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Cytotoxic effects of new MTA-based cement formulations on fibroblast-like MDPL-20 cells

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Abstract: The present study aimed at evaluating the cytotoxic effects of a novel cement called CER on periodontal fibroblast-like cells of mice (MDPL-20), in comparison with different formulations of Mineral Trioxide Aggregate (MTA), by means of the cell viability test (MTT) and cell morphology analysis. Thirty-two round-shaped samples were fabricated with the following cements: white MTA, white and gray CER and experimental white MTA. The samples were immersed in serum-free culture medium for 24 hours or 7 days (n = 16). The extracts (culture medium + components released from the cements) were applied for 24 hours to previously cultured cells (40.000 cells/cm^2) in the wells of 24-well plates. Cells seeded in complete culture medium were used as a negative control. Cell viability was assessed using the MTT assay. Two samples of each cement were used for cell morphology analysis by Scanning Electron Microscopy (SEM). The extracts obtained at the 7-day period presented higher cytotoxicity compared with the 24-hour period ($p < 0.05$). The gray CER obtained at 24 hours presented the highest cytotoxic effect, whereas the experimental white MTA presented the lowest, similar to the control ($p > 0.05$). However, at the 7-day period, the experimental white MTA presented no significant difference in comparison with the other cements ($p > 0.05$). At the 7-day period, CER cement presented cytotoxic effects on fibroblast-like cells, similar to different MTA formulations. However, the immersion period in the culture medium influenced the cytotoxicity of the cements, which was greater for CER cement at 24 hours.

Keywords: Biocompatible Materials; Dental Materials; Endodontics; Silicate Cement.

Introduction

Mineral Trioxide Aggregate (MTA) cement was initially used as a root-end filling material and for root perforation treatment;[1] however, due to its favorable attributes, it has also been used for apexification,[2] root resorption treatment,[3] pulpotomy[4] and pulp capping in conservative procedures.[5]

Studies have reported that the success rate of pulp therapy using MTA is higher than that using calcium hydroxide-based materials.[6] Considering that MTA cements are applied in direct contact with connective tissues,

such as periodontal ligament and pulp, and that moisture from the surrounding tissue also acts as an activator of its bioactivity, the cytotoxic effects and the biological properties of this material have been the focus of extensive research.[7,8,9]

The ability of tissue to regenerate and the antibacterial property of MTA-based cements are related to ionic dissociation in calcium and hydroxyl ions.[10] The calcium ions released by the cement react and produce calcite granules, when the cement comes into contact with the carbon dioxide and carbonic acid from the cell catabolism.[7] In addition, there is a tendency for fibronectin to accumulate. This is a glycoprotein found in the tissue, and synthesized by fibroblasts and endothelial cells.[11] Glycoproteins allow adhesion, cell differentiation and growth, leading to mineralized tissue deposition.[11]

Despite the clinical success of MTA as a sealing cement, it has some disadvantages, such as a sandy consistency and a long setting time.[12] MTA takes approximately 180 minutes to set; however, its working time it less than 4 minutes.[12] A long exposure period of cells to the cement before its complete hardening may compromise its biological property, leading to an inflammatory reaction, which may affect its physical characteristics.[10]

A novel experimental cement, called CER, was developed to improve some undesirable characteristics of MTA cement, such as poor handling from porosities formed after manipulation,[1] making it highly unstable, high solubility,[9] long setting time,[10] and dental staining.[3,12] The name CER is the Portuguese acronym for "*cimento endodôntico rápido*", or "quick-setting endodontic cement" in English. This cement is basically composed of clinker (raw material used in the manufacture of Portland cement), barium sulfate (radiopacifier), water and an emulsifier (proprietary) to increase the handling characteristics of CER.[12]

Studies have reported that calcium and hydroxyl ions released by CER cement were similar to those released by conventional MTA-based cements.[12,13] In addition, CER presented greater ability to release calcium ions than MTA, when in contact with an aqueous environment. This feature could accelerate tissue repair in endodontic therapy.[12,13] The authors of these studies also reported that CER has a shorter setting time and improved handling, in comparison with MTA, in addition to a coefficient of thermal expansion similar to that of dentin, thus making it more suitable for sealing root perforations.[12,13] However, *in vitro* and *in vivo* studies are needed before a new dental material can be safely recommended for clinical practice, observing the research levels established by the associations and federations responsible for biological test standardization.[14]

In vitro cytotoxicity protocols, performed to compare materials and predict their safe clinical application, have been considered the initial tests needed to support further *in vivo* studies in animals.[14] The aim of this *in vitro* study was to evaluate the cytotoxic effects of CER cement on fibroblast-like MDPL-20 cells, in comparison with different MTA-based cement formulations, by means of the cell viability (MTT) test and cell morphology analysis. The null hypothesis tested was that there would be no difference in cytotoxicity promoted by the different cements tested.

