Retractability of 2 Mineral Trioxide Aggregate–based Root Canal Sealers: A Cone-beam Computed Tomography Analysis

Prasanna Neelakantan, MDS, Deeksha Grotra, BDS, and Subash Sharma, BDS

Abstract

Introduction: The retractability of recent calcium silicate or mineral trioxide aggregate (MTA) sealers has not yet been assessed. The aim of this study was to evaluate the removal of 2 MTA-based sealers (MTA Fillapex [Angelus Soluções Odontológicas, Londrina, PR, Brazil] and MTA Plus [Prevest-Denpro, Jammu City, India]) using a rotary retreatment system, considering an epoxy resin sealer (AH Plus [Dentsply Maillefer, Ballaigues, Switzerland]) as the standard for comparison.

Methods: Root canals in 45 single-rooted teeth were instrumented using a rotary nickel-titanium system (MTtwo; VDW GmbH, Munich, Germany) and obturated with gutta-percha using one of the following sealers (n = 15): group 1, MTA Fillapex; group 2, MTA Plus; and group 3, AH Plus. The teeth were scanned using a cone-beam computed tomography scanner. After 2 months, the root canals were retreated with a rotary retreatment system (ProTaper Universal Retreatment; Dentsply Maillefer, Ballaigues, Switzerland) and a second cone-beam computed tomography scan was performed to assess the amount of remaining root filling material (in percentage) and dentin removal (in cubic millimeters). The time taken to reach the working length was calculated in minutes. Group comparisons were performed using 1-way analysis of variance and the Student-Newman-Keuls post hoc test (P = .05).

Results: There was a significant difference in the amount of remaining root filling material between the 3 groups (P < .05), with group 1 showing the least amount of root filling material (1.8% ± 0.22%) and group 3 showing the highest (10.4% ± 0.71%). The amount of dentin removal and the time taken to reach the working length was significantly higher in group 3 than in groups 1 and 2 (P < .05). There was no significant difference between groups 1 and 2 in these outcome variables (P > .05).

Conclusions: The rotary retreatment system evaluated was not able to completely remove any of the sealers. MTA Fillapex showed less remaining root filling material than MTA Plus. (J Endod 2013;39:893–896)

Key Words
Calcium silicate, computed tomography, cone-beam computed tomography, mineral trioxide aggregate, retreatment, root filling, sealer

Non-surgical revision of endodontic treatment (orthograde retreatment) aims at complete removal of root canal filling material to regain access to the apical foramen in order to facilitate cleaning and shaping of the root canal system. Several cross-sectional epidemiologic studies have shown radiographic signs of apical periodontitis in root-filled teeth, thereby indicating the need for the revision of root canal treatment (1, 2).

Gutta-percha in conjunction with sealers is the most common root filling material (3). More recently, calcium silicate–based materials such as mineral trioxide aggregate (MTA)-based sealers have been developed, and these materials have been claimed to be biocompatible, to stimulate biomineralization, and to offer a superior seal (4). Furthermore, these materials have been shown to exhibit better bond strengths to dentin than zinc oxide–eugenol–based cements (5) and a sealing ability similar to epoxy resin–based sealers (6). There are also indications for obturating the entire canal with MTA-based materials (7, 8).

MTA Fillapex (Angelus Soluções Odontológicas, Londrina, PR, Brazil) is a 2-paste resin sealer that contains MTA, salicylate resin, natural resin, bismuth, and silica. MTA Plus (Prevest-Denpro, Jammu City, India) is a calcium silicate–based material that is available as a powder-liquid formulation. This material has a finer particle size than other commercially available versions of MTA (50% of the particles finer than 1 μm). A salt-free water-soluble gel is provided as the mixing vehicle to improve the washout resistance of the material (9). Although studies have been performed that address the removal of gutta-percha and zinc oxide–eugenol and resin sealers, no study has addressed the removal of MTA-based sealers from root canals. This is important considering the widespread use of MTA-based materials in contemporary endodontics because of its ability to undergo biomineralization (10–12). Because MTA-based materials are known to be hard upon setting (13), the ability to retreat canals obturated with these sealers is an area of concern that needs to be addressed.

The aim of this study was to evaluate the retractability of 2 MTA-based sealers (ie, MTA Fillapex and MTA Plus) using cone-beam computed tomography (CBCT), considering an epoxy resin sealer (AH Plus; Dentsply Maillefer, Ballaigues, Switzerland) as the standard for comparison. The null hypothesis was that there is no significant difference in the removal of these materials from the root canal system.

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Materials and Methods

Specimen Preparation and Root Filling

Human single-rooted maxillary canines (N = 45) were collected and thoroughly cleaned by removing the hard deposits using curettes and the soft deposits by soaking in 5.25% NaOCl for 10 minutes. This was done as a routine protocol to disinfect the teeth for operator safety. The teeth were decoronated at the cementoenamel junction using a diamond disc under water cooling. The root lengths were standardized to 17 mm. The teeth were radiographed (DSX 730; Owandy Dental Imaging, Champs sur Marne, France; and Kodak 2100 X ray unit, Kodak Dental Systems, Atlanta, GA) at different angulations to confirm the presence of a single canal. The working length was established using a size 10 K-file (Mani Inc, Tochigi, Japan) to the root canal terminus and subtracting 0.5 mm from this measurement. The root canals were instrumented using MTtwo nickel-titanium rotary instruments (VDW GmbH, Munich, Germany) up to size 35/0.06 taper. Irrigation was performed using a 5-mL disposable plastic syringe (Ultradent Products Inc, South Jordan, UT) with a polypropylene capillary tip (Ultradent) placed passively into the canal, up to 2 mm from the apical foramen without binding. Irrigation was performed applying 5 mL 3% sodium hypochlorite during hypromolysis. After this, all specimens were irrigated with 5 mL 17% EDTA for 2 minutes. Canals were rinsed with 5 mL distilled water and dried using paper points (Dentsply Maillefer).

The roots were inserted into moistened foam to simulate the soft tissues and randomly divided into 3 groups (n = 15) based on the material used for obturation: group 1 (gutta-percha with MTA Fillapex), group 2 (gutta-percha with MTA Plus), and group 3 (gutta-percha with an epoxy resin sealer [AH Plus]). The root canals were dried with sterile paper points and obturated with gutta-percha and sealer using the lateral compaction technique. A standardized gutta-percha master cone size 30 was fitted with tug back at the working length. The sealers were introduced into the canal using a Lentulo spiral (Dentsply Maillefer). Cold lateral compaction with accessory gutta-percha cones size 15 was performed until these could not be introduced more than 5 mm into the root canal. The excess gutta-percha was removed with a heated plugger to ensure a standardized length of the root fillings (15 ± 1 mm). Subsequently, the quality and apical extent of the root canal filling were assessed with digital radiographs in the buccolingual and mesiodistal directions. The teeth were radiographed (DSX 730; Owandy Dental Imaging, Champs sur Marne, France) at different angulations to verify the quality of the filling procedure. Specimens exhibiting voids were discarded, and fresh specimens were prepared. All endodontic procedures were performed by the same operator.

CBCT Scans

All the teeth were scanned by a CBCT scanner (i-CAT; Imaging Sciences International, LLC, Hatfield, PA) with constant thicknesses of 125 µm/slice. The teeth were viewed both cross-sectionally and longitudinally. Volume rendering and multiplanar volume reconstruction were performed to calculate the volume of root filling material.

The specimens of groups 1 and 2 were wrapped in pieces of gauze soaked in phosphate-buffered saline (PBS) solution (pH = 8.4) and introduced into plastic vials with foam moistened with 10 mL PBS. The obturated roots of all 3 groups were stored in 100% humidity at 37°C for 1 month. After 2 months of storage, the specimens of each group were retreated as described later.

Retreatment Techniques

ProTaper instruments were used with 300 rpm. The D1 ProTaper file (size 30, 0.09 taper) was used for the removal of the coronal third of the root canal filling followed by the D2 ProTaper instrument (size 25/0.08 taper) for the middle third of the root canal. Finally, the D3 ProTaper instrument (size 20/0.07 taper) was used to the working length. Apical preparation was performed with ProTaper instruments F2 (size 25/0.08 taper), F3 (size 30/0.09 taper), and F4 (size 40/0.06 taper). Final irrigation was performed with 5 mL 17% EDTA (Pulpdent, Watertown, MA) and 5 mL 3% NaOCl (Prime Dental Products, Mumbai, India), and root canals were dried with paper points. All instruments were used only for 1 specimen, and the removal of filling materials was judged complete when the working length was reached and no more filling material could be seen on the last instrument used. A second CBCT scan was performed, and the volume of the remaining material in each tooth was estimated as mentioned earlier. The time required to retreat each canal was also recorded. Pre- and postoperative volumes of the root filling material and dentin were measured (in cubic millimeters) by 2 blinded observers, and the mean values for each specimen were calculated. The mean percentage of residual filling material and the mean amount of dentin removal during retreatment procedures were calculated.

Data Presentation and Analysis. The mean residual filling material was expressed as percentage (± standard deviation) values of the total filling material, and the time used to reach the working length was expressed in minutes (± standard deviation). The mean dentin removal was expressed in cubic millimeters (± standard deviation).

Group comparisons were performed using 1-way analysis of variance and the Student-Newman-Keuls post hoc test. The alpha-type error was set at 0.05 for all statistical analyses.

Results

Residual Root Filling Material

The percentage of residual root filling material was lowest in group 1 (1.8% ± 0.22%), which was significantly less than in the other 2 groups (P < .05). Group 3 showed a significantly higher percentage of remaining root filling material (P < .05) (Table 1).

Dentin Removal

The amount of dentin removed in samples of group 3 was significantly higher than in group 1 (P < .05), whereas this difference was not significantly different from group 2 (P > .05) (Table 1).

| TABLE 1. Means and Standard Deviations for Residual Root Canal Filling (in %) and Dentin Removal (in mm³) and the Time Taken to Reach the Working Length (in minutes) for Each Group (n = 15) |
|---------------------------------|---------------------------------|---------------------------------|
| Residual root filling material  | Dentin removal                  | Time taken to reach working length |
| Group 1 (gutta-percha with MTA Fillapex) | 1.8 ± 0.22 a                    | 5.5 ± 0.55 a                     |
| Group 2 (gutta-percha with MTA Plus)   | 6.0 ± 0.65 b                    | 6.0 ± 0.65 a                     |
| Group 3 (gutta-percha with epoxy resin sealer) | 10.4 ± 0.71 c                  | 6.85 ± 0.51 b                    |

Mean values that share a superscript letter were not significantly different at the 5% level within the same root third (1-way analysis of variance and the Student-Newman-Keuls post hoc test).
Time Taken to Reach the Working Length

The time taken to reach the working length in the groups restored with gutta-percha and MTA-based sealers was significantly less than the time taken to reach the working length in the group obturated with gutta-percha and epoxy resin sealer \((P < .05)\). However, there was no statistically significant difference in the time taken to reach the working length between groups 1 and 2 \((P > .05)\) (Table 1).

Discussion

The causes for persistent periapical disease after root canal treatment include necrotic tissues, bacterial biofilms, coronal leakage, recurrent caries, and tooth fractures \((14)\). To re-establish an environment conducive to repair and healing, these etiologic factors must be removed. Immaterial of the etiologic factor, nonsurgical root canal treatment requires removal of the root filling material present in the root canal system \((15)\).

This study evaluated the retreatability of 2 MTA-based sealers using CBCT scanning, considering an epoxy resin sealer (AH Plus) as the standard for comparison. The method used to evaluate the filling remnants plays an important role in the results obtained in each study. Previous studies on retreatability of root filling materials involved longitudinal sectioning of teeth followed by either digital imaging of surfaces or scanning electron microscopic analysis. However, these methods are 2-dimensional and do not offer accurate measurement of the root filling remaining inside root canals.

Recently, micro-CT (microCT) has been used as a research tool in endodontics for studying root canal anatomy \((16)\), the assessment of root canal preparation techniques \((17)\), the efficacy of obturating methods, and the removal of root filling materials \((18)\). However, microCT is not suitable for clinical use, and it is imperative to evaluate methods of removal of root filling materials using a technique that can show accuracy similar to microCT yet is clinically feasible. A recent study showed that only high-resolution CBCT instruments allow dentists to detect the full length of the root canal and, hence, can be suggested as a clinical alternative to microCT \((19)\). It has also been established that CBCT scanning is more accurate than other forms of CT, namely spiral CT and peripheral quantitative computed CT \((20, 21)\). To the best of our knowledge, this is the first study to use CBCT scanning to study the retreatability of MTA-based root filling materials.

The results of this study showed that none of the root filling materials could be removed completely. This is in accordance with previous studies \((15, 22, 23)\). In the present study, the removal of MTA Fillapex consumed the least amount of time followed by MTA Plus and AH Plus. Furthermore, the least amount of remaining root filling material after retreatment was observed with MTA Fillapex; this was significantly less than the other groups. This may be attributed to 2 reasons: the low bond strength of MTA Fillapex to root dentin and the questionable biomineralization of MTA Fillapex. The low bond strength of MTA Fillapex confirms the results of 2 recent studies that reported a low adhesion capacity of MTA Fillapex \((24, 25)\). The concept of biomineralization demands more explanation. The superior performance widely reported by the literature for the use of MTA was recently attributed to its biomineralization ability. The interaction of MTA with a phosphate-containing fluid produces calcium-deficient B-type carbonated apatites via an amorphous calcium phosphate phase. The apatite formed by the MTA-PBS system deposits on the collagen fibrils, thereby triggering the formation of an interfacial layer with tag-like structures at the MTA-dentin interface. Furthermore, this biomineralization process is claimed to enhance the resistance to the dislodgement of MTA from dentin \((10–12)\). However, the low bond strength of MTA Fillapex has been attributed to the low adhesion capacity of these tag-like apatites \((24)\). However, this supposition is in contrast to the findings of Salles et al \((26)\), who noted that MTA Fillapex showed increased alkaline phosphatase activity after 7 days, thereby stimulating hydroxyapatite crystal nucleation. Nevertheless, a comparison of MTA Fillapex with other commercial brands of MTA is yet to be performed.

