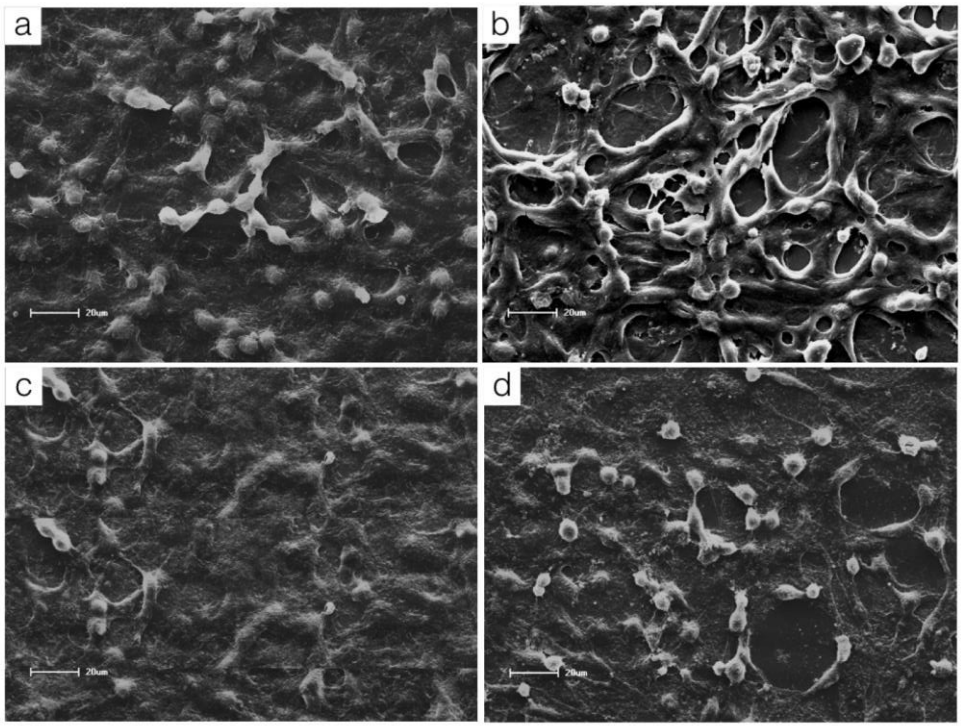
Source: Authors.

MTA, MTA Repair HP and Biodentine materials promoted cytoprotection to the 3T3 cells at the same magnitude (*P>0.05*). Their results showed an improved cell growth and were considered statistically different from the obtained cells treated with peroxide solution (positive control) (*P=0.046*). Also, viability results of the tested root canal materials were close to that of the negative control (cells treated only with culture media) (*P=0.046*) (Figure 2).

Morphological analysis by SEM technique

Cell morphological changes by treatments performed with bioceramic materials are likely to be observed through the SEM technique. Vacuolization, changes in fibroblast shape and size represent morphological damage to cells due to the treatment performed. Figure 3 demonstrates the images obtained by the SEM technique of the treated cells.

Figure 3: Panel of SEM micrographs of 3T3 fibroblasts (a) control group; (b) MTA, (c) MTA HP and (d) Biodentine. Representative images at 500x magnification.



Source: Authors.

Scanning electron microscopy revealed no significant morphological changes comparing to the control group analysis of fibroblasts exposed to MTA, MTA Repair HP, and Biodentine eluates. Cells from the control group (Figure 3a) or those treated with MTA (Figure 3b), MTA-HP (Figure 3c), and Biodentine (Figure 3d) revealed a consistent fibroblast growth, covering most of the surface and numerous mitoses are occurring. Cells were generally spindle-shaped with flattened and extended cellular processes that attached to the substrate.