Methodology

Cell culture

Periodontal ligament fibroblasts (MDPL-20) were cultivated in 75 cm^2 sterilized plastic bottles (Costar Corp. Cambridge, USA) in Dulbecco's Modified Eagle's Medium (DMEM - Sigma Chemical Co., St. Louis, USA) containing 10% fetal bovine serum (FBS, GIBCO, Grand Island, USA), 100 mg/mL of penicillin, 100 µg/mL of streptomycin, and 2 mol/L of glutamine (GIBCO), in a humidified atmosphere at 37°C, containing 5% CO_2 and 95% air. The cells were sub-cultured every three days, at a density of 3×10^4 cells/cm^2, to obtain the number of cells required to perform this *in vitro* study.

Cements extracts

Four endodontic sealing cements were selected to be evaluated in the present study: white MTA (Ângelus, Londrina, Brazil), white and gray CER, and the following formulation called Experimental White MTA (association of conventional white

MTA - Ângelus - powder with CER cement emulsifier). Since the emulsifier improves the handling characteristics of CER cement,[12] the cytotoxicity of Experimental White MTA was also assessed. Complete DMEM (Ambion - Life Technologies, Grand Island, USA) was applied to the cell and used as the control. The composition, the powder/liquid ratio and the setting time of the cements assessed in this study are shown in Table 1.

The tested cements were manipulated according to the manufacturer's recommendations to fabricate thirty-two standardized round-shaped samples (2 mm thick and 4 mm in diameter) of each dental material. After setting, the thirty-two samples of each cement were randomly distributed according to the periods (24 hours or 7 days) (n = 16) of DMEM immersion. These specific periods of immersion were used to correlate the calcium and hydroxyl ion release promoted by the cements throughout their setting process with their potential cytotoxic effects. Since the setting time of the tested cements may extend from 24 hours (initial setting time) up to 7 days (final setting time), the rate of calcium and hydroxyl ions released from the cements may range significantly, thus allowing for an increase in cement cytotoxicity.

The extracts were obtained from the tested cements by individually placing the round-shaped samples in the wells of 24-well plates containing 1.1 mL of DMEM culture medium without fetal bovine serum (DMEM-FBS), and incubating them for 24 hours or 7 days, at 37°C, 5% CO_2 and 95% air.

The fibroblast-like MDPL-20 cells were seeded (40.000 cells/cm^2) in the wells of 24-well sterile acrylic plates (Costar Corp., Cambridge, USA) and maintained in an incubator at 37°C, 5% CO_2 and 95% air, for 48 hours. After this period, the DMEM of each well was aspirated, and an aliquot of 400 µL from the extract of each cement and the negative group control (DMEM only) was applied to the cells for 24 hours. The protocols performed in this study followed the ISO 10993-5 (2009) recommendations.[15]

Cell viability assay (MTT)

Cell viability was assessed with the methyl tetrazolium colorimetric assay (MTT), which is characterized by the cytochemical demonstration of succinic dehydrogenase produced by the cells. After 24-hour exposure to the extracts or to the DMEM (negative control), the cultured fibroblasts were replaced by 900 mL of DMEM and 100 mL of MTT solution (5 mg/mL in phosphate buffered saline - PBS) (SIGMA Chemical Co., St. Louis, USA). After a 4-hour incubation period, the solution was replaced by 600 mL of isopropanol acidified with hydrochloric acid (0.04 N HCl) to dissolve the formazan crystals. Then, three aliquots of 100 µL from each compartment were transferred to 96-well plates (Costar Corp.) and the cell viability was assessed proportionally to the specific absorbance at 570 nm, in an ELISA reader (ELX 800 - Universal Microplate Reader, BioTek Instruments, Winooski, USA). The experiments were performed in triplicate, according to ISO 10993-5 (2009) recommendations.[15]

Table 1. Composition, powder/liquid ratio and setting time of cements evaluated in this study.

