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Analysis of radiopacity, pH and cytotoxicity of a new bioceramic material

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ABSTRACT

Objective: RetroMTA® is a new hydraulic bioceramic indicated for pulp capping, perforations or root resorption repair, apexification and apical surgery. The aim of this study was to compare the radiopacity, pH variation and cytotoxicity of this material to ProRoot® MTA. Material and Methods: Mixed cements were exposed to a digital x-ray along with an aluminum stepwedge for the radiopacity assay. pH values were verified after incubation period of 3, 24, 48, 72 and 168 hours. The cytotoxicity of each cement was tested on human periodontal ligament fibroblasts using a multiparametric assay. Data analysis was performed using ANOVA and Tukey's *post hoc* in GraphPad Prism. Results: ProRoot® MTA had higher radiopacity than RetroMTA® ($p<0.001$). No significant differences were observed for the pH of the materials throughout experimental periods ($p>0.05$) although pH levels of both materials reduced over time. Both ProRoot® MTA and RetroMTA® allowed for significantly higher cell viability when compared with the positive control ($p<0.001$). No statistical difference was observed between ProRoot® MTA and RetroMTA® cytotoxicity level in all test parameters, except for the ProRoot® MTA 48-hour extract media in the NR assay ($p<0.05$). Conclusion: The current study provides new data about the physicochemical and biological properties of Retro® MTA concerning radiopacity, pH and cytotoxic effects on human periodontal ligaments cells. Based on our findings, RetroMTA® meets the radiopacity requirements standardized by ANSI/ADA number 57[2], and similar pH values and biocompatibility to ProRoot® MTA. Further studies should be performed to evaluate additional properties of this new material.

Keywords: Dental materials. Endodontics. Physical and chemical properties. Cytotoxicity.

INTRODUCTION

Mineral trioxide aggregate (MTA) is considered a gold standard material for several endodontic applications such as pulp capping, perforations or root resorption repair, apexification and apical surgery[9]. The main elemental components of MTA are Portland cement, bismuth oxide and gypsita[23,24]. MTA is commercially available as ProRoot® MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) and MTA-Angelus® (Angelus, Londrina, PR, Brazil) in white and gray colors. However, studies have reported on MTA as having difficult handling characteristics[18], delayed setting time (around 150 min)[14], elevated cost[15,18] and tooth discoloration[19].

The possibility of tooth discoloration associated with the use of MTA has been attributed to the presence of bismuth oxide (radiopacifying agent) in its composition, and raises a major concern in clinical practice as it may negatively impact the patients' anterior esthetics. Marciano, et al.[19] (2014) analyzed the color change in the tooth structures induced by bismuth oxide and white MTA-Angelus®, as well as the interaction of the radiopacifying agent (bismuth oxide) with collagen, the main constituent of the dentin. Spectrophotometer was used for the color assessment of the tooth structure, and visual observation was used for the color assessment of the chemical interaction between bismuth oxide and collagen. The authors concluded that the white MTA-Angelus® caused discoloration and dentin stain. Further, they showed that collagen reacts

with bismuth oxide, resulting in a grayish color, and therefore suggested the use of an alternative radiopacifier in MTA formulations[19].

RetroMTA® (BioMTA, Seoul, Korea) has recently been introduced in the market as a new hydraulic bioceramic material proposed for use in similar endodontic applications as MTA (pulp capping, perforations or root resorption repair, apexification and apical surgery). However, unlike MTA, this material does not contain Portland cement, and hydraulic calcium zirconia is included as a radiopacifying agent. According to the manufacturer, RetroMTA® is ideal for aesthetic repair, since it has no discoloration and has a fast setting (initial setting time of 150 seconds), which would be beneficial considering the moist environment of the oral cavity.