MTA is a bioactive material that can form a layer of hydroxyapatite or carbonated apatite on its surface when it comes in contact with a phosphate-containing fluid for 2 months. Formation of this interfacial layer develops a chemical bond between MTA and dentinal walls \((27)\). A previous study showed that the greatest mean bond strength values were observed after exposure of MTA to a pH of 8.4. At this pH level, MTA showed higher surface hardness as well as less porosity \((28)\). Therefore, samples were exposed to PBS at a pH of 8.4 for a period of 2 months to bring about biomineralization \((29)\). MTA Plus is a material that presents basically the same composition of the original MTA formulation, whereas MTA Fillapex is mainly composed of a combination of resins, silica, and MTA. The total content of MTA in Fillapex is only 13.2%. Whether this quantity of MTA is substantial enough for inducing biomineralization equivalent to MTA Plus, which is 100% MTA, may be questioned. This may be further evidenced by recent works that have shown that MTA Fillapex showed a significantly inferior sealing ability than ProRoot MTA and AH Plus \((30)\). Further analysis of the interface is needed to determine the mechanism of bioactivity of these sealers.

Conclusions

Under the conditions of the present study, it was impossible to completely remove the root filling materials from the root canal systems. The least remaining root filling material was demonstrated by MTA Fillapex.

Acknowledgments

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

References

Genotoxicity of three endodontic sealers by single cell gel-electrophoresis/comet assay

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Abstract

Introduction: The aim of this study was to compare the genotoxicity of two MTA-based sealers (MTA Fillapex and MTA Plus) and a RealSeal self etch (SE) sealer.

Methods: Twenty discs of each sealer were constructed, dissolved in cell culture media and sealer extracts were diluted into two different concentrations 100% and 50%. Thereafter Baby Hamster kidney fibroblast cell cultures were treated with each concentration of tested sealers for two exposure periods 24 h and 5 days. Comet assay was used to evaluate DNA damage by measuring tail length and intensity, and the results were statistically analyzed by Kruskal–Wallis and Mann–Whitney U tests (\(P < 0.05\)).

Results: After 24 h, 100% medium extracts of RealSeal SE showed significantly the highest DNA damage followed by MTA Fillapex. After 5 days, MTA plus induced the least DNA damage at both tested concentrations. The DNA damage of both RealSeal SE and MTA Fillapex was significantly dose and time dependent.

Conclusions: MTA Plus was the least genotoxic sealer in this study and its genotoxicity is not significantly affected by exposure time or its concentration.

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Keywords: Genotoxicity; MTA-based sealers; RealSeal SE sealer; Comet assay

1. Introduction

Root filling materials usually remain in close contact with living periapical tissues over a long period of time via the apical foramen and occasional lateral foramina. The tissue's response to these materials is important and may influence the outcome of endodontic treatment [1]; therefore, an ideal endodontic material should be biocompatible with the periradicular tissues [2].

Genotoxicity is one of the important factors influencing biocompatibility. Genotoxic damage will not
necessarily lead to cell death; it may damage the cell genome that may significantly diminish the tissue’s self-repairing potential or in the long term cause the development of neoplasia [1]. A variety of genotoxicity assays assess DNA breakage such as metaphase chromosomal aberrations, micronuclei, and sister chromatid exchange. Over the past decade, the single-cell gel (comet) assay was developed as a rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells [3].

Mineral trioxide aggregate (MTA) is a cement composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium sulfate, bismuth oxide, and small amounts of other mineral oxides that modify its chemical and physical properties. It is widely used as a root-end filling material [4], in vital pulp therapy [5] and as an apical barrier in immature teeth with necrotic pulps [6]. Lately, it has been successfully used in regenerative endodontic procedures in immature teeth with apical periodontitis [7]. MTA is biocompatible [8], noncytotoxic [9], nonmutagenic, neither genotoxic nor carcinogenic [10] and has excellent sealing properties [8].

Based on these favorable characteristics and for the purpose of improving the drawbacks of the conventional MTA as long setting time and the difficulty in handling, a new formulation of MTA-based sealer has been introduced. MTA Fillapex is a resin sealer based on MTA in its composition in addition to salicylate resin, natural and diluting resins, nano-particulated resin, bismuth trioxide, nano-particulated silica, and pigments. It has low solubility, and easy handling [11], however the results related to its biological response are conflicting. Several researches revealed that this material showed high cytotoxicity and genotoxicity [12] even after 90 days [13]. Despite others showed that the cytotoxicity of MTA Fillapex decreases by time [14].

MTA Plus material has been introduced in the market that has basically the same composition of the original MTA formulation. It has a finer particle size than other commercially available versions of MTA (50% of the particles finer than 1 mm). The material being proposed would bond to tooth structure thus providing a hermetic seal [15].

Moreover, RealSeal self-etch (SE) is the simplified dual-cured version of RealSeal methacrylate resin-based sealers [16]. It has hydrophilic characteristics that enable them to wet canal wall, penetrate dentinal tubules [17], bond to radicular dentin [18], and to root-filling materials [19]. In spite of the cytotoxic effect of RealSeal SE sealer that was clarified by some studies [20–22], it has been approved for endodontic use [23–24].

Genotoxicity of RealSeal SE and MTA Fillapex sealers is scarcely studied and yet for MTA Plus. However studying the genotoxicity of some dental materials it was concluded that there was an evidence of dose-dependent response [1]. Consequently, this study was conducted to evaluate the genotoxicity of MTA Fillapex, MTA Plus, and RealSeal SE sealers using Comet assay to detect genomic damage expressed in two parameters including tail length and tail intensity at two concentrations (100% and 50%) after 24 h and 5 days exposure periods.

2. Materials and methods

2.1. Sample preparation

Twenty discs of each tested material; MTA Plus,1 MTA Fillapex2, and RealSeal SE3 sealers representing groups I, II, and III respectively were fabricated according to the manufacturer’s instruction under aseptic conditions in sterile cylindrical Teflon blocks, 5 mm in diameter and 2 mm in height. Samples of MTA Plus and MTA Fillapex were allowed to set at 37 °C for the time given by the manufacturer. RealSeal SE samples were light cured using the Elipar TriLight halogen curing unit4. Immediately after setting, excess flash material was removed with a sterile scalp and the hardened discs were sterilized with ethylene oxide after exposure to ultraviolet light for 2 h.

2.2. Preparation of extracts

Samples from each tested material were dissolved in cell culture Dulbecco’s Modified Eagle medium5 (DMEM) (1 g/5 mL) and incubated at 37 °C. The obtained extracts were sterile filtered using Millex-GS sterile filter6. To observe a dose-response relationship, the extracts were diluted with DMEM to achieve 100% and 50% concentrations V/V.

2.3. Cell cultures

BHK-21; Baby Hamster kidney fibroblast cells (clone CCL-10) was supplied from cell culture

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1 Prevest-Denpro, Jammu City, India.
2 Angelus, Londrina, PR, Brazil.
3 SybronEndo, Orange, CA.
4 3M ESPE, St. Paul, MN, USA.
5 Sigma–Aldrich, St Louis, MO, USA.
6 Millipore S.A.S., Molsheim, Cedex, France.
department (VACVSE RA—EGYPT) and maintained in an incubator until use. Growth medium from mother bottle was decanted, cells were washed with Trypsin (0.25%) containing 0.1 mm ethylene diamine tetra-acetic acid (EDTA)\textsuperscript{5}, in phosphate buffered saline (PBS) for 5 min at 37 °C for cell dissociation. Washing trypsin was decanted and cells were kept in contact with the residual trypsin for 10 min at 37 °C with periodic observation under the inverted Nikon microscope\textsuperscript{7} till complete cell dissociation. Detached cells were dispensed in growth medium to maintain $2 \times 10^5$ cell/mL. Cells were dispensed into tissue culture flasks, 25 ml TV\textsuperscript{8} with periodic examination under the inverted microscope till confluence sheet detected. Then cultures were treated with each concentration of tested material extract for 24 h and 5 days at 37 °C\textsuperscript{25}.

2.4. Slide preparation and comet assay

The comet assay was performed according to a standard protocol \textsuperscript{26}. All the chemicals needed to perform the comet assay were obtained from Sigma\textsuperscript{5}. The culture medium was carefully removed, and 5 μL of the sedimented fibroblasts was suspended in 100 μL of 0.5% low melting agarose to obtain 10,000 of fibroblasts per slide. This agarose layer was sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were coded and kept on ice during the polymerization of each gel-layer. After the solidification of the 0.5% agarose layer, the slides were immersed in a lysis solution [1% sodium sarcosinate, 2.5 mm sodium chloride (NaCl), 100 mm Na₂EDTA, 10 mm Tris-hydrochloric acid. (HCl), 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)] at 4 °C. After 1 h, the slides were placed in an electrophoresis buffer [0.3 mm sodium hydroxide (NaOH), 1 mm Na₂EDTA, pH 13] at 0 °C for 20 min to unwind the DNA. The electrophoresis was performed at 300 mA and 1.0 V cm\textsuperscript{−1} in a horizontal electrophoresis platform for 20 min.

The slides were neutralized with a Tris-HCl buffer (pH 7.5) and stained with 10% ethidium bromide for 10 min. Each slide was analyzed using an inverted Nikon fluorescence microscope. A hundred comets per slide were analyzed by the Comet assay V analysis system\textsuperscript{9} to determine DNA damage. Two parameters were estimated: tail length (the distance of DNA migration from the body of the nuclear core which recorded as the distance from the perimeter of the comet head to the last visible point in the tail) and tail intensity (percentage of DNA in the tail). During the analysis, the edges and eventually damaged parts of the gel as well as debris, superimposed comets, comets of uniform intensity and comets without a distinct head (‘clouds’, ‘hedgehogs’ or ‘ghost cells’) were excluded.

3. Statistical analysis

Data were presented as mean and standard deviation (SD) values and explored for normality using Kolmogorov—Smirnov and Shapiro—Wilk tests. Data showed non-parametric distribution; so Kruskal—Wallis test was used to compare the three materials. Mann—Whitney U test was used for pairwise comparisons between the materials when Kruskal—Wallis test is significant; to compare both tested concentrations (100% and 50%) as well as the two time periods of evaluation (24 h and 5 days). The significance level was set at $P \leq 0.05$. Statistical analysis was performed using IBM\textsuperscript{10} SPSS Statistics Version 20 for Windows.

4. Results

Comet assay results revealed that all tested sealers (MTA Plus, MTA Fillapex and RealSeal SE) recorded tail length and intensity measurements exhibiting a genotoxic effect at both tested exposure periods and concentrations as shown in Table 1.

4.1. Inter-material comparison (Table 1)

4.1.1. After 24 h exposure period

Considering the 100% medium extracts, the significantly highest mean values of DNA damage, tail length and intensity, were associated with group III (RealSeal SE) while the lowest were associated with group I (MTA Plus). There was no statistical significant difference between groups I and II regarding tail length, however group I showed statistically lower mean value of tail intensity compared to group II (Fig. 1). Alternatively, no significant differences were recorded among tested materials for both tail length and tail intensity at 50% medium extracts.

\textsuperscript{7} Nikon—Japan.
\textsuperscript{8} TPP-Swiss.
\textsuperscript{9} Perceptive Instruments Ltd, Halstead, UK.
\textsuperscript{10} SPSS, Inc., IBM Company.
4.1.2. After 5 days exposure period

At 100% concentrations there were significant differences among all tested groups regarding the tail length with the highest mean value for group III and the lowest for group I. While regarding the tail intensity, group I recorded the significantly lowest mean value versus both groups II and III.

Also, at 50% medium extracts, group III recorded the highest significant mean value of tail length compared to groups I and II. Regarding tail intensity, there was no statistical significant difference among the three tested materials ($P = 0.603$).

4.2. Intra-material comparison (Table 2)

4.2.1. Comparison between concentrations of sealer extract

The concentrations of the studied sealers had a significant effect on DNA damage, tail length and intensity, at both 24 h and 5 days exposure periods for groups II and III. The significantly higher mean values were noted at 100% concentrations compared to those at 50% and this was obviously shown in Fig. 2. Whereas, there was no significant difference in DNA damage between both tested concentrations of group I at both exposure periods.

4.2.2. Comparison between exposure periods

The effect of tested periods was statistically significant on both DNA damage parameters for all tested sealers types and concentrations. It demonstrated significantly higher values of genotoxicity at 5 days exposure versus 24 h (Fig. 3) except for MTA Plus medium extracts.

5. Discussion

DNA damage in cells could have an important implication on health because they are cumulative and may in turn affect cell functions leading to cell death or slow onset disease overtime [27]. To avoid unwanted side effects following the use of root canal sealers, only materials exerting no or minimum deleterious effects on living cells should be used [1].

Comet assay is a standard, non-invasive, and a powerful technique that directly measures DNA damage in cells. It provides a direct measure of single-strand DNA breaks, suggesting that exposure to root canal sealers may cause DNA damage, particularly at higher concentrations and longer exposure periods.
damage in almost all kinds of individual cell types. This assay is based on the principle that, DNA damage reduces the size of DNA fragments which is detected by applying an electrophoretic field to lysed cells where damaged cellular DNA fragments are separated from intact DNA, yielding a classic “comet tail” shape under the microscope. The extent of DNA damage is usually estimated by comet tail measurements where image analysis software is available for measuring various parameters [28].

According to the previous reports [12,13], only a few studies on putative genotoxicity of MTA-based root canal sealers and methacrylate resin-based sealers have been conducted and the results are conflicting. Thus, the genotoxicity of these materials at different concentrations was assessed in this study.