Commercial name	Composition	Powder/liquid ratio	Setting time
White MTA	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide and calcium sulfate dihydrate. Liquid: Distilled water.	1 g/30 µL	15 minutes
White CER	Powder: Clinker (dicalcium sulphate, tricalcium silicate) and barium sulphate. Liquid: Emulsifier.	600 mg/170 µL	7 minutes
Gray CER	Powder: Clinker (dicalcium sulphate, tricalcium silicate, tricalcium aluminate, tetracalcium ferroaluminate) and barium sulfate. Liquid: Emulsifier.	600 mg/170 µL	7 minutes
Experimental White MTA	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, calcium sulfate dihydrate. Liquid: Emulsifier.	1 g/30 µL	10 minutes

Cell morphology (SEM)

Two representative specimens from each group were submitted to cell morphology analysis, using a Scanning Electron Microscope (SEM) (ZEISS DSM-940A model, Oberkochen, Germany). For this purpose, 12 mm diameter glass coverslips (Fisher Scientific, Pittsburg, USA) were placed at the bottom of each well of a 24-well plate before seeding the fibroblasts. After 24-hour exposure of the cells to the extracts or to the DMEM, the solutions were aspirated and 1 mL of 2.5% buffered glutaraldehyde was applied to them. After 120 minutes, the fixative solution was aspirated and the cells were rinsed three times with 1 mL PBS (5 minutes each), and post-fixed with 1% osmium tetroxide solution for 60 minutes. Next, the cells were dehydrated by ascending exchanges of ethanol solutions (30%, 50%, 70%, 95% and 100%), and washed with solvent of low surface tension - 1,1,1,3,3,3-hexamethyldisilazane - (HMDS - Acros Organics, Fair Lawn, USA). The glass coverslips containing the attached cells were affixed to metal stubs and maintained overnight in a desiccator device. Afterwards, the samples were gold-sputtered, and cell morphology was carried out blindly by a single examiner.

Statistical analysis

The dataset for the viability of MDPL-20 cells did not present a normal distribution (Kolmogorov-Smirnov, $p < 0.05$). The data were then compiled and submitted to the non-parametric Kruskal-Wallis statistical test, complemented by the Mann-Whitney test (level of significance = 5%), using SPSS 19.0 software (SPSS Inc., Chicago, USA).

Results

Cell viability assay (MTT)

The cell viability data are shown in Table 2.

There was a significant reduction in the production of the SDH enzyme by the cells after 7 days of contact with the extracts ($p < 0.05$), in all the experimental groups. At the 24-hour period, white MTA produced the lowest toxicity, statistically comparable to the control group ($p > 0.05$), whereas the white and gray CER cements presented the highest cytotoxic effects on the fibroblast-like MDPL-20 cells. However, at the 7-day period, white MTA presented a toxic potential similar to that of white and gray CER cements, with no statistical difference among them ($p > 0.05$).

Cell morphology (SEM)

The morphology of MDPL-20 cells can be observed in Figure.

Two representative specimens of each cement and of the control group were selected to assess the morphology of the fibroblasts that remained adhered to the glass substrate. In the negative control group, a number of cells remained adhered to the glass substrate during both periods evaluated, displaying numerous long and slender cytoplasmic processes, characteristic of typical fibroblasts (Figure E/F). Similar morphology was observed in the cells exposed to the extracts from the treated groups, irrespective of the analysis period. No cytoplasmic shrinkage was seen; however, a noteworthy decrease was observed in the number of cells attached to the glass substrate, for all treated groups, in comparison with the negative control group (Figure A-D).

Table 2. Mean values of succinate dehydrogenase enzyme (SDH) production by cells exposed to the cement extracts (24 hours or 7 days) or to DMEM.