There are only two studies in literature reporting on some of the physicochemical and biological properties of RetroMTA®. Kang, et al.[15] (2015) compared the discoloration of ProRoot® MTA with MTA-Angelus®, ENDOCEM Zr® (Maruchi, Wonju, Korea) and RetroMTA®. Test samples of the four materials were analyzed regarding changes in color after being irradiated with light for 15 and 30 minutes. *In vitro* tooth discoloration was also evaluated after filling the pulp chambers with the materials and measured for a 16-week period. From their results, these authors concluded that RetroMTA® and ENDOCEM Zr® showed less discoloration than ProRoot® MTA and MTA-Angelus® in both experiments[15]. Ghorbanzadeh, et al.[11] (2015) evaluated the marginal adaptation of ProRoot® MTA, OrthoMTA® (BioMTA, Seoul, Korea) and RetroMTA® when used as retrofilling materials. Single-rooted teeth were retrofilled with either ProRoot® MTA, OrthoMTA® or RetroMTA® and stored in phosphate buffer saline for one week or for two months until evaluation of the marginal adaptation of each test material to dentin under scanning electron microscope. Results showed that all of the tested materials presented similar marginal adaptation for both time periods[11].

The composition of RetroMTA® seems to be promising in several aspects, such as fast setting time and no discoloration, hence it could be a possible substitute to MTA. Therefore, the aim of this study was to evaluate the radiopacity, pH variation and cytotoxicity of RetroMTA® in comparison to ProRoot® MTA.

MATERIAL AND METHODS

Material

The chemical composition of ProRoot® MTA and RetroMTA® are presented in Figure 1. Both materials were mixed according to the manufacturer's instructions. Briefly, the manufacturers' instructions for RetroMTA® manipulation cite mixing 0.3 g of powder with 3 drops of the liquid for 20 seconds with the use of a plastic spatula.

Radiopacity assays

Mixed samples (n=3 *per* group) were placed into stainless steel rings (10 mm in diameter and 1 mm in height) and incubated at 37±1°C and 95% relative humidity for 24 hours. The samples were placed onto an occlusal phosphor plate along with an aluminum stepwedge with 1 mm of increments (1 to 9 mm). Radiographic images were taken with Focus™ X-ray (Instrumentarium Dental, Tuusula, Finland) at 70 kVp and 7 mA, and a 30 cm focus-film distance. Images were analyzed with AxioVision Rel. 4.6 Software (Zeiss, Jena, Germany). The gray pixel values of three points from each sample image and the aluminum step from the stepwedge were measured, and the averages calculated. The average pixel values were converted into millimeters of Aluminum (mm Al), as previously described[6]. Then, a graph of the radiographic density versus the thickness of the aluminium stepwedge was plotted to all obtained radiographs, and a calibration curve was generated using logarithmic regression. The obtained equation was used to calculate the radiopacity of the materials in mm of Al[6].

pH analysis assays

Mixed samples (n=5 *per* group) were placed into plastic tubes (1.0 mm of internal diameter and 1 cm of length) using an endodontic file. Samples

Material	Composition*	Manufacturer
ProRoot MTA	Portland cement 75% Calcium sulfate dehydrate 5% Bismuth oxide 20%	Dentsply Tulsa Dental, Tulsa, OK, USA
RetroMTA	Calcium Carbonate 60%-80% Silicon dioxide 5%-15% Aluminium oxide 5%-10% Calcium zirconia complex 20%-30%	BioMTA, Seoul, Korea

*According to the manufacturer's material safety datasheet

Figure 1- Chemical composition of the materials used in the study

were immersed in a glass vial with 10 ml of distilled water and incubated for 3, 24, 48, 72 hours and 7 days. After each experimental period, the pH was evaluated with a pH meter (Accumet basic AB 15, Fisher Scientific, Pittsburgh, PA, USA) and samples were placed in a new vial with fresh water.