The current results indicated that RealSeal SE root canal sealer was the one that has induced more genotoxic effect on BHK-21 cells at both tested concentrations and periods of exposure. This might be explained by the inherently high resin content of the sealer; UDMA, EBPADMA, PEGDMA, BIS-GMA, which accounts for more than 60% [29]. Additionally, methacrylate-based sealer usually sets in about 30 min inside the root canals in the anaerobic environments. In the present study, the sealer was not fully

<table>
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<th>Comet parameter</th>
<th>Sealer concentration</th>
<th>100%</th>
<th>50%</th>
<th>P-value</th>
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<td>Tail Length</td>
<td>Group I (MTA Plus)</td>
<td>24 h</td>
<td>33.8 ± 6.9</td>
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<td>14.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>139.9 ± 4.3</td>
<td>55 ± 0.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>&lt;0.001*</td>
<td>0.004*</td>
<td></td>
</tr>
<tr>
<td>Tail intensity</td>
<td>Group I (MTA Plus)</td>
<td>24 h</td>
<td>27.3 ± 2.7</td>
<td>14.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>29 ± 1.6</td>
<td>21 ± 1</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.072</td>
<td>0.152</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group II (MTA Fillapex)</td>
<td>24 h</td>
<td>49.1 ± 1.3</td>
<td>15.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>72.1 ± 0.7</td>
<td>34.3 ± 2.8</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.015*</td>
<td>0.037*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group III (RealSeal SE)</td>
<td>24 h</td>
<td>59.9 ± 1</td>
<td>12.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>84.5 ± 2.3</td>
<td>35 ± 1.9</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.001*</td>
<td>0.042*</td>
<td></td>
</tr>
</tbody>
</table>

Mann–Whitney U test: *Significant at P ≤ 0.05.

Fig. 2. Photographs from comet assay showing cells exposed for 5 days to medium extracts of 50% RealSeal SE sealer (a) and 100% of the same sealer (b) with more tail length and more DNA damage.
isolated from air, and oxygen may inhibit free radical polymerization of resins that might not be completely polymerized [22]. This was supported by Tyagi et al. [30] who related the toxicity of methacrylate resin-based sealers to the presence of unpolymerized hydrophilic monomers (such as 2-hydroxyethyl methacrylate (HEMA)) that can easily diffuse into the cell and elicit significant toxicity. A conflicting result by Brzovic et al. [1] who demonstrated that Epiphany sealer was not capable of inducing DNA damage might be related to the different assay evaluating genotoxicity where they used chromosomal aberration analysis.

However, after 24 h exposure the genotoxicity of 50% RealSeal SE sealer extracts was not statistically different from other two tested sealers for both comet assay parameters. It was suggested that the concentration of residual monomers leached at this short exposure time and 50% concentration of the polymerized material under the experimental conditions was too low to exhibit genotoxic activity. This finding concurs with other studies which examined genotoxicity of either Epiphany or RealSeal (which is basically Epiphany) sealers with different cytogenetic assays including the number of micronuclei formation [31] and cytotoxicity action [29].

According to the present data, it was found that MTA Fillapex root canal sealer produced more DNA damage and genotoxicity compared to MTA Plus sealer. The genotoxicity of MTA Fillapex was most likely related to the composition of this material where it contains only 30% MTA and resin components such as salicylate which has a potential concern in cellular genotoxicity [12–14]. Furthermore, according to Bramante et al. [32], MTA Fillapex contains high levels of arsenic, heavy metal, element as a contaminant. The release of this arsenic element reacts with protein thiols of the cells and may induce genotoxicity. This finding is consistent with the results of other researches which confirm the genotoxic effect of MTA Fillapex sealer [12–14].

It has been difficult to discuss and compare the present results obtained with MTA Plus sealer because of the lack of previous studies. The current findings showed that MTA Plus has the least genotoxic effect compared to tested sealers. The good compatibility of this sealer might be a sequence of its main components including tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, bismuth oxide, and calcium sulfate which are the main components of dentin tissue. Additionally, this type of sealer doesn't include Iron (III) Oxide (Fe_{2}O_{3}) even in low concentration compared to other classic MTA that might evoke DNA damage [12].

The present data demonstrated that genotoxicity of both RealSeal and MTA Fillapex significantly increased with time. Additionally it was found that their genotoxicity is dose-dependent where there was significantly more DNA damage associated with 100% concentration compared to that of 50% for both sealers. It could be suggested that the concentration of toxic materials that are leached from the set material of RealSeal and MTA Fillapex (residual monomer and/or arsenic material) at 10% concentration and 5 days exposure was large enough to induce more genotoxic effect. The significant role of sealer concentration and/or exposure time confirmed the findings of some authors [1,13,31,33]. In contrary, Marques et al. [34] revealed that the toxic effect of MTA Fillapex decreased with time. This difference may be attributed to different methodology since they didn't use the Comet assay in evaluating the toxic effect of MTA Fillapex on rat subcutaneous tissue.
On the other hand, in the present study, the genotoxicty of MTA Plus was not found to be dose dependent. This was in consistent with Ribeiro et al. [35] who noted that there was no difference in DNA damage at different concentrations of MTA as depicted by the single cell gel assay, and Aminozarbian et al. [36] who found that MTA genotoxicity did not vary by increasing its concentration.

6. Conclusion

Under the conditions of this study, all tested sealers showed variable degrees of genotoxicity. The DNA damage observed by RealSeal SE and MTA Fillapex sealers was dose and time-dependant. Given the diversity of root canal sealers on the market, additional investigations, especially for MTA Plus, using other parameters of genotoxicity as chromosomal aberration and sensitization tests are recommended to establish an overview on the real potential of these sealers. In vitro results might not be directly extrapolated to in vivo conditions; therefore, it is essential to have long term in vivo studies and clinical investigations to assess the biocompatibility of tested sealers.

Acknowledgment

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References


Ion Release, Porosity, Solubility, and Bioactivity of MTA Plus Tricalcium Silicate

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Abstract

Introduction: The aim of this study was to evaluate MTA Plus (Prevest Denpro Limited, Jammu, India, for Avalon Biomed Inc) material’s properties, namely calcium release, the pH change, solubility, water sorption, porosity, surface morphology, and apatite-forming ability after immersion in simulated body fluid. Methods: Two tricalcium silicate powders (MTA Plus and ProRoot MTA; Dentsply Tulsa Specialties, Tulsa, OK) and Dycal (Dentsply Caulk, Milford, DE) were tested. After incubation at 37°C and 99% relative humidity, calcium and hydroxyl ion release were tested up to 28 days in deionized water at 37°C. Water absorption, interconnected pores, apparent porosity, and solubility were measured after 24 hours of immersion in deionized water at 37°C. The morphologic and elemental analysis of the materials’ surfaces were examined using an environmental scanning electron microscope/energy dispersive x-ray analysis after 24 hours in simulated body fluid using the ISO 23317 method. Results: All 3 materials created an alkaline pH within 3 hours, which continued for 28 days. MTA Plus had a higher ion release than ProRoot MTA and Dycal; the use of the MTA Plus gel enhanced the initial calcium release and the increase of the pH. Both MTA materials were more porous, water soluble, and water sorptive than Dycal and more bioactive. After aging in simulated body fluid, MTA Plus material caused precipitation of an apparent calcium phosphate layer. Conclusions: MTA Plus showed improved reactivity and prolonged capability to release calcium and increase the local pH to alkaline values in comparison with ProRoot MTA. These pronounced ion-releasing properties are interlinked with its noticeable porosity, water sorption, and solubility and with the formation of calcium phosphorus minerals. The finer calcium silicate powder may explain the higher ion release, water sorption, porosity, and solubility of MTA Plus compared with ProRoot MTA. For clinicians, MTA Plus represents a lower-cost bioactive tricalcium silicate material with interesting chemical-physical properties that could be a convenient alternative to the conventional calcium silicate mineral trioxide aggregate-like cements. (J Endod 2014;40:1632–1637)

Key Words

Calcium phosphate formation, ion releasing, mineral trioxide aggregate, MTA Plus, porosity, ProRoot MTA, root-end filling materials, solubility, tricalcium silicate, water sorption

Mineral trioxide aggregate (MTA) products are hydrophilic powders composed of tri- and dicalcium silicates. These hydraulic materials set with the addition of water to form calcium silicate hydrate gel (1–5). Portland cement is composed of primarily tri- and dicalcium silicates and was used in dentistry in 1878 (4); it was patented in 1995 when it was combined with bismuth oxide powder (3, 5).

Gray (5) and tooth-colored (also known as white) (6) ProRoot MTA (Dentsply Tulsa Specialties, Tulsa, OK) has revolutionized root-end therapy and pulp capping procedures in relation to the property to set in moist/bloody environments or sites contaminated by biological fluids (2, 7), the ability to stimulate the formation of hydroxyapatite (1, 2), and the generation of a flux of calcium and hydroxyl ions through dentin as recently shown by Gandolfi (8).

MTA Plus (Prevest Denpro Limited, Jammu, India, for Avalon Biomed Inc) is a finer powder, lower-cost product that has a composition similar to tooth-colored ProRoot MTA (9) and is proposed for treating dental pulp (pulp capping, cavity lining, and pulpotomies) and root canals (root-end filling, perforation repair, root resorption, apexification, and obturation in pulpectomy).

The MTA Plus kit includes 2 mixing liquids: a proprietary salt-free polymer gel and water. MTA Plus is indicated as a root canal sealer as well as a root-end filling material and a pulp capping cement. By using the gel and varying the powder to gel ratio, different setting times and physical-rheologic properties can be obtained. The gel has been formulated to confer washout resistance, whereas its fine powder particle size improves handling and placement (10). Recent studies showed the possibility to perform retreatment of teeth filled with MTA cements (10, 11) although it has been suggested to avoid filling procedures using MTA-like cements to completely obturate the root canal because the collagen and flexural strength of the dentin can be negatively affected (12, 13).

The aim of the present study was to compare the properties, namely calcium release, pH, solubility, water sorption, porosity, surface morphology, and apatite-forming ability after immersion in simulated body fluid (SBF), of 3 materials.
Materials

MTA Plus (lot 41001), tooth-colored ProRoot MTA (lot 09003850), and Dycal (Dentsply Caulk, Milford, DE; lot 81007) were tested. ProRoot MTA and Dycal represented the comparison commercial materials for root-end filling and pulp capping procedures, respectively.

MTA Plus and ProRoot MTA were mixed for 30 seconds on a glass slab with their liquids using a liquid to powder ratio of 0.31 for ProRoot MTA and 0.37 for MTA Plus. MTA Plus powder was also mixed with its gel at 0.33. To prepare Dycal, base and catalyst pastes were mixed equally. Dycal (14) contains calcium hydroxide (Ca(OH)₂) in a salicylate base. Freshly mixed pastes were compacted into polyvinyl chloride molds (8 ± 0.1 mm diameter × 1.6 ± 0.1 mm), and the excess was removed. The exposed upper surface area of each sample was 50.24 ± 0.01 mm².

Ion Release

The molds were placed on the bottom of cylindrical polystyrene containers (3-cm high with a 4-cm diameter) with 10 mL deionized water, sealed, and stored at 37°C. The water was collected and renewed after 3 hours and 1, 7, 14, and 28 days. Calcium ion and pH measurements were performed. The water of the measurement stabilized. Deionized water was the negative control.

pH. The pH measurements were performed on the collected water with a pH probe (Sen Tix Sur WTW, Weilheim, Germany) connected to a multiparameter meter (inoLab 750; WTW, Weilheim, Germany).

Calcium Release. After the pH measurement, the solution was supplemented with 200 μL (2%) ionic strength adjuster (4 mol/L KCl, WTW). Measurements were made using a calcium ion selective probe (Eutech Instruments Pte Ltd, Singapore). The cumulative release of calcium was calculated.

Porosity, Solubility, and Water Sorption

Another set of disks was set at 37°C and 99% relative humidity for 70% of their setting time (55 minutes for MTA Plus and 250 minutes for ProRoot MTA). The specimens were unmade, dried with filter paper, and weighed (initial mass, Dᵢ). Each disk was immersed vertically in 20 mL distilled water at 37°C. After 24 hours, the mass while suspended in water (S) was determined. Excess water was removed, and the saturated mass (M) was recorded. Samples were dried at 37°C to a stable weight (dry mass [Dᵢ]). Each weight measurement was repeated 3 times to the nearest 0.001 g using an analytical balance (Bel Engineering series M, Monza, Italy).

The exterior volume (V [M – S]), the volume of open pores (V₀p [M – Dᵢ]), the volume of impervious portion (V₀p [V₀p = Dᵢ – S]), and the apparent porosity (P [(M – Dᵢ)/(M – S)] × 100) were calculated following Archimedes’ principle (American Society for Testing and Materials C373 [2006]). The water sorption (A [(M – Dᵢ)/Dᵢ] × 100) and the solubility (S S = (Dᵢ – Dₚ)/Dᵢ) were calculated (15, 16).

Statistical Analysis

Two-way repeated measures analysis of variance statistical analysis was performed with the Student-Newman Keuls test (P < .05).

Bioactivity Test

The ISO 23317 (Implants for surgery—In vitro evaluation for apatite-forming ability of implant materials) method was used to evaluate layers precipitated on the materials soaked in SBF. Molds filled with freshly prepared cement pastes (set for about 10 minutes) were placed vertically in 20 mL Hank’s Balanced Salt Solution (HBSS; Cambrex Bio Science Verviers Sprl, Verviers, Belgium [cat. n.10-527]) for use as SBF. Each sample’s exposed surface was 100.48 ± 0.01 mm² (including upper and lower surfaces), and the surface/volume ratio was 100.48/20 = 5.024 (15). HBSS was replaced weekly.

Freshly prepared samples (approximately 5 minutes after mixing) and samples aged in HBSS for 1, 7, or 28 days were examined “wet” using an environmental scanning electron microscope (ESEM; Zeiss EVO 50; Carl Zeiss, Oberkochen, Germany) connected to a secondary electron detector for energy dispersive x-ray analysis (EDX; Oxford INCA 350 EDS, Abingdon, UK) (2). EDX provided qualitative and semi-quantitative measurements of atomic calcium and phosphorus to calculate the superficial calcium to phosphorus (Ca/P) atomic ratios (17). The formation of calcium carbonate (CaCO₃) was presumed when the Ca/P ratio exceeded 1.67.

Results

Ion Release

Table 1, sections A and B, contain the pH values and calcium ion values (mean ± standard deviation, n = 10) for all soaking times. All 3 materials created an elevated pH (alkaline) after 3 hours of soaking, with the highest value for MTA Plus mixed with gel (12.0). Over 28 days, all materials gradually decreased in their rate of release of hydroxyl ions, and the pH diminished. After 28 days, the pH was the highest for Dycal solutions (9.8) and the lowest for ProRoot MTA eluate (7.1).