Cement	Period	
	24 hours	7 days
White MTA	0.2421 (0.2271-0.2587) ^{a,C}	0.0988 (0.0878-0.1039) ^{b,A}
White CER	0.2090 (0.1884-0.2273)* ^{a,AB}	0.1034 (0.0965-0.1127) ^{b,AB}
Gray CER	0.1857 (0.1807-0.1956) ^{a,A}	0.1014 (0.0995-0.1063) ^{b,A}
Experimental White MTA	0.2137 (0.1952-0.2228) ^{a,B}	0.1135 (0.1133-0.1170) ^{b,B}
Negative Control (DMEM)	0.2507 (0.2273-0.2921) ^{a,C}	0.1728 (0.1670-0.1749) ^{b,C}

*Median (interquartile range P25/P75).
Different lowercase letters in lines and uppercase letters in columns indicate a statistically significant difference (Mann-Whitney test, $p < 0.05$). n = 16.

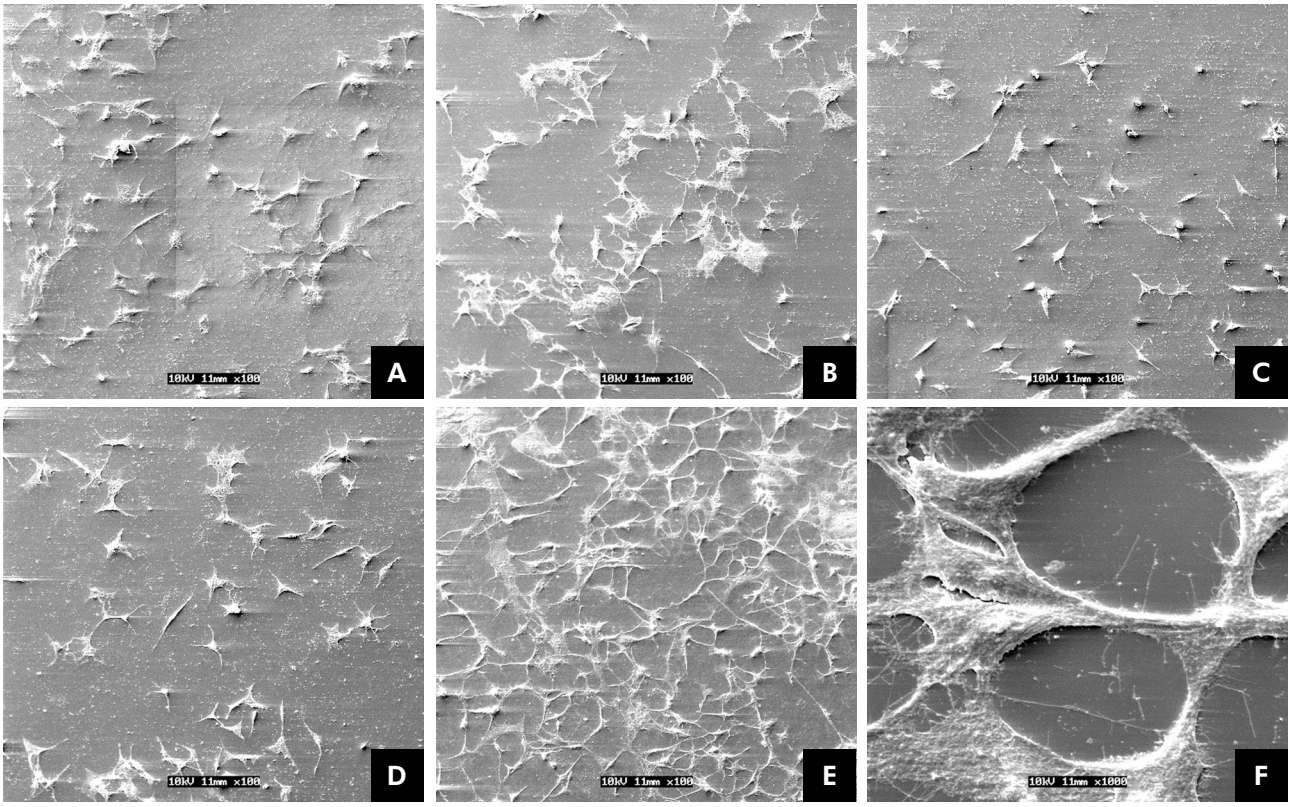

Figure. Representative SEM images of MDPL-20 cells exposed to the extracts from the experimental cements or the DMEM at 24-hour and 7-day periods. (A) White MTA: Only a few cells remained adhered to the glass substrate, in comparison with the negative control group, irrespective of the period of analysis. Note the typical morphology of fibroblasts - wide cytoplasm with several small cytoplasmic processes. (B) White CER: Significant decrease in the number of cells adhered to the glass substrate, in comparison with the negative control group. (C) Gray CER: Observe the similar morphology of cells exposed to the control extracts, despite the lower number of adhered cells. (D) Experimental White MTA: As found in the other experimental groups, a reduction was observed in the number of cells adhered to the glass substrate, in comparison with the negative control group, with no cell morphology alterations. (E) Negative control group: Note the greater number of cells adhered to the substrate than in the experimental groups. (F) High magnification of (A): the cells adhered to the glass exhibit long and slender cytoplasmic processes, typical of MDPL-20 fibroblasts, irrespective of the period of analysis.

Discussion

This study aimed at evaluating the cytotoxic effects of a novel cement called CER on periodontal fibroblast-like cells, in comparison with different formulations of MTA. Based on the results obtained, it can be stated that the null hypothesis tested was partially accepted, since there was a significant difference in the cytotoxicity of the cements, but only at the 24-hour period.

Since MTA cement is recommended for diverse endodontic therapies, its biological properties have been evaluated by several authors using different cell lines, such as MDPC-23 and OD-21 pulp cells, periodontal ligament fibroblasts (MDPL-20) and MG-63 osteoblasts.[16,17] These studies have reported a reduced toxic effect of the different types of MTA cements available in the dental market.[16,17]

In general, the present study demonstrated greater cell viability at the 24-hour period, in comparison with the 7-day period. This may be explained by the longer exposure period of cells to the extracts obtained from the cements. Fibroblasts exposed for 24 hours to the extracts from conventional white MTA showed less cytotoxicity in comparison with the other cements. Despite the intense cytotoxicity caused by the gray version of CER cement at the 24-hour period, both gray and white versions presented a toxic potential similar to that of the different MTA formulations assessed at the 7-day period.