Multi-parametric cytotoxicity assays

Preparation of cement elutes (extract media)

Cement elutes were prepared as previously described[29]. In short, cements were mixed inside a laminar flow hood and inserted into 1000 µl pipette tips (VWR, Radnor, PA, USA) that were cut at 2.2 cm from their final segment. The tip with the cement was attached to the lid of a microcentrifuge tube using an o-ring (5 mm in diameter and 2 mm in height). Upon closing of the lid, the tip containing the cement was immersed in the tube containing 0.5 ml of Dulbecco's Modified Eagle Medium (DMEM) (ATCC, Manassas, VA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 U/mL penicillin (PEN) and 100 mg/mL streptomycin (STREP) (Sigma-Aldrich, Saint Louis, MO, USA). Each cement sample was incubated at 37°C, 95% humidity and 5% CO_2 for 24 and 48 hours. After each incubation period, the samples were removed from the media and the extract media were briefly vortexed, transferred to a new microcentrifuge tube and stored at -20°C until further use.

Cell culture

Human periodontal ligament fibroblasts (HPDL) were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL PEN and 100 mg/mL STREP and incubated at 37°C, 95% humidity and 5% CO_2.

Cell viability assays

HPDL fibroblasts were seeded at a density of $2x10^4$ cells/well in a 96-well plate and incubated for 24 hours. Next, 200 µl of extract media of each test material was added to each well and incubated for an additional 24 hours. Fresh culture media was used as a negative control, and 0.1% sodium dodecyl sulfate (SDS) (Bio-Rad, Hercules, CA, USA) as a positive control. All experiments were performed in triplicate and in three independent reactions.

The cytotoxicity of the test materials was evaluated using a multi-parametric assay kit (In Cytotox XTT-NR-CVDE, Xenometrix, Allschwill, Switzerland) as follows: first, the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfopheny)-2H-tetrazolium-5-carboxyanilide inner salt] assay measures the ability of viable cells to convert XTT, a tetrazolium salt, into formazan by the succinate dehydrogenase, which belongs to the mitochondrial respiratory chain; second, the Neutral Red assay (NR) measures the ability of viable cells to incorporate and bind NR within lysosomes; and finally, the Crystal Violet Dye Elution (CVDE) assay stains viable DNA and provides quantitative information about the cell density[8]. The results were analyzed using an ELISA plate reader (Dynex Tecnologies, Chantilly, VA, USA). Cell viability was calculated in function of the negative control [cell viability % = mean optical density (OD) of test sample × 100 / mean OD of negative control]. According to the ISO standard number 10993-5[13], a reduction in cell viability by more than 30% is considered to have a cytotoxic effect.

Statistical analysis

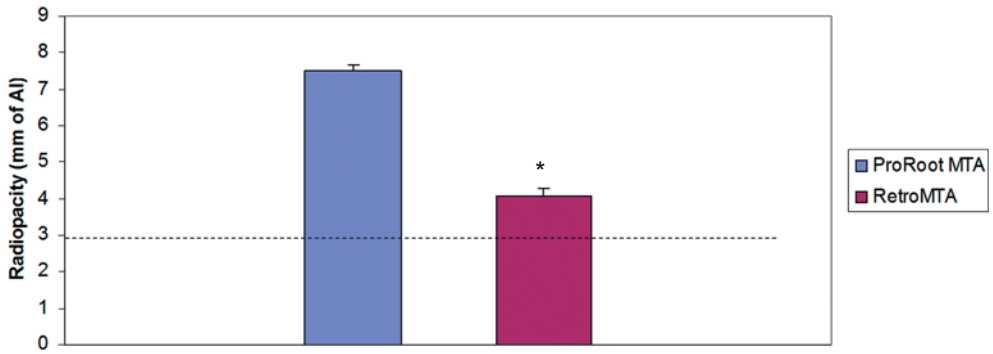
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as implemented in GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The significance level was established at 5% ($p \leq 0.05$).

RESULTS

Figure 2 shows the mean values and standard deviations of the radiopacity levels for ProRoot® MTA and RetroMTA® in mm of Al. While both materials achieved the minimum required radiopacity value of 3 mm of Al, as recommended by ANSI/ADA[2], ProRoot® MTA showed significantly higher radiopacity values (7.52±0.15 mm of Al) when compared to RetroMTA® (4.07±0.20 mm of Al) ($p<0.001$).