The calcium ion release after 3 hours was highest from MTA Plus, mixed with water (43 ppm) or gel (119 ppm), and lowest for Dycal (25 ppm) and ProRoot MTA (24 ppm). The calcium ion release diminished for all materials over 28 days. At 28 days, MTA Plus with gel had a release of 19 ppm compared with ProRoot MTA, Dycal, and MTA Plus with water (16, 16, and 8 ppm, respectively).

Porosity, Solubility, and Water Sorption

The apparent porosity varied from 9% for Dycal to 40% for MTA Plus mixed with water or gel (39%) as shown in Table 1, section C. The water sorption and the solubility followed the same trends. Water sorption was only 5% for Dycal and highest for MTA Plus with gel (20.5%). The solubility was lowest for Dycal and highest for MTA Plus powder with water (18.5%).

Bioactivity Tests

The ESEM/EDX results for freshly mixed MTA Plus with gel (Fig. 1) revealed compounds of calcium, silicon, bismuth, aluminum, and carbon on the surface. From this, we concluded that MTA Plus contains calcium silicates, bismuth oxide, a minor amount of calcium aluminate, and a carbon-containing polymer. After 7 days, a precipitate had formed on MTA Plus that contained compounds of calcium, sodium, magnesium, phosphorus, and chloride with a Ca/P ratio of 4.45. The carbon peak was no longer observed, indicating the precipitate formed from HBSS completely covering the surface. The atomic percentage of calcium and phosphorus increased for the 28-day sample, whereas all other elemental peaks diminished, except for sodium and chlorine, which is consistent with the precipitation of NaCl and a calcium phosphate phase. The Ca/P ratio was 2.47, which is higher than that of hydroxyapatite.

The freshly mixed and unexposed MTA Plus mixed with water (Fig. 2) had major EDX peaks for calcium, silicon, and bismuth and lesser peaks for sodium, sulfur, aluminum, and potassium, whose origins can be attributed to minor constituents of cement but may have been obscured in the gel sample. When soaked in HBSS, the bismuth
TABLE 1. (A) Calcium Release in the Soaking Water; (B) pH of the Soaking Water; and (C) Porosity, Water Sorption, and Solubility of the Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Ca Release (ppm)</th>
<th>pH 1 day</th>
<th>pH 3 days</th>
<th>pH 7 days</th>
<th>pH 14 days</th>
<th>pH 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProRoot MTA</td>
<td>3.95</td>
<td>8.55</td>
<td>8.39</td>
<td>8.62</td>
<td>8.57</td>
<td>8.54</td>
</tr>
<tr>
<td>ProRoot MTA gel</td>
<td>4.35</td>
<td>8.98</td>
<td>8.69</td>
<td>8.95</td>
<td>8.89</td>
<td>8.88</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.81</td>
<td>7.01</td>
<td>7.00</td>
<td>7.16</td>
<td>7.02</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Different letters represent statistically significant differences (2-way repeated measures analysis of variance followed by the Student-Newman-Keuls test, \(p < .05\) in the same line (capital letters) or in the same column (small letters).}

Discussion

These *in vitro* tests of the 2 MTA products and Dycal highlight the similarities of the MTA products and the contrast with Dycal, a Ca(OH)\(_2\)_2-based product. All 3 materials released calcium and hydroxide ions. The MTA products are known to form Ca(OH)\(_2\) in a calcium silicate hydroxide matrix. EDX disclosed calcium, silicon, aluminum, and bismuth in the MTAs, indicative of calcium silicates, calcium aluminate, and bismuth oxide. The MTA products appeared to contain bismuth oxide, and the Dycal material was deduced to contain its stated calcium tungstate zinc oxide and titanium dioxide for radiopacity. MTA Plus had a composition similar to ProRoot MTA, but MTA Plus with water had a small peak for sulfur, indicating calcium sulfate, which was also presumed to be present in ProRoot MTA but not observed with it or MTA Plus and gel.

The beneficial release of Ca(OH)\(_2\) diminished for all materials over 1 month. The precipitated calcium phosphate layer reduced the diffusion of calcium or hydroxide ions. MTA Plus with gel had high calcium release and pH. MTA Plus and gel slightly reduced the porosity, sorption, and solubility. ProRoot MTA had a calcium release lower than previously reported \(15, 16\) despite the same methodology but was similar to Dycal. The Dycal data were similar to that reported in other studies (eg, low solubility \(4.94\%\)) \(15, 16, 18–20\).

High porosity, solubility, and water sorption were observed for the MTA compounds. However, these measurements were initiated after as little as 55 minutes after mixing. The solubility was calculated as weight loss, with a method different from the ISO 6876 method for root canal sealers. The present solubility values are much higher than measured in other studies in which the samples tested were set for 21–24 hours at 37°C and 98%–100% relative humidity before immersion in water \(21–23\).

In these tests, the high surface to volume ratio also differs from the clinical situation in a root-end filling in which the surface to volume ratio is lower. The solubility of ProRoot MTA \(14.7\%\) measured here seems inconsistent with its excellent clinical performance; however, the solubility is reduced in biological fluids \(15, 24\) by the rapid formation of a calcium phosphate layer. The ongoing hydration reactions of dicalcium silicate (lasting about 28 days) undoubtedly interfered with these measurements.
Solubility is less for calcium silicates after a longer setting time. In vivo, the exposed surface to volume ratio is less than tested here. The elution of the calcium and hydroxide ions apparently initiated the formation of calcium phosphate phases but also increased the porosity. The Dycal material appeared less bioactive; its precipitate did not mask the underlying material from the electron beam, suggesting/confirming a thin apatite layer. This was true to a lesser degree for the ProRoot MTA. Both MTA products produced thicker precipitates with a higher Ca/P ratio exceeding 1.67, indicating a coprecipitation of calcium carbonate formed from the Ca(OH)₂. MTA Plus formed a thick calcium phosphate layer (good bioactivity) after soaking in simulated body fluid forming large calcium phosphate–based spherules. This material has shown dentin remineralization in the presence of SBF (25). The high calcium and OH ion releases are connected with the formation of calcium phosphate deposits.

MTA Plus had a higher ion release than ProRoot MTA and Dycal; the use of the MTA Plus gel enhanced the initial calcium release and raised the pH. The finer calcium silicate powder may explain the higher ion release, water sorption, porosity, and solubility of MTA Plus compared with ProRoot MTA. Both MTA products were more water soluble and water sorptive than Dycal and more bioactive.

The ability to release calcium ions able to diffuse through dentin (8) and inside the surrounding tissues is a key factor for successful endodontic and pulp capping therapies because of the action of calcium on the differentiation of mineralizing cells as dental pulp cells (26, 27), cementoblasts (28), osteoblasts (29, 30), periodontal fibroblasts (31, 32), mesenchymal stem cells (33), and hard tissue mineralization. Alkaline pH values accelerate apatite nucleation because apatite solubility decreases and OH⁻ may be a component of apatite (17). Also, hydroxide ions stimulate the release of alkaline phosphatase and bone morphogenetic protein 2, which participate in the mineralization process (31). The MTA cements’ apatite-forming ability has favorable clinical implications as root canal filling materials in association with gutta-percha because their sealing is improved with time by the deposition of calcium phosphates at the interface and inside the dentinal tubules (34).

The mechanism of apatite formation on calcium silicate MTA cements in phosphate-containing solutions was summarized by Gandolfi et al (1) in 11 steps. The growth of a layer of apatite is an ideal environment for stem cell and osteoblast differentiation and colonization to support new bone formation. Apatite together with the epigenetic signals correlated to ion release may well explain...
the excellent clinical outcomes of MTA cements (35). Moreover, the apatite-forming ability may provide clinical advantages by improving their sealing by the deposition with the time of calcium phosphates at the interface and inside the dentinal tubules of the root canal when MTA cements are used as root canal filling materials in association with gutta-percha (34).

MTA Plus had a prolonged capability to release calcium and increase the local pH in comparison with ProRoot MTA. These ion-releasing properties are interlinked with its noticeable porosity, water sorption, and solubility and with the formation of a calcium phosphate layer. For clinicians, MTA Plus represents a lower-cost, bioactive tricalcium silicate material as a convenient alternative to the conventional calcium silicate MTA-like cements.

Acknowledgments

Dr Primus is the inventor of MTA Plus and white ProRoot MTA. She is not part of Prevest Denpro or Dentsply and did not participate in the data collection nor statistical analysis.

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

References


Capping a Pulpotomy with Calcium Aluminosilicate Cement: Comparison to Mineral Trioxide Aggregates

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Abstract

Introduction: Calcium aluminate cements have shown little affinity for bacterial growth, low toxicity, and immunogenicity when used as a restoration material, but calcium aluminate cements have not been tested in vivo in pulpotomy procedures. Methods: To address this question, a calcium aluminosilicate cement (Quick-Set) was tested along with 2 mineral trioxide aggregates, ProRoot MTA and MTA Plus. These cements were used as a capping agent after pulpotomy. Control rats had no pulpotomy, or the pulpotomy was not capped. Proinflammatory cytokines interleukin (IL)-1β and IL-1α were measured, and histology was performed at 30 and 60 days after capping. The nociceptive response was determined by measuring the lengthening of the rat’s meal duration. Results: and Conclusions: IL-1β and IL-1α concentrations were reduced in the capped teeth; but no differences were observed among the 3 cements. Dentinal bridging could be detected at both 30 and 60 days with each of the 3 cements, and the pulps were still vital 60 days after capping. Meal duration significantly shortened after placement of the 3 different cements, indicating a nociceptive response, but there were no differences among the materials. Calcium aluminosilicate cement had similar properties to mineral trioxide aggregates and is a viable option for pulpotomy procedures. (J Endod 2014;40:1429–1434)

Key Words
Calcium aluminate/aluminosilicate cement, calcium silicate, endodontic, mineral trioxide aggregate, pain, tricalcium silicate

C}alcium aluminate cements show little immunogenicity, toxicity, or affinity for bacterial growth when tested as a restoration material (1–3). Unfortunately, calcium aluminate cements have a higher failure rate than commonly used restoration materials (4–6). Recently, calcium aluminate cements have been tested in vitro and in vivo as an endodontic material (7–9). In these studies calcium aluminate cement showed little immunogenicity or affinity for bacterial growth, but to date, no study has tested calcium aluminate cements as a pulp-capping material in vivo. In addition to studying immunogenicity and bacterial static properties, pain after capping pulpotomies with mineral trioxide aggregates (MTAs) was measured in patients (10). To our knowledge, no study has compared the nociceptive response after capping pulpotomies with different hydraulic cements (a cement that hardens on hydration), and a pain study has not included calcium aluminate cement. To address this knowledge gap, we hypothesized that calcium aluminosilicate cement is a viable pulp-capping material that shows low immunogenicity and little pain and has a high biocompatibility when in contact with tooth pulp.

In this study, a calcium aluminosilicate cement and MTAs were used as a capping material after pulpotomy, and the inflammatory, biocompatibility, and nociceptive responses were measured after placement of these cements. Inflammation was measured by quantifying proinflammatory cytokines interleukin (IL)-1β and IL-1α in the pulp of the treated teeth. Histology was also performed to assess dentinal bridging, the presence of bacteria, and pulp vitality. The nociceptive response was measured by using a behavioral assay, specifically the rat’s meal duration. Previous studies have shown that a lengthening of the rat’s meal duration correlates to orofacial pain in rats (11–16), and that meal duration has been shown to be significantly longer after pulpotomy (12).

Materials and Methods

Animals

All animal experiments were approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee in accordance with the guidelines of the United States Department of Agriculture, National Institutes of Health Office of Laboratory Animal Welfare, and National Research Council’s “Guide for Care and Use of Laboratory Animals.” Male Sprague-Dawley rats (250–300 g) were purchased from Harlan Industries, Houston, TX. On arrival, the animals were housed individually in a temperature-controlled room (23°C) and kept on a 14:10 light/dark cycle with lights on at 6:00 AM. The rats were given chow (Harlan Industries, Indianapolis, IN) and water ad libitum.

Pulpotomy and Cement Placement

After administering ketamine (90 mg/kg) and xylazine (9 mg/kg), an occlusal pulpotomy on the 6 maxillary molars was completed by a board-certified endodontist by using a ½-round carbide bur. The teeth were immediately capped with 1 of 3 cement materials: ProRoot MTA (Dentsply, Tulsa Dental Specialties, York, PA) mixed 3:1 powder:water, Quick-Set (Avalon Biomed Inc, Bradenton, FL, patent pending) (17), or MTA Plus (Avalon Biomed Inc) mixed at 2.5:1 powder:gel by weight with their respective gels. The mixed cements were placed in the cavity created by the iatrogenic pulpotomy. A self-adhering flowable composite resin (VertiseFlow; Kerr Corporation, Orange, CA) was placed immediately over the cements, and the resin was ultraviolet cured (18). The negative control group had no pulpotomy, and an untreated pulpotomy was used as a
positive control. Previous studies had groups with exposed untreated pulp for 28 days or longer (19, 20). Treatment groups in this study included the control, the exposed pulps, the exposed pulps capped with ProRoot MTA, the exposed pulps capped with MTA Plus, and the exposed pulps capped with Quick-Set. Twelve animals were in each of these treatment groups. Six animals in each treatment group were killed at 30 days, and 6 animals were killed at 60 days after capping. Death was completed by exposure to CO₂. After breathing was observed to stop, the animals were decapitated. The maxillae were isolated and fractured along the midline; half of the maxilla was placed in 4% paraformaldehyde and 1 × phosphate-buffered saline for histology, and the other half was placed in liquid nitrogen for storage and later analysis. The maxilla that was either fixed or frozen was randomly chosen for each animal in each treatment group.

Enzyme-linked Immunosorbent Assay

IL-1α was quantified because this cytokine was elevated after pulpotomy, whereas the common proinflammatory cytokine IL-1β was not found to be elevated in some instances (21).