The calcium and hydroxyl ion diffusion promoted by the MTA cement increases the pH adjacent to the periodontal tissues, possibly acting on osteoclastic activity to promote alkalinization of the medium, which favors the healing process.[17,18] It is known that CER cement releases calcium and hydroxyl ions, and increases the pH of the medium in a manner similar to that of MTA cement.[13] After 24 hours, the calcium and hydroxyl ions released by CER cement are greater than those released by MTA; however, following this initial period, the values become similar.[13]

Despite the important role of calcium ion release in the tissue repair process, the high rate of calcium and hydroxyl ions released in the culture medium probably resulted in irreversible damage to the cells,[16,17] demonstrating that the association of cells to the interaction period is significant,[13] as can be seen in the present study. Since the setting time of the tested cements may extend from 24 hours (initial setting time) up to 7 days (final setting time), the rate of calcium and hydroxyl ions released from the cements may vary significantly, thus allowing for an increase in cement cytotoxicity.[16,17] On the other hand, a previous *in vivo* study[18] reported a favorable histological response promoted by CER and MTA at the 7-day period. However, it is worthwhile stressing that *in vitro* conditions are not homeostatic, and that there is no tissue elimination of toxic substances, such as that reported for *in vivo* assays.[19] In addition, a living host possesses defense mechanisms and a lymphatic system that removes the toxic substances released.[19]

Several changes in the surface composition and the morphology of MTA cement can lead to variations in its biological response and may result in clinically relevant differences.[20] The conflicting results observed in this *in vitro* study for the different cements could be also related to the emulsion added, which probably modified the chemical properties of the materials.[12,13] With this in mind, the chemical changes made in the cement composition were designed to yield dental materials with good handling characteristic and adequate working time, associated with low cytotoxicity.[21]

The present study demonstrated that CER and the experimental MTA formulation not only presented better physical and chemical properties than the conventional white MTA,[12,13] but also exhibited greater toxicity when applied to fibroblast-like MDPL-20 cells than the reference material, at the initial period. The cytotoxicity of the tested cements at the final period of analysis was similar. Since time is a relevant factor in determining cement cytotoxicity, future studies are needed to analyze these new MTA-based cement formulations over longer periods of time.

Conclusion

According to the experimental conditions and the methodology employed in this *in vitro* study, it was concluded that CER cement presented cytotoxicity similar to that of different MTA formulations at a 7-day period. However, the immersion period influenced the cytotoxicity of the cements, which was greater for CER cement at 24 hours.

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