There were no significant differences in the pH levels of ProRoot® MTA and RetroMTA® throughout the experimental periods ($p>0.05$). The pH of ProRoot® MTA varied from 9.93 to 8, while the values for RetroMTA® varied from 9.93 to 7.9. It is worth noting that the pH of both materials tended to decrease over time (Figure 3).

Both ProRoot® MTA and RetroMTA® allowed for significantly higher cell viability when compared with the positive control ($p<0.001$). When comparing the XTT, NR and CVDE values for ProRoot® MTA and RetroMTA® among the experimental periods, no significant differences were found, except for the 48-hour extract media in the NR assay, in which ProRoot® MTA showed significantly lower cell viability ($p \leq 0.05$) (Figure 4).



*Significantly different p<0.001

Figure 2- Radiopacity of ProRoot® MTA and RetroMTA® in mm of Al. Dashed line represents the radiopacity of 3 mm of Al as recommended by ANSI/ADA No. 57[2].

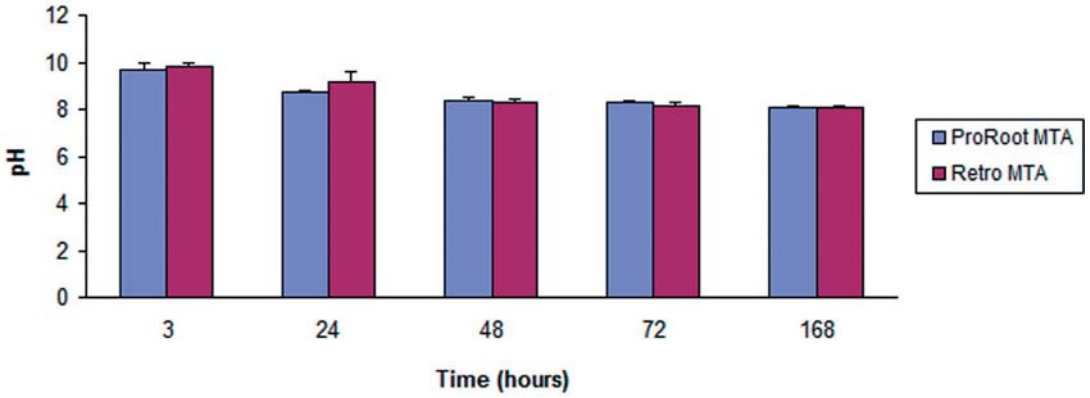


Figure 3- Results of pH analysis over time

DISCUSSION

In this study, we evaluated the radiopacity, pH variation and cytotoxicity of RetroMTA® and ProRoot® MTA, and provided novel information on some physicochemical and biological properties of this new material.

ANSI/ADA specification number 57[2] recommends that an endodontic material must have a radiopacity value higher than 3 mm of Al. Both ProRoot® MTA and RetroMTA® met the established criteria, however ProRoot® MTA showed significantly higher radiopacity when compared to RetroMTA®. Our results for the radiopacity of ProRoot® MTA are in agreement with previous reports using similar methodology[4,5,14]. Although this is the first report on the radiopacity of RetroMTA® in the scientific literature, our values observed for radiopacity are similar to the values presented by the manufacturer (5 mm of Al).

Moreover, according to the manufacturers, the pH of ProRoot® MTA is ~12, and the pH of RetroMTA® is initially 12.5, decreasing to pH 8 after four weeks. To evaluate the pH of the materials at different time periods, distilled water was changed after each reading, thus the reading would reflect the increase over that period[27]. In the present study, the pH of the tested materials was alkaline, varying from 9.93 to 8 for ProRoot® MTA and from 9.93 to 7.9 for RetroMTA®. The lower pH levels observed here when compared to the manufacturers values may be explained by the different methodological approaches used. In this study, to simulate the surface area of the material exposed during clinical use, the cements were placed in plastic tubes for the analysis, thus decreasing the surface area of the material in contact with the liquid[20,27], which may have contributed to the lower pH values observed. Our findings corroborate previous studies with similar sample sizes in which lower pH values for the ProRoot® MTA were observed when compared to the manufacturer's product specifications[10,22,26]. It is worth noting that both materials showed a decrease in the pH over time. The cements were immersed in distilled water while fresh, allowing the material surface to be exposed before setting of the material. Further, this allows the release of hydroxyl ions, thus increasing the pH. Over time, the pH decreases, probably because the setting is complete[27].