Three to four out of a total of 6 animals from each treatment group were chosen randomly for measurement of the cytokine levels (pg/mL) by enzyme-linked immunosorbent assay (ELISA). To quantitate the cytokines in the pulp tissue, a maxilla from the rat was removed from the liquid nitrogen, and the molars (3 molars per maxilla) were extracted. The teeth were ground and placed in 300 µL T-Per tissue protein extraction reagent containing Halt Protease Inhibitor (Thermo Scientific, Rockford, IL). The lysates were frozen, thawed, vortexed, and centrifuged for 10 minutes at 4°C, and the supernatant was decanted. Total protein in the supernatant was determined in each sample by using a BCA protein assay (Thermo Scientific, Waltham, MA). Quantitation was completed on duplicate 50-µL samples of supernatant by using ELISA (R&D Systems, Minneapolis, MN) following the manufacturer’s directions. Values were given as pg IL-1α or IL-1β per µg total protein.

Histology

Histology was performed by using a randomly chosen maxilla from 3 rats per treatment group. First, the maxilla was immersed in 4% paraformaldehyde and 1 × phosphate-buffered saline continuously for 1 week, and then the samples were demineralized in 0.5 mol/L EDTA until radiographic examination revealed an absence of radiopaque structures. The demineralized samples were dehydrated and embedded in paraffin blocks. Serial sections, 6 µm thick, were sliced with a Leitz 1512 rotary microtome (Leica, Buffalo Grove, IL) in a buccolingual longitudinal orientation. Every 20th section was collected and stained with hematoxylin-eosin or a Brown and Hoppins stain. Briefly, paraffin was removed with xylene, and the slides were hydrated in a series of ethanol/water treatments. A portion of the slides were immersed in hematoxylin, rinsed, immersed in eosin, rinsed, and dehydrated, and a non-aqueous mounting medium was added. Alternate slides were immersed in crystal violet, rinsed, immersed in Gram iodine, rinsed, and finally immersed in fuchsin and then rinsed and mounted. For the Brown and Hoppins stain, gram-positive bacteria were blue, and the gram-negative bacteria were red. Two examiners blinded to tooth position and treatment scored the teeth for immune cell infiltrate, presence of bacteria, or dentinal bridging. Imaging was completed with a Zeiss Axioplan microscope (Thornwood, NY) and an Insight 2 Spot camera (SPOT Imaging Solutions, Sterling Heights, MI). Images were captured and analyzed with Spot Advanced software (SPOT Imaging Solutions).

Bacteria and Pulp Vitality Measurements

Every 20th section was collected for the maxillae of 3 rats in each treatment group, and the sections were stained with either Brown and Hoppins stain or hematoxylin-eosin stain. After the Brown and Hoppins staining, the bacteria on each slide were counted, and all the bacteria for all the sections of a single maxilla were added together. This total bacteria count per maxilla was reported for each of the 3 rats per treatment group. After the hematoxylin-eosin staining, the percentage of pulp vitality remaining after treatment for each rat was calculated by dividing the area of the vital pulp in the maxillary molars by the average area of vital pulp in the 3 control rats, and then the result was multiplied by 100.

Meal Duration

Meal duration was measured before and after pulpotomy by placing rats in individual, sound-attenuated chambers equipped with computer-activated pellet feeders (Med Assoc Inc, East Fairfield, VT). The feeder units dispensed 45 mg rodent chow pellets (product no. FO 165; Bioserv, Frenchtown, NJ). When a rat removed a pellet from the feeder trough, a photo beam placed at the bottom of the trough was no longer blocked, signaling the computer-controlled system to drop another pellet. The computer recorded the date and time each pellet was dropped, and the computer kept a running tally of the total daily food consumption. A record of the pellets dropped over time established the meal duration. Meal duration was monitored in this study 2 days before and for 9 days after the pulpotomy. Nine days after
each pulpotomy there was no significant difference compared with the control group. Meal duration was measured in the 12 animals in each treatment group.

Statistics

Meal duration and cytokine data were analyzed by two-way analysis of variance, with treatment and time as independent variables and meal duration and cytokine concentration as dependent variables. Bonferroni post hoc tests were completed on the groups that showed significant main effects. Values were the means ± standard error of the mean.

Results

Proinflammatory cytokines were quantified from 3 extracted teeth per rat. Thirty days after capping and composite overlay, IL-1β and IL-1α levels were significantly higher in the rats with exposed pulps versus rats that had the pulps capped or the control group (Fig. 1A: $F_{4,28} = 6.7, P < .001, n = 4$ and Fig. 1B: $F_{4,24} = 10.95, P < .01, n = 3$). Sixty days after placing the cement, all the groups had significantly higher amounts of proinflammatory cytokines versus the controls (Fig. 1). The control group was not significantly different when comparing the 30-day and 60-day groups for either IL-1β or IL-1α. Bacteria and immune cells that can stimulate and produce these cytokines were present in the exposed pulps (Fig. 2A–F). Few bacteria were observed in the capped teeth (Fig. 2G); however, clusters of immune cells were present in the margins, and this was noted at the interface between the hydraulic cement and the dentin of less than 20% of the teeth (Fig. 2D–F, open arrows).

After 60 days, 1.6 ± 0.26 of the 6 teeth that were capped with the hydraulic cement ProRoot MTA, on average, lost this material (5 animals were analyzed in each treatment group, each with 6 molars for analysis). On average, 2.6 ± 0.71 of the 6 teeth that were capped with MTA Plus lost this material ($n = 5$), and 2.0 ± 0.44 of the 6 teeth capped with Quick-Set lost the material ($n = 5$). Loss of material was likely because of physical forces applied during mastication. Histology of the teeth indicated that pulp cells were viable when in contact with the hydraulic cements at both 30 and 60 days (Fig. 3, arrows; Table 1). The finer particle size of the MTA Plus and Quick-Set groups compared with the ProRoot MTA cement group can be observed (compare regions labeled “m” in Fig. 3). Exposed teeth exhibited large areas of dead pulp at 30 days, and often these teeth exhibited the absence of viable pulp tissue after 60 days (Fig. 3, second row of panels; Table 1).
Dentinal bridging appeared as early as 30 days after placing the hydraulic cements (Fig. 3, open arrows), and this new dentin was present after capping with each of the 3 cements at 30 and 60 days (Fig. 3).

Meal duration (ie, the nociceptive response) was significantly reduced, $F_{4,66} = 10.8, P < .001$ ($n = 12$), after capping with hydraulic cement versus animals with exposed pulp (Fig. 4). Comparison between the 3 hydraulic cements showed no significant difference in meal duration (Fig. 4); however, the nociceptive response significantly increased for 3 days ($P < .05$) in animals that received a cement pulp cap compared with untreated control animals (Fig. 4).

**Discussion**

Despite the differences in composition, the 3 hydraulic cements had equivalent inflammatory (IL-1β, IL-1α), cellular (dentinal bridging and cellular viability), and nociceptive responses (meal duration). This suggests an equivalent efficacy when the cements were used for capping a pulpotomy procedure in the clinic. In a previous study, pain was reported to be similar for MTA and zinc oxide–eugenol cement when placing a retrofill (22), and similarly in our study, we found that different materials do not affect the pain response. Pain has also been measured in humans when placing MTA cement in a pulpotomy procedure, but to date, no study has measured pain associated with the multiple cements used in endodontic procedures (10).

A very relevant danger for dental pulp tissue after direct pulp capping is a bacterial infection, and bacterial infections of vital pulp tissue can cause inflammation and pulp necrosis (23–25). In rats, the oral bacterial flora is more comparable to that in humans than other commonly used research species (26–29). An improper seal resulting in greater microorganism infiltration at the margins of the material and dentin interface would be indicated by the presence of immune cells. Quick-Set is a calcium aluminoosilicate cement with a...
lower pH (10.9 ± 0.5) than MTA (11.6 ± 0.5), and it demonstrates greater infiltration of the dentinal tubules by the cement (17). Tubule penetration by MTA blocks bacterial access to the nutrients of the pulp, and this blockage can inhibit bacterial growth, reducing inflammation and possibly infection (24, 25, 30, 31). Thus, we expected Quick-Set would have improved bacteriostatic properties compared with MTAs. Thirty days after capping, Quick-Set did not show improved infection control when compared with the other cements. It is more difficult to compare the materials (ie, infection/vitality) after 60 days because on average, 2 of the 6 capped teeth lost the cement, leading to bacterial access and infection of the pulp. Bacterial infection caused by a poor cement/dentin interface and loss of cement likely resulted in the high inflammatory cytokine levels 60 days after capping. Minimizing this infection would enhance dentinal bridging and improve pulp-capping success (23). A future experiment would be to place a permanent restorative material such as amalgam after pulp capping. Permanent restorative materials have been shown to improve the seal after pulp capping and reduce infection and inflammation (32).

When vital pulp tissue is exposed in the rat, an inflammatory reaction results that is very similar to the reaction in humans; however, the biological response is faster in rats than in humans (33). Determining the inflammatory and healing properties after pulp capping a mechanical lesion in a rat model would be expected to provide results representative of human patients. The hydraulic cements all decreased inflammation 30 days after pulp capping a mechanical lesion. This decrease in inflammation was associated with a decrease in orofacial pain, which is consistent with previous studies (11,13–16). Future studies could determine whether a reduction in postoperative pain, an indicator of reduced pulpal damage, can be correlated to a reduction in the 30% failure rate resulting from pulp capping of mechanical lesions (32). Note that no differences were measured in the nociceptive response between the 3 cements in this study; therefore, we could not correlate the quality of pain to differences in healing.

Reparative dentin found in the tested rats after pulp capping was shown to be identical to the reparative dentin found in human teeth (34). In this study we observed signs of dentinal regeneration for all 3 materials at 30 and 60 days. These results are consistent with previous work that showed that reparative hard tissue formation can be detected in 83% of rat molar teeth 28 days after pulp capping (35). Consistent with our results, dentinal bridging has been shown to occur with tricalcium silicates (36), and bioactivity has been shown for calcium aluminates (37) such as the calcium aluminosilicate Quick-Set (18).

Another factor measured in this study was the nociceptive response. The response was significantly higher in rats that had cement placed over the exposed pulps compared with the control teeth. One explanation for the higher nociceptive response would be compression and pressure on the pulp that resulted when the cement was placed. Such compression (eg, vasoconstriction) might lead to inflammation, although studies suggest this pressure should be reduced by fluid leaving the pulp chamber through the vasculature (38, 39).

In conclusion, hydraulic cements improve the inflammatory and nociceptive responses after pulp caps, and the newly developed cement, calcium aluminosilicate or Quick-Set, has equivalent properties to older MTAs that are used for endodontic pulp-capping procedures.

Acknowledgments

The authors thank Connie Tillberg, Mallika Prakesh, Jose Aldana, Alicia Cox, and Brent Herrington for their technical support. These studies were supported by NIDCR grant DE020204.

The authors report the following potential conflict of interest: Dr Primus owns the intellectual property for the dental materials MTA Plus and Quick-Set materials and has a financial interest in Avalon Biomed Inc.

References


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**Table 1. Vital Cells after Pulp Treatment**

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>30 days after pulpotomy</th>
<th>60 days after pulpotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Exposed</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>ProRoot MTA</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>MTA Plus</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>Quick-Set</td>
<td>Medium</td>
<td>Medium</td>
</tr>
</tbody>
</table>

High: >95% of pulp vital; low: <10% pulp vital; medium: >10% but <95% pulp vital.

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**Figure 4.** Twenty-four meal duration of rats treated with 3 different hydraulic cements. Meal duration was measured 2 days before pulpotomy, and these pretreatment days were numbered –2 and –1. Days after pulpotomy were numbered 1–9. Figure 1 shows treatment groups. Significant differences of P < .05 are indicated on the graph with * and by letters.


In Vitro Biocompatibility and Oxidative Stress Profiles of Different Hydraulic Calcium Silicate Cements

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Abstract

Introduction: MTA Plus is a new calcium silicate cement with unknown cytotoxicity characteristics. The objectives of this study were to examine the effect of MTA Plus on the viability, apoptosis/necrosis profile, and oxidative stress levels of rat odontoblast-like cells.

Methods: MDPC-23 cells were exposed to gray and white MTA Plus (GMTA, WMTA), gray and white ProRoot MTA (GTA, WMTA) cements, or their eluents. The cells were evaluated for (1) cell viability by using XTT assay, (2) apoptosis/necrosis by using flow cytometry and confocal laser scanning microscopy, and (3) oxidative stress by measuring reactive oxygen species.

Results: XTT assay showed that all test cements exhibited marked initial cytotoxicity that decreased with time. By the end of the third week, GMTA and GTA were comparable to untreated cells (negative control) in terms of cell viability, whereas WMTA and WMTA were significantly lower than the untreated cells. Apoptosis/necrosis profiles of cells exposed to WMTA and GMTA were not significantly different from untreated cells, whereas cells exposed to WMTA and GMTA showed significantly less viable cells. All experimental groups exhibited reduction of intracellular reactive oxygen species formation compared with untreated cells, although cells exposed to WMTA were not significantly different from untreated cells. Conclusions: Both the gray and white versions of MTA Plus possess negligible in vitro cytotoxic properties that are time and dilution dependent. They enrich the spectrum of hydraulic calcium silicate cements currently available to clinicians for endodontic applications. (J Endod 2014;40:255–260)

Key Words

Apoptosis, cell viability, hydraulic calcium silicate cements, necrosis, reactive oxygen species

Hydraulic calcium silicate cements (HSCCs) have become an integral component of endodontists’ armamentarium because of their bioactivity (1), biocompatibility (2), and osteogenicity (3). These cements have successfully replaced traditional dental cements in endodontic surgeries, apexification, vital pulp therapy, perforation repair, and regenerative endodontic procedures (4). Despite the favorable biological properties of current HSCCs, high operational cost, suboptimal handling properties, relatively long setting times (5), initial cytotoxicity (6), incompatibility with other restorative materials (7), and potential staining of tooth structure (8) are some of the drawbacks of contemporary HSCCs that hinder their use in endodontics. Although some of these hurdles have been addressed in more recent formulations (9, 10), none of the currently available HSCCs address all of the above challenges.