4. Discussion

The materials developed to root canal filling that are in direct and permanent contact with the periradicular tissue must have the highest biocompatibility, since any product of cytotoxic degradation may cause cell and tissue damage, affecting the healing of the wound, as well the development of the root channel treatment (Waltimo et al., 2001). In vitro cytotoxicity and

cytoprotection tests are suitable tools for evaluating the basic biological characteristics related to biocompatibility. In fact, other factors should be considered, such as the structure and physical characteristics of the material (Gomes-Filho et al., 2009). This study was carried out to determine the cytotoxicity, surface morphology, cell adhesion and proliferation in calcium silicate-based endodontic cements. In this study, the test methodology used to determine viability was the MTT, which consists of the ability of mitochondrial enzymes present in living cells to convert tetrazolium yellow salt to blue formazan crystals. The advantages of this method are simplicity, fastness, and accuracy (Gomes-Filho et al., 2009).

The MTA is recommended extensively for vital pulp therapies, protecting scaffolds during regenerative endodontic procedures, apical barriers in teeth with necrotic pulps and open apices, perforation repairs as well as root canal filling and root-end filling during surgical endodontics (Torabinejad et al., 2018; Aravind et al., 2021; Dal-Fabro et al., 2021). The main components of the MTA include tricalcium silicate, tricalcium aluminate, tricalcium oxide and silicate oxide. In this study, the statistical analysis of the MTT test data did not show any differences between the three materials in 24 hours. The results obtained in this study are consistent with those obtained previously (Duarte et al., 2018; Espaladori et al., 2018), that showed non-cytotoxic effect of the MTA. The tested materials herein demonstrated a significant cytoprotection effect against the damage caused by hydrogen peroxide. Both MTA and MTA HP have demonstrated no cytotoxic effects, but considering to cytoprotection, MTA presented higher efficiency compared to MTA HP.

Biodentine is a bioactive material that has similar indications of MTA and in addition to being indicated as a tooth repairer, also works with temporary enamel restoration and definitive dentin replacement (Hasweh et al., 2018). In its composition, there is tricalcium silicate, calcium carbonate, zirconium oxide and calcium chloride (Rajasekharan et al., 2018). The results obtained from the statistical analysis of the Biodentine cytotoxicity in contact with fibroblastic cells did not show a cytotoxic effect, presenting the same results as the MTA, comparable to those found in other studies (Zbou et al., 2013; Attik et al., 2014; Valentim et al., 2021). Its viability was efficient, but did not exceed the result obtained by the MTA. Attik et al. (2014) showed in its study that the viability between the compounds remains the same up to 24 hours of incubation.

To conclude, the cytotoxicity, cytoprotection and structural morphology tests with the endodontic materials showed biocompatibility of the materials in contact with the fibroblast cells. In the cytotoxic environment, the response of the cell in contact with calcium silicate materials was positive, not affecting its metabolism. Statistically, this metabolic response was lower than 5%, comparing the control group and the cells treated with MTA, MTA Repair HP and Biodentine. The cytoprotective response of the 3T3 cells treated with the endodontic cements in contact with the hydrogen peroxide showed an efficiency greater than 95% compared to the positive control (cells treated with H_2O_2 only), however, the viability of the cements was close to the negative control (cells treated with culture media only). The methodology employed in the preparation of the MEV assay consisted of culturing 3T3 cells in coverslips and treating them with MTA, MTA Repair HP and Biodentine. After treatment, they were fixed, dehydrated, sputtered with gold and then taken to MEV (Shimadzu, Kyoto, Japan) for morphological evaluation. The micrographs did not present significant morphological alterations between the control group and the cells treated with the materials. The tests performed with the endodontic bioceramic materials presented positive results, highlighting the MTA for being more efficient compared to MTA Repair HP and Biodentine in the ambit of cytoprotection after 24 hours.

5. Conclusion

The use of endodontic materials MTA, MTA Repair HP and Biodentine for root canal treatment has demonstrated high bioactivity and biocompatibility, as presented in cytoprotection and morphological trials. We suggest future research with bioceramic materials, in vivo.

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