We also evaluated the cytotoxicity of ProRoot® MTA and RetroMTA® using a multiparametric cytotoxicity assay which allows the simultaneous evaluation of the toxic effects of tested materials

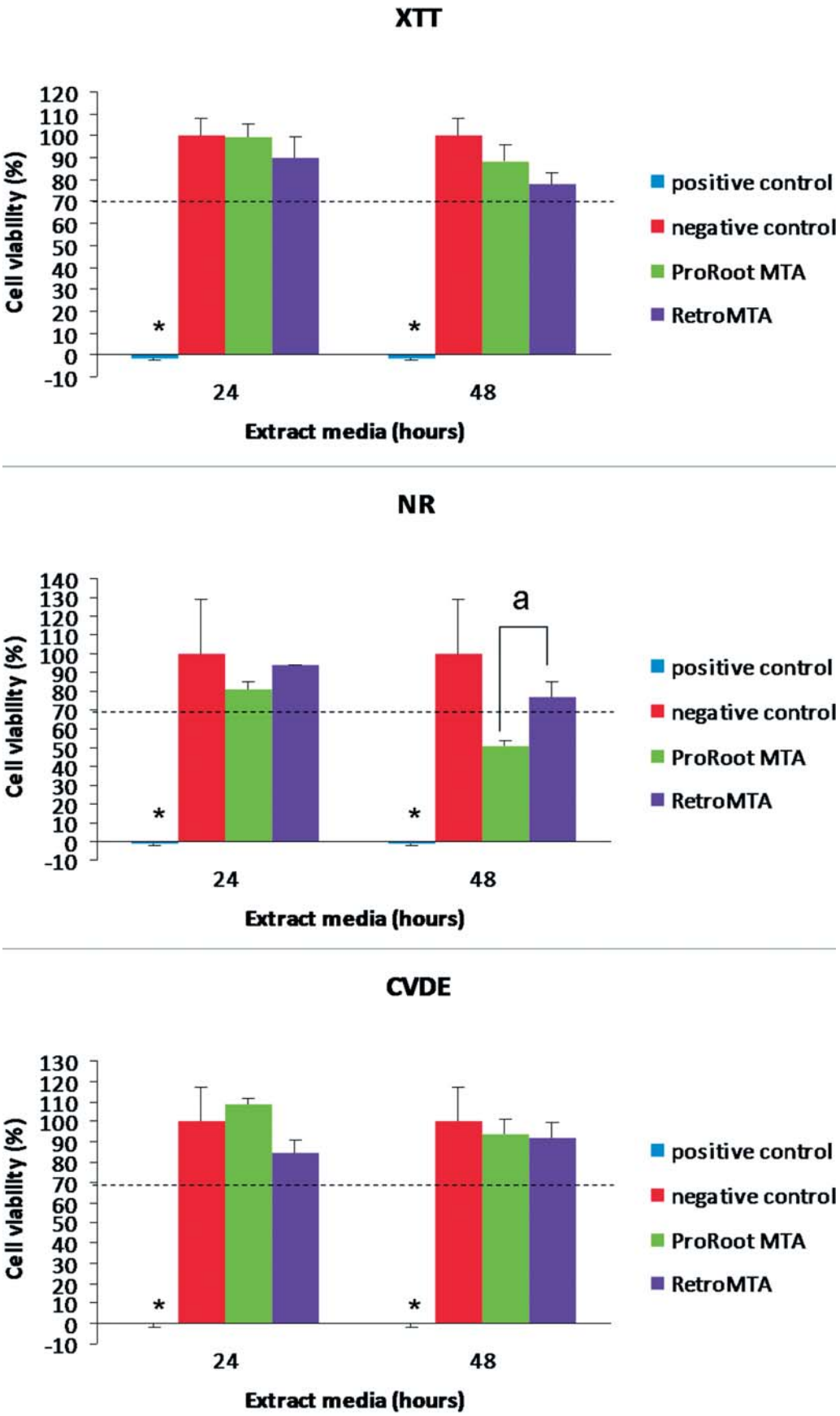


Figure 4- Cell viability (%) after exposure to each material extract media. *Significantly different from all groups ($p<0.0001$). a, significantly different between test materials ($p<0.05$). Dashed line represents the 70% cut-off level established by ISO 10993-513

in the same sample through three different parameters: mitochondrial metabolism and respiratory toxicity (XTT), lysossomal integrity and membrane permeability (NR), and cell proliferation and presence of DNA (CVDE). Our results showed that both ProRoot® MTA and RetroMTA® promoted significantly higher cell viability when compared to the positive control. No statistically significant differences were observed when comparing XTT, NR and CVDE values for ProRoot® MTA and RetroMTA®, except for the 48-hour period in the NR assay. At this experimental period in the neutral red assay, the cell viability decreased in the ProRoot® MTA group, although no major decrease in the XTT and CVDE values were observed, corroborating the findings of De Deus, et al.[8] (2009). The observed lower viability in the neutral red assay may indicate some adverse effect of ProRoot® MTA on membrane integrity that could contribute to possible cell toxicity *in vitro*. Nonetheless, the effect alone may not be enough to provoke damage to normal cell function, as mitochondrial activity values were within normal limits and no effects of DNA damage were suspected in the XTT and CVDE assays. Moreover, the excellent *in vivo* biocompatibility of ProRoot® has been reported on several studies[17,21,25,30]. The main advantage of this multiparametric assay in comparison to other commonly used cytotoxicity assays [i.e. MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] is that it provides information about the specific biological mechanisms through which the materials may be cytotoxic[8,9]. Although the MTT assay measures the mitochondrial metabolic activity rate as an estimate of cell viability, an observed excess of metabolic activity may