MTA Plus (Avalon Biomed Inc, Bradenton, FL, gray and white versions) is an HSCC with finer particle size that mixes with a proprietary water-based gel for enhanced handling and application as well as improved washout resistance (11). Because of its variable stoichiometric properties (12), the powder/gel ratio of MTA Plus may be adjusted to enable more diverse applications, ranging from perforation repair, root-end filling, direct pulp capping (thicker consistency) to sealing of the cleaned-and-shaped root canal space (thinner consistency).

Because HSCCs are usually applied in intimate contact with pulpal or peritubal tissues, in vitro testing of biocompatibility and cellular responses is an important preliminary step in assessing the overall biocompatibility of such cements. Thus, the objective of the present study was to evaluate the cellular viability, apoptosis and necrosis profiles, and oxidative stress levels exhibited by a rat odontoblast-like cell line after their exposure to the gray and white versions of MTA Plus.

Materials and Methods

The main constituents and primary phases of HSCCs included in the present study are summarized in Table 1. White and gray MTA Plus (WMTAP, GMTAP) were mixed with the proprietary hydrogel by using a liquid/powder ratio of 0.3. For comparison, white and gray ProRoot MTA (GTA, WMTA) (Dentsply Tulsa Dental Specialties, Tulsa, OK) were also examined; these HSCCs were mixed with deionized water by using the same liquid/powder ratio. The mixed materials were placed in pre-sterilized Teflon molds (5-mm diameter and 3-mm thick), covered with pre-sterilized Mular sheets, and allowed to set in a 100% humidity chamber for 24 hours. Untreated cells were used as the negative control. Disks of similar dimensions to the test cements and prepared from a zinc oxide–eugenol cement (Intermediate Restorative Material
[IRM]; Dentsply Caulk, Milford, DE) were assigned as the positive control. All set materials were sterilized with ultraviolet light for 4 hours before testing.

**Cell Culture**

Rat odontoblast-like cells derived from the apical papilla (MDPC-23) were used (13). The cells were plated in complete growth medium and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 hours until fully established. The growth medium consisted of Dulbecco modified Eagle medium (Lonza, Walkersville, MD) and 10% fetal bovine serum (Invitrogen Corp, Carlsbad, CA) supplemented with 2 mmol/L L-glutamine and 100 U/mL penicillin/streptomycin.

**Cell Viability**

An XTT Cell Viability Assay Kit (Biotium Inc, Hayward, CA) was used to determine cell viability on the basis of the cleavage of the yellow tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) by mitochondrial enzymes in metabolically active cells to form a soluble orange formazan product. Production of formazan is directly proportional to the number of vital cells and is quantified by measuring its absorbance at 490 nm. The viability of untreated cells was used as control, and absorbance of the control was adjusted to 100%, with which the relative dehydrogenase activities of the other groups were compared (N = 12). The XTT assay was performed on cells that had been directly exposed to test cements and on eluents derived from those materials.

Direct evaluation of the test materials was performed on a weekly basis according to a cycling regimen (14, 15). A weekly cycle consisted of direct evaluation of the toxicity of the cement disks over the plated cells for 3 days and indirect evaluation of the effect of eluents derived from the set cements on the plated cells. The latter was achieved by immersion of the disks in complete growth medium for 4 days to collect eluents. Accordingly, during the first part of each weekly cycle, cement and control disks were placed individually in Transwell inserts with a 3-mm pore size (BD Falcon, Franklin Lakes, NJ) to prevent direct contact of the cells by the specimen. After the inserts were placed over the plated cells, an additional 2 mL growth medium was added to each well to ensure that the level of the culture medium was above the sides of the Transwell insert. The disks were exposed to the plated cells for 3 days, without further change in culture medium, before testing for mitochondrial dehydrogenase activity. During the second part of each weekly cycle, the disks were retrieved and incubated at 37°C with complete growth medium (1 disk/2 mL) for 4 days to collect the eluents from the set cement before using the same disks for the next cycle. For each disk, the same growth medium was used for eluent collection throughout the entire testing period. This cycling regimen was repeated weekly for 3 weeks (ie, 3 cycles) until the material disks were rendered noncytotoxic (ie, >85% of the mean dehydrogenase activity exhibited by the untreated control).

For indirect evaluation of the eluents, each eluent concentrate collected after the 2-week aging period was diluted with fresh growth medium to 1:1, 1:5, and 1:10 of its original concentration to achieve a final volume of 2 mL (N = 12). Each diluted, eluent-containing growth medium was then used as the respective culture medium for freshly plated rat dental papilla–derived odontoblast-like cell line (MPDC-23) cells for testing cell viability.

**Apoptosis/Necrosis**

**Flow Cytometry.** After MDPC-23 cells were exposed to test materials for 5 days, the cells were detached, centrifuged, and resuspended at 1 × 10⁶ cells/mL in 1X binding buffer (Biotium Inc). The cells were

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>White ProRoot MTA</th>
<th>Gray ProRoot MTA</th>
<th>ProRoot MTA</th>
<th>MTA Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>Water</td>
<td>Water-based gel with water-soluble thickening agents and polymers*</td>
<td>Water</td>
<td>Water-based gel with water-soluble thickening agents and polymers*</td>
</tr>
<tr>
<td>Powder:liquid ratio (by weight)</td>
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<td>Variable from 1:1 to 4:1 depending on indication</td>
<td>3:1</td>
<td>Variable from 1:1 to 4:1 depending on indication</td>
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<tr>
<td>Primary phases</td>
<td>3CaO$\cdot$SiO₂, 2CaO$\cdot$Al₂O₃, CaSO₄, Bi₂O₃, 3CaO$\cdot$Al₂O₃</td>
<td>3CaO$\cdot$SiO₂, 2CaO$\cdot$Al₂O₃, CaSO₄, Bi₂O₃, 3CaO$\cdot$Al₂O₃</td>
<td>3CaO$\cdot$SiO₂, 2CaO$\cdot$Al₂O₃, CaSO₄, Bi₂O₃, 3CaO$\cdot$Al₂O₃</td>
<td>3CaO$\cdot$SiO₂, 2CaO$\cdot$Al₂O₃, CaSO₄, Bi₂O₃, 3CaO$\cdot$Al₂O₃</td>
</tr>
</tbody>
</table>

*Contents are generally regarded as safe.

Calcium aluminoferrite (Ca₂(Al,Fe)₂O₅) is only present in the gray version of both cements. After setting, hydrated calcium silicates form with calcium hydroxide.

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Calcium aluminoferrite (Ca₂(Al,Fe)₂O₅) is only present in the gray version of both cements. After setting, hydrated calcium silicate
stained with fluorescein isothiocyanate (FITC)—annexin V (AnV) (λ_{abs}/λ_{em} = 492/514 nm, green fluorescence) and ethidium homodimer-III (EtD) (λ_{abs}/λ_{em} = 528/617 nm, red fluorescence) and incubated for 15 minutes in the dark. The stained cells were subjected to fluorescence-activated cell sorting by using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) to determine the percentage distribution of vital (AnV/Etd negative), early apoptotic (AnV positive, Etd negative), late apoptotic (secondary necrosis, AnV/Etd positive), and necrotic (AnV negative, Etd positive) cell populations. Experiments were performed in triplicates.

**Confocal Laser Scanning Microscopy.** Cells were plated at 2500 cells/cm² onto coverslips in 6-well plates and exposed to the test materials for 3 days. The cells were triple-stained with Hoechst 33342 (λ_{abs/lem} = 350/461 nm, blue fluorescence), EtD (red fluorescence), and FITC-AnV (green fluorescence). The coverslips were mounted on slides for qualitative evaluation of cell death (apoptosis versus necrosis) after exposure to the materials. A 2-photon confocal laser scanning microscope (CLSM) (LSM 510 META; Carl Zeiss Microscopy, Thornwood, NY) coupled to an MIRA 900 Ti:Sapphire laser (Coherent Inc, Santa Clara, CA) was used for imaging.

**Oxidative Stress**

Detection of oxidative stress in MDPC-23 cells was performed by measuring intracellular reactive oxygen species (ROS) formation by using the CellROX Orange Oxidative Stress Reagent (Invitrogen). After the cells were exposed to test materials for 3 days, they were detached, centrifuged, and resuspended in 1% phosphate-buffered saline. CellROX Orange (a fluorescent redox cytoplasmic stain, λ_{abs}/λ_{em} = 545/565 nm) was added to the cells at a final concentration of 5 μmol/L and incubated at 37°C for 30 minutes. The FACSCalibur flow cytometer was used to detect the percentage of ROS-positive cells in each group (N = 6). The experiment was run in triplicates; untreated cells were used for comparisons with the results derived from cells exposed to different materials. Additional cells were plated on coverslips, double-stained with CellROX Orange and Hoechst 33342, and examined with a fluorescent microscope (Axioplan 2 Imaging; Carl Zeiss) for qualitative evaluation of intracellular ROS distribution.

**Statistical Analysis**

The IRM positive control group was excluded from all statistical analyses to increase the robustness of the respective test. For XTT assay of the effect of materials on cell viability, results derived from each weekly cycle were analyzed separately by using one-factor analysis of variance (ANOVA) and post hoc Tukey test or their nonparametric equivalents. Similar tests were used for analyzing the effect of dilution of the eluents derived from the test cements on cell viability. Parametric versions of these tests were used after evaluation of the normality (Shapiro–Wilk test) and equal variance assumptions (modified Levene test) of the individual data tests. If those assumptions were violated, the data were non-linearly transformed to satisfy those assumptions before using parametric testing methods. If those assumptions remained violated after non-linear transformation, the original data set was analyzed by using Kruskal–Wallis ANOVA and Dunn multiple comparison tests. For apoptosis/necrosis, the numbers of vital, non-apoptotic, non-necrotic cells in each group were analyzed by using one-factor ANOVA and post hoc Tukey test. For oxidative stress evaluation, the numbers of ROS-positive cells in each group were also analyzed by using one-factor ANOVA and post hoc Tukey test. Statistical significances for all analyses were set at α = 0.05.

**Results**

**Cell Viability**

Relative mitochondrial dehydrogenase activities of MPDC-23 cells after their direct exposure to the test cements are delineated in 3 weekly cycles and summarized in Figure 1A. For the first cycle, all cements were significantly cytotoxic (P < .001). For the second cycle, all cements still exhibited significant cytotoxicity except for GMTAP; the latter caused the cells to produce a similar level of dehydrogenase activity as the untreated cells (P = .07). For the third cycle, GMTAP and GMTA were not significantly different (P = .762 and P = .128, respectively), whereas WMTAP and WMTA were significantly different (P = .009 and P < .001, respectively) from the activities of the untreated cells. Nevertheless, the 4 HSIC groups were not significantly different from each other at each weekly cycle.

Indirect evaluation of the effect of eluent concentrations on dehydrogenase activities of MPDC-23 cells is depicted in Figure 1B. At 1:1 dilution, cells cultured with growth medium containing eluents derived from...
from different HSCSs were significantly more cytotoxic than cells cultured in the eluent-free medium ($P < .001$). At 1:5 dilution, only WMTA was significantly more cytotoxic than the untreated control ($P < .05$). At 1:10 dilution, there was no difference among the HSCSs and untreated control ($P = .565$).

**Apoptosis/Necrosis**

A representative CLSM image of vital, apoptotic, and necrotic cells is illustrated in Figure 2A. A 2-dimensional plot of the distribution of vital, early apoptotic, late apoptotic, and necrotic cells in a typical cell-sorting procedure is shown in Figure 2B. Cells exposed to WMTAP and GMTAP were not significantly different from unexposed (untreated) cells in the percentage of vital cells, whereas cells exposed to WMTA and GMTA had a significantly lower percentage of vital cells ($P < .001$ and $P = .002$, respectively). There was no difference between WMTAP and GMTAP ($P = .992$), as well as between WMTA and GMTA ($P = .962$) (Fig. 2C). The flow cytometry results were qualitatively confirmed by CLSM imaging. Cells exposed to the HSCSs were mostly vital and exhibited blue fluorescent nuclei with minimal signs of apoptosis/necrosis. These cells exhibited comparable fluorescence characteristics as the untreated cells. By contrast, cells exposed to IRM were mostly apoptotic, with prevalence of green fluorescent cytoplasm and occasional necrotic pink nuclei (merging of blue and red fluorescence) that are characteristic of late apoptosis and necrosis.

**Oxidative Stress**

A fluorescent microscopic image of MDPC-23 cells with Hoechst 33342–stained, blue fluorescent nuclei and diffuse orange cytoplasmic fluorescence that is indicative of the production of ROS is shown in Figure 3A. Cells exposed to IRM had very high levels of ROS (Fig. 3A). Untreated cells exhibited significantly higher ROS levels compared with cells that were exposed to GMTA, GMTAP, and WMTAP ($P < .001$), whereas there was no difference between the oxidative stress levels of untreated cells and cells that were exposed to WMTA ($P = .515$) (Fig. 3B).

**Discussion**

The use of MDPC-23 cells for the present work was based on their potential clinical significance with the use of HSCSs for direct pulp capping and for their superior sensitivity, while maintaining the same cytotoxicity ranking, when compared with transformed fibroblast and osteoblast cell lines (16, 17). The XTT assay evaluates cell viability through colorimetric quantification of formazan produced via reduction of tetrazolium salts by mitochondrial dehydrogenases (18). These enzymes are expressed only in vital cells and are inactivated shortly after cell death. Accordingly, formation of highly colored formazan dyes is indicative of a metabolically active cell population. In the present work, a modified cycling regimen (14, 15) was used to test the direct effects of test materials on cell viability at different time points as well as the indirect cytotoxic effects through eluents derived from the HSCSs (19). Results from direct and indirect evaluations were in agreement and suggest that under the experimental conditions, gray versions of both cements appear to be less cytotoxic than the white versions. The results further support previously reported findings that gray MTA is more biocompatible than white MTA (20, 21).

Analysis of the mode of cell death (apoptosis/necrosis) via flow cytometry and fluorescent microscopy is helpful in better understanding

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**Figure 2.** (A) Representative CLSM image of MPDC-23 cells that were triple-stained with Eid (red fluorescent nonvital DNA dye), Hoechst 33342 (blue fluorescent nuclear counterstain), and FITC-AnV (green fluorescent phosphatidylserine-binding cytoplasmic dye) after their exposure to the test cements. Healthy cell nuclei are stained blue, apoptotic cells show green cytoplasm, and necrotic cells show red or pink nuclei. (B) Representative 2-dimensional flow cytometry dot plot of data derived from FITC-AnV and Eid-stained MDPC-23 cells after their exposure to test cements for 5 days. (C) Bar graph comparing the percentage of vital cells (lower left quadrant of B) after exposure to test cements ($N = 5$). Groups labeled with the same letter designators are not significantly different ($P > .05$).

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the cytotoxic effects of the test materials on cell permeability. Exposure to MTA Plus (both gray and white versions) resulted in more vital cells than ProRoot MTA (both gray and white versions). Judging by the apoptosis/necrosis profile of all test cements, it seems that the initial cytotoxicity of MTA Plus and ProRoot MTA is more likely to be attributed to apoptosis rather than necrosis, as evidenced by detection of phosphatidylserine expression on the cell surfaces via the use of AnV (22). This suggests that the initial sites of irreversible damage by the cytotoxic agents are extracellular, whereas the nuclear membranes of those cells still remain intact.

Evaluation of intracellular ROS formation provides another perspective toward understanding of cellular responses to the test materials. ROS is a natural by-product of normal oxygen metabolism and has been found to play important roles in cell signaling, proliferation, and survival (23). Increase in ROS levels occurs during stress, results in significant damages to cell structures, and has been implicated in diseases such as cancer, aging, neurodegenerative diseases, and diabetes (24–27). Interestingly, exposure to HCSCs resulted in decreasing the ROS levels in GMTA, WMTAP, and GMTAP when compared with levels identified from unexposed cells. A plausible explanation is that the pH elevation caused by release of calcium hydroxide from HCSCs may decrease ROS formation. These findings support earlier reports relating the production of ROS to decreases in extracellular pH (28–30). It is of interest to see whether the decreased level of oxidative stress when MDPC-23 cells are exposed to MTA Plus may enhance the ability of these cells to differentiate and deposit mineralized matrix when they are cultured in osteogenic differentiation medium. Research in this direction is in order.

Within the limits of the present study, it may be concluded that the cytotoxic effects imposed by MTA Plus on MDPC-23 cells are both time and concentration dependent, and that they possess negligible cytotoxic risks after the elution of their cytotoxic components. Because these risks are significantly lower than those imposed by zinc oxide-eugenol–based cement, a favorable perspective toward understanding of cellular responses to the test materials is in order. Research in this direction is in order.

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**References**

A quantitative method for determining the antiwashout characteristics of cement-based dental materials including mineral trioxide aggregate

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Abstract

Aim To introduce and assess a novel method for measuring washout resistance of cement-based dental materials, including mineral trioxide aggregate (MTA), to qualitatively verify the results with a clinical simulation and to evaluate the washout resistance of a new root-end filling material.

Methodology A method for assessment of washout resistance of root-end filling materials was developed by adapting the CRD-C 661-06 (a method for evaluating the resistance of freshly mixed concrete to washout in water), to permit testing of dental cements. White Portland cement (PC), MTA-Plus mixed with either water or a polymer-based antiwashout gel (MTA-AW), MTA-Angelus, IRM and amalgam were tested with either distilled water or HBSS as washout media. Additionally, the washout resistance was tested qualitatively by spraying the test materials at the terminus of simulated canals with a metered jet of water.

Results A mass loss of 2–7% for PC, 0.4–4% for MTA-Plus, −0.9% for MTA-AW, 5–10% for MTA-Angelus and 0% for IRM and amalgam was recorded with the modified CRD-C 661-06 method. No significant difference was found between using water and HBSS as washout media for the same material. The results of the modified CRD-C 661-06 method were similar to those obtained on the simulated canals.

Conclusions The modified CRD-C 661-06 method provided repeatable results that were comparable to the simulated clinical method. The antiwashout gel used with MTA-Plus reduced the material washout and was similar to IRM and amalgam.

Keywords: antiwashout, dental materials, mineral trioxide aggregate, root-end filling materials.

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Introduction
Mineral trioxide aggregate (MTA) is a Portland-cement-based material (Camilleri et al. 2005) with numerous applications in endodontics such as pulp capping, apexification, repair of root perforations, root-end filling (Torabinejad & Chivian 1999) and others (Parirko & Torabinejad 2010). However, one of the drawbacks of MTA is washout (Bortoluzzi et al. 2006), which refers to the tendency of freshly prepared cement paste to ‘disintegrate upon early contact with blood or other fluids’ (Wang et al. 2007).

Following a survey of the literature, no standardized method for evaluating washout resistance of cement-based dental materials was evident. A number...
of researchers have resorted to employing diverse quantitative and qualitative methods for evaluating washout resistance. These include visual observation after immersion in water (Chen et al. 2010, Lin et al. 2010) and measuring the change in mass after injecting cement in water (Wang et al. 2007, Kai et al. 2009). Other investigators (Porter et al. 2010) employed water sprayed from a known distance at a specified flow rate on samples consisting of cement paste. The resultant specimens were then photographed and visually examined, and washout resistance was evaluated by allowing two independent, blinded evaluators to determine the percentage of the original margin remaining from the photographs.

Further methods used for the investigation of washout resistance involved the injection of the cement into distilled water, and after immersion for 24 h, the nondecayed part of the cement was freeze-dried. Its mass, expressed as a percentage of the original mass of the cement, was used to determine the washout resistance (Kai et al. 2009). A similar method (Wang et al. 2007) involved injecting the cement into a container filled with water, shaking the container for a set number of minutes and measuring the mass of cement remaining, expressed as a percentage of the original mass of cement injected.

The materials tested were calcium silicate (Wang et al. 2007, Kai et al. 2009) and calcium phosphate (Chen et al. 2010, Lin et al. 2010) bone cements. Dental and endodontic materials (white MTA, Generex-A, Capasio and Ceramicrete-D) have also been investigated (Porter et al. 2010). A visual method was used to assess the loss of material caused by washout of the cement in solution.

The diverse number of washout testing methods available reveals the necessity of a standardized method for measuring the washout resistance of dental materials. The ideal method would be one that gives quantitative, objective and reproducible results. One possibility is to adapt the method described in the CRD-C 661-06 specification, section 16, which provides a test method for determining the resistance of freshly mixed concrete to washing out in water, to make it applicable for small volumes of dental materials. This method is based on the older CRD-C 61-89A specification and is currently used for testing the washout resistance of concrete. Briefly, this method involved placing the test material into a perforated cylinder, allowing it to sink freely through a column of water and then raising it back up. The test cycle was repeated a number of times. The mass of material lost following each cycle was measured. Washout was then expressed as a percentage of the initial mass of the sample. The original method uses concrete samples with a mass of 2 kg. This is prohibitively large amount for dental cements, and not representative of the small volumes of material typically employed in dentistry.

Although there are several formulations of materials based on tricalcium silicate cement, there are two main mineral trioxide aggregates namely ProRoot MTA (Dentsply, York, PA, USA) and MTA-Angelus (Angelus Soluções Odontológicas, Londrina, PR, Brazil). The materials have a similar chemical composition and are composed of Portland cement and bismuth oxide. A difference in the texture and in the particles of each material exists. MTA-Angelus does not contain the calcium sulphate phase, which results in a shorter setting time of the material (Oliveira et al. 2007). In addition, MTA-Angelus is less radiopaque (Camilleri & Gandolfi 2010). Recently, another MTA has been introduced on the market. According to the manufacturer, MTA-Plus (Avalon Biomed Inc., Bradenton, FL, USA) is similar in composition to ProRoot and MTA-Angelus but is ground finer. MTA-Plus is marketed accompanied by water or a hydrosoluble gel aimed at reducing washout.

The purpose of this study was to introduce and assess a method to quantitatively measure the washout resistance of cementitious dental materials and to verify these results qualitatively by comparing them with the results of a simulated clinical situation. In addition, the washout resistance of a novel root-end filling material is also assessed.

**Materials and methods**

The materials used in this study included MTA-Plus (compound by Prevest Denpro, Jammu, India for Avalon Biomed Inc.) lot #2011022801, Portland cement (PC: CEM 1, 52.5 N: Lafarge Cement, Birmingham, UK), MTA-Angelus (Angelus, Londrina, PR, Brazil), IRM (Dentsply, Konstanz, Germany) and amalgam (AB Ardent, Arlandastad, Sweden). The MTA-Plus was mixed with either water (MTA-Plus) or an antiwashout gel (MTA-AW; compounded by Prevest Denpro, Jammu, India for Avalon Biomed Inc. Bradenton, FL, USA) at a water-to-cement ratio of 350 μL g⁻¹ and gel-to-cement ratio of 350 μg g⁻¹, respectively. White Portland cement was mixed at a water to cement ratio of 350 μL g⁻¹. MTA-Angelus, IRM and amalgam were mixed as directed by the
manufacturer. The fluids used for washout testing included distilled water and Hank’s balanced salt solution (HBSS; H6648, Sigma Aldrich, St. Louis, MO, USA).

**Drop method using an adaptation of the CRD-C 661-06 method**

The test set-up (Fig. 1) consisted of a standard-sized test tube with an internal diameter of 14.5 mm, which was filled to a height of 120 mm with distilled water or HBSS at room temperature (23 °C). A cylindrical container with a 9.0 mm diameter and a height of 17 mm was constructed from two pieces of woven brass mesh (60 wires per inch) with a wire diameter 0.18 mm. The seams on the side and around the bottom had an overlap of 1.0 mm and were bonded with light-curing resin (Heliobond, Ivoclar Vivadent AG, Schaan, Liechtenstein). The empty mesh cylinder was weighed on an analytic balance with an accuracy of ±0.0001 g (Sartorius AG, Gottingen, Germany), and a quantity of material to be tested was prepared and transferred to the cylinder. In the case of PC and MTA-based materials, approximately 1.35 g of cement paste was prepared, using 1.00 g of each cement powder, by mixing with the appropriate quantity of distilled water or antiwashout gel on a glass slab with a spatula. In the case of IRM, two scoops of powder and three drops of fluid were mixed and transferred to the basket. Amalgam was prepared by triturating one 600-mg capsule for 10 s and transferring the material immediately to the mesh cylinder. The material under study was packed into the cylinder using a dental plugger, and the top surface of the cement was flattened (Fig. 2a). The outer part of the cylinder was lightly patted with absorbent paper to remove any extruded material. The mass of the cylinder, filled with material, was then measured, and the exact mass of cement in the cylinder was calculated. The cylinder was released just above the surface of the fluid in the test tube (Fig. 2b) and allowed to sink unhindered as specified in the standard (CRD-C 661-06). The cylinder was left at the bottom of the tube for 15 s and then brought out of the water in 5 ± 1 s and allowed to drip for 2 min. The cylinder was patted dry with absorbent paper to remove any remaining water and weighed. The complete procedure (Fig. 1b,c) was repeated in the same fluid (as specified in the standard) to give a total of three drop cycles per specimen.

The materials were tested in distilled water and HBSS. Two replicate tests per material per fluid were conducted using fresh solution for each replicate. Washout (or loss of mass of the sample) was expressed as a percentage of the initial mass of the sample and calculated using Equation 1:

\[
D = 100 \times \frac{M_i - M_f}{M_i}
\]

where: \(D\) = washout (%); \(M_i\) = Mass of sample before initial drop; \(M_f\) = Mass of sample after each drop

Analysis of variance (ANOVA) with \(P = 0.05\) and Tukey’s post hoc test were used to perform multiple comparison tests.

**Figure 1** (a) Test set-up to determine washout resistance and (b) Procedure for one drop cycle.
Metered spray testing using simulated canals

The set-up for the simulated clinical situation is shown in Fig. 3a. A syringe with a 21-gauge needle was mounted in a retort stand and filled with 10 cc of distilled water. An Endovue block (Dentsply, Konstanz, Germany) with a pre-prepared root-end cavity was used. The canal was prepared to 0.5 mm short of the canal terminus using the crown-down technique with ProTaper rotary nickel–titanium instruments (Dentsply Maillefer, Ballaigues, Switzerland) under copious irrigation. The canals were dried and filled with gutta-percha and AH Plus sealer (Dentsply Maillefer, Montigny de Bretonneux, France) using the warm vertical condensation technique with System B (Sybron Endo, Orange, CA, USA).

The Endovue blocks were then weighed, filled with the material to be tested, re-weighed and placed in a ceramic dish beneath the needle, such that the jet of water from the needle impinged on the edge of the block and flowed down the side, thus washing over the material under test but not directly spraying into it (Fig. 3b). In this way, the material is washed out by the same mechanism responsible for washout in vivo when the root end is irrigated with a stream of water perpendicular to the root-end cavity. The distance from the tip of the needle to the edge of the block was set to $35 \pm 1$ mm. A 1-kg weight was placed on the syringe plunger to provide a constant force. This combination resulted in the syringe emptying entirely within $15 \pm 1$ s, giving a mean flow rate of $0.667 \text{ cc s}^{-1}$. Following the test, the block was dried with filter paper, taking care not to disturb the material in the cavity, and weighed. The loss in mass of the block was then expressed as a percentage of the mass of dental material initially placed in it.

Figure 2 (a) Preparation of cement specimen for washout testing; (b) cylinder filled with cement paste held over solution in tube; (c) cylinder allowed to free-fall in solution.

Figure 3 (a) Experimental set-up for verification of washout results; (b) Detailed view of endo block showing orientation of filled cavity and area struck by water jet.
Results

Drop method using an adaptation of the CRD-C 661-06 method

The results of the drop tests are presented in Fig. 4. Three of the materials tested exhibited washout. In order of increasing washout resistance, these included MTA-Angelus, Portland cement and MTA-Plus. MTA-AW displayed mass gain after the first washout test cycle, but then its mass remained unaltered after subsequent test cycles. IRM and amalgam did not change mass after any of the three drops. The standard deviation was relatively small compared with the mean values, indicating an acceptable confidence level and repeatability between samples. Thus, the number of repeats conducted was deemed satisfactory.