also represent a response to increased cellular stress due to toxicity, thus caution is recommended with the use of MTT as its estimation of cell viability may be misleading[28,29]. Moreover, the MTT requires the cells to be killed, making it impossible to follow up on the cells in culture[1], and yet requires an extended incubation time with lower sensitivity than the XTT[3]. Our experimental model designed to obtain extract media for the cell viability assay and the use of human periodontal ligament cells resembles a clinical scenario, simulating the amount and surface area of the material that usually comes in contact with surrounding tissues in most clinical applications[8,9,12,29]. Further, the use of the extract media also simulates the scenario in which toxic elements released from the materials could be leaching into the periodontal ligament[16]. Recently, Chung, et al.[7] (2015) also investigated the cytotoxicity of calcium-silicate cements including ProRoot® MTA and RetroMTA® on human pulp-derived cells using a XTT assay. Human pulp-derived cells were grown in direct contact with the material, Dycal, or no cement for seven days. Initial cell attachment, viability, calcium release, and the levels of vascular endothelial growth factor (VEGF), angiogenin, and basic fibroblast growth factor (FGF-2) were evaluated. The cell viability was tested with freshly mixed and set materials after three and seven days. These authors reported that the overall biocompatibility of RetroMTA® was similar to those of the control and ProRoot® MTA, corroborating our findings.

CONCLUSION

The current study provides new and important data about the physicochemical and biological properties of RetroMTA® concerning radiopacity, pH and cytotoxic effects on human periodontal ligaments cells. Based on our findings, RetroMTA® meets the radiopacity requirements stipulated by ANSI/ADA[2], and presents similar pH values and biocompatibility to ProRoot® MTA. Further studies should be performed to evaluate additional properties of this new material.

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The authors deny any conflicts of interest related to this study.

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