No statistically significant differences were observed between the washout percentage in distilled water and HBSS for the same material at the same test cycle number (\( P > 0.05 \)) in all cases. This indicates that washout was not affected by the different constitution of the two media tested. No statistically significant differences were observed between IRM and amalgam for all tests. For the first test cycle in water, no statistically significant differences were observed amongst PC, MTA-Plus and MTA-AW, whilst statistically significant differences were observed between MTA-Angelus and MTA-Plus (\( P = 0.001 \)), amalgam and MTA-Angelus (\( P < 0.001 \)) and similarly between IRM and MTA-Angelus (\( P > 0.001 \)). For the first cycle in HBSS, more material was washed out from PC than from MTA-AW (\( P < 0.001 \)), and more was washed out from MTA-Angelus than MTA-Plus (\( P = 0.003 \)). For the second test cycle, more material was washed out from PC than from MTA-AW in water, whilst in HBSS the loss in weight was observed with the numerical order being MTA-Angelus > PC > MTA-Plus > amalgam/IRM > MTA-AW. There was a statistically significant difference between the PC and MTA groups (\( P = 0.022, P < 0.001 \) respectively), between MTA-Plus and MTA-Angelus (\( P < 0.01 \) in all cases) and between the MTA-Plus groups with and without antiwashout gel (\( P = 0.027 \)). In the third test cycle, the pattern of weight loss was similar to that of the second test cycle.

Metered spray testing using simulated canals

The results of the simulated canal tests are presented in Fig. 5. All the water-based materials (PC, MTA-Plus and MTA-Angelus) were washed out of the cavity in their entirety. In the case of the other materials (MTA-AW, IRM and amalgam), the mass did not change following spraying with water and drying, indicating that none of the material was washed out. These results indicate that the antiwashout gel successfully inhibited washout even in a stream of water. The results from this test concur with the trend seen in the plunge tests, where a positive washout percentage was recorded for PC and the two MTA
preparations, and zero or a negative washout percentage was recorded for MTA-AW, IRM and amalgam.

**Discussion**

Washout poses a clinical problem during root-end surgery. Prior to closing an apical flap, it is necessary to irrigate the area well to avoid postoperative complications. In addition, the flow of blood in the surgical site once the suction keeping the area dry is ceased will also cause washout to a certain degree. Washout resistance of root-end filling materials is thus important. Enhanced washout resistance avoids loss of the material placed at the root end.

The Specification CRD-C 661-06 (2006) suggests the test method for testing washout resistance of concrete in the construction industry. Portland cement, which is the main constituent compound in MTA (Torabinejad & White 1995), is also used as a binder in concrete. The washout test determines the relative amount of cement paste lost when the concrete is exposed to a large volume of water. Washout resistance in concrete can be determined by the stream, drop, pH factor, plunge and the spray tests (Sonebi et al. 1999). The stream and drop tests are based on visual inspection of loss of material when exposed to a liquid normally water and thus are operator dependent. The pH factor test measures the rise in pH of the storage liquid when fresh concrete is dropped. This method is relevant for Portland cement type materials, which leach out calcium hydroxide thus causing a rise in pH of the surrounding media, but may have limited use when testing other materials. The plunge test is specified by CRD-C 661-06, and both this test and the spray test measure the change in mass of concrete when subjected to water. The conditions set by these tests are standard and reproducible. In addition, the measurements undertaken are not subjective.

In this study, the plunge test was selected to measure the washout resistance of a variety of dental materials, including newly introduced variety of mineral trioxide aggregate (MTA-Plus) mixed with water or an antiwashout gel, MTA-Angelus, intermediate restorative material (IRM) and amalgam. All these materials are used as root-end filling materials. Portland cement was tested as a control material. The plunge test developed was based on the CRD-C 661-06 specifications using smaller dimensions of the basket and water container to be able to accommodate the testing of a dental material. The results obtained indicate that the method exhibits good levels of repeatability and precision. As the result is a value, and does not rely on personal judgment as visual inspection methods do, it is quantitative and objective. Because the method involves multiple drops of the same sample, it gives an insight into the behaviour of the material.

The materials were tested both in distilled water and HBSS as previous studies have shown a link between physical properties and curing conditions of MTA-like systems (Formosa et al. 2012). The Portland cement lost a relatively large percentage of mass after the first test cycle and continued losing mass in subsequent cycles. The MTA-Plus lost a smaller amount of mass in the first test cycle, and the rate of mass loss started to taper off by the third cycle. This tapering-off effect was also observed for MTA-Angelus in distilled water, but not in HBSS. The increased resistance of MTA-Plus compared with PC may be due to its finer particle size. This would give MTA-Plus particles a greater surface area and thus greater cohesive force of attraction between adjacent particles; hence, the slightly greater resistance to washing out as this is the only force keeping the freshly mixed paste intact in the minutes immediately after mixing, as the C-S-H network has not had time to develop any strength. With regards to washout, MTA-Angelus

![Figure 5](image.png)
fared worst amongst the materials tested. The MTA-Plus mixed with antiwashout gel exhibited a gain in mass after the first cycle. The enhanced antiwashout resistance may be due to the water-soluble polymer present in the gel as claimed by the manufacturer. However, the capacity to absorb water to some degree when the surface of the sample was placed in contact with the water. The washout resistance of the samples incorporating antiwashout gel was confirmed visually as the liquid in the test tube remained clear after each test. In contrast, the fluid in the test tube was visibly turbid after testing with PC, MTA-Plus and MTA-Angelus, both with water and HBSS. IRM and amalgam did not change mass in any of the tests. This was a predictable result as neither material is water based, and in fact, the eugenol in the liquid component of IRM is hydrophobic, as are the metals in the amalgam.

The results of the drop tests were confirmed with the simulated canal tests. Preliminary tests using root filled teeth were performed, but the results obtained were not reliable because of the tooth absorbing a portion of the water and thus distorting mass measurements. For this reason, simulated canals in resin blocks were substituted for the teeth as they are made of a material that does not absorb water and thus provided a means of eliminating water uptake as a source of error. The simulated canal results were consistent with the plunge test results. In particular, MTA-AW resisted washing out in both tests, but did not gain any appreciable mass. This may be due to the much smaller surface area exposed, compared with the drop test, and the very small mass of material that could be placed into the root-end cavity (around 0.04 g in the tests conducted). Comparing the two methods, more material loss was observed with the simulated canals than with the plunge test. The reason for this may be that material in the simulated canal had its surface directly exposed to the water, whilst in the plunge test, the mesh cylinder partially isolated the cement from the water. In the simulated canal, the water jet was able to mechanically dislodge large quantities of cement, whilst in the plunge test a mass loss could only be achieved as a result of cement particles migrating into the washout medium and being washed out through the spaces in the mesh. In addition, the 5 mm diameter of the exposed cement placed in the cavity prepared in the blocks was significantly larger than the space between wires in the mesh (approximately 0.4 mm).

This method appears to address the main shortcomings of the other methods used in literature. Visual observation of degree of washout (Chen et al. 2010, Lin et al. 2010, Porter et al. 2010) is subjective and does not give a quantitative measure of washout resistance. Measurement of mass change after injecting cement in water (Wang et al. 2007, Kai et al. 2009) requires that the nondecayed part be taken out of the fluid and weighed. This introduces a potential source of variation as there may be some subjectivity in picking what constitutes the ‘nondecayed’ part and it is difficult to pick out the nondecayed part without losing further mass in the process. In contrast, in the plunge method, the mesh cylinder conveniently isolates the nondecayed part of the cement and makes mass measurement unambiguous. Finally, injection of the cement into distilled water, immersing for 24 h, freeze-drying and weighing (Kai et al. 2009) is more time-consuming and requires additional specialized equipment (and its associated costs) compared with the plunge method.

The main shortcoming of the plunge method is that the immersion speed was only controlled by the fluid resistance encountered by the falling cylinder. The immersion speed is dependent on the mass and geometry of the mesh cylinder. The latter is negated by using the same cylinder dimensions for each test. In the case of the fluid resistance, a greater mass of cement would result in a higher terminal velocity of the cylinder through the water. However, this is offset by the greater volume of cement present, which thus exposes more area to the washout in the test tube and results in a greater mass of cement being washed out. In practice, this gave comparable washout percentages between replicates of the same material, for the range of sample masses tested. However, testing of materials with large differences in density is not catered for and might result in the denser cements having a higher apparent washout percentage because of their higher falling speed.

Conclusions

The method presented has been verified as a quantitative, objective way in which the washout resistance of cementitious dental materials may be investigated and compared. The standard deviation in percentage washout between replicate runs with the same material was found to be relatively small, on the order of 10% of the average value, and thus the method exhibits acceptable repeatability. The results
were found comparable to the simulated clinical method. Portland cement, MTA-Plus mixed with water and MTA-Angelus exhibited significant washout, whilst the antiwashout gel used with MTA-Plus reduced the material washout and made its washout resistance similar to IRM and amalgam.

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References


Push-out bond strength of MTA with antiwashout gel or resins

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Abstract

Aim Assessment of the push-out bond strength of four MTA-based formulations for use as root-end filling materials.

Methodology MTA Plus mixed with (i) water (‘MTA-W’); (ii) a proprietary water-based antiwashout gel (‘MTA-AW’); (iii) Superbond C&B chemically curing resin (‘MTA-Chem’); and (iv) Heliobond light-curing resin (‘MTA-Light’) was tested. Root slices 3 mm thick human had a 1.5 mm diameter hole drilled centrally and were treated with 17% EDTA for 60s. Forty specimens divided into groups 1–4 were prepared and filled with MTA-W, MTA-AW, MTA-Chem and MTA-Light, respectively. Groups 3 and 4 were etched with 37% phosphoric acid for 60s, and bonding agent was applied to the dentine surface. Specimens were stored for 28 days in Hanks’ Balanced Salt Solution at 37 °C. Push-out strength was tested with a punch and die (punch diameter 1.3 mm, die diameter 2.0 mm, punch speed 1 mm min⁻¹). Stereomicroscopy was used to classify failure mode (adhesive, cohesive or mixed type).

Results The resulting push-out strengths were 5.1 MPa (MTA-W), 4.3 MPa (MTA-AW), 4.7 MPa (MTA-Chem) and 11.0 MPa (MTA-Light). MTA-W had higher push-out strength than MTA-AW (P = 0.022). The same was noted for MTA-Light relative to the other materials (P < 0.05). All materials exhibited adequate push-out strengths compared with MTA-W. Failure was predominantly mixed, except for MTA-Chem (predominantly adhesive).

Conclusions All materials exhibited adequate push-out strength. Previous studies have shown the new formulations have additional advantages including increased washout resistance and faster setting time, making them promising for future dental applications.

Keywords: antiwashout, bond strength, composite resins, mineral trioxide aggregate, push out, root-end filling materials.

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Introduction
Mineral trioxide aggregate (MTA) is a dental cement with numerous applications including pulp capping, apexification, repair of root perforations, root-end filling and others (Torabinejad & Chivian 1999, Bogen & Kuttler 2009, Parirokh & Torabinejad 2010). The main clinical limitations of this material are its long setting time (Gomes-Filho et al. 2009, Porter et al. 2010) and consequent risk of washout (Bortoluzzi et al. 2006), which refers to the tendency of freshly prepared cement paste to disintegrate upon contact with blood or other fluids (Wang et al. 2007). Washout can occur when rinsing an osteotomy site (Porter et al. 2010) resulting in a compromised root-end seal.

A number of approaches can be taken to minimize this problem, such as the use of washout-resistant MTA. MTA Plus (Avalon Biomed Inc. Bradenton, FL,
USA) is supplied with either water or an antiwashout gel. MTA Plus powder is similar to ProRoot MTA, but it is ground finer (Camilleri et al. 2013). The antiwashout gel was shown to be a water-based solution containing minor quantities of silicon, potassium, calcium and chlorine. The presence of an organic additive could be inferred from the FT-IR plots. No crystalline phases were detected in the dried gel (Formosa et al. 2013b). The gel significantly reduced washout when compared to the same MTA mixed with water (Formosa et al. 2013a). MTA Plus mixed with antiwashout gel exhibited lower levels of calcium ions in solution and reduced fluid uptake in the early stages of reaction. The antiwashout gel reduced the setting time of the cement and enhanced the compressive strength (Formosa et al. 2013b).

Alternatively, incorporation of resin into MTA has been proposed as a way of reducing setting time, which may extend the material’s clinical use (Gandolfi et al. 2011a). In this application, combinations of MTA with light-curing resin (Gomes-Filho et al. 2010, Gandolfi et al. 2011a,b) and chemical curing resin (Chung et al. 2011) have shown promising results, promoting remineralization, releasing calcium ions and producing an alkaline pH in physiological solution. Composites based on MTA Plus exhibited calcium ion release, alkalinizing pH and formation of apatite. These composites are recommended for applications where bioactivity is desirable but not critical, and only they have a significant advantage over MTA in some other aspect (Formosa et al. 2013c). The setting time of MTA with light-curing resin was analogous to the light exposure time, that is, 30–100 s (Gandolfi et al. 2011a), whilst for MTA mixed with chemical curing resin, the actual setting time was 11.2 min (Chung et al. 2011). The resin-modified materials are indicated for use as dressings over pulpotomies and as pulp-capping agents where the tooth can be etched and the reduction in setting time would be beneficial as the extended setting time could result in increased number of appointments and the risk of coronal leakage (Hong et al. 2010) and also reaction of unset MTA with materials used to temporize the tooth being treated (Camilleri 2011).

As leakage from the apical or coronal direction is a possible cause of root treatment failure (Madison & Wilcox 1988), an important factor for the success of various endodontic procedures is the marginal adaptation (Reyes-Carmona et al. 2010) and bond strength (Wennberg & Ørstavik 1990) of the material with dentine. A frequently used test for measuring dislocation resistance and bond strength of a restorative material to dentine is the push-out test.

Push-out tests have been successfully conducted in the past on numerous brands of MTA under various environmental conditions (Frankenberger et al. 2000a, Gancedo-Caravia & Garcia-Barbero 2006, Reyes-Carmona et al. 2010, Saghir et al. 2010, Shokouhinejad et al. 2010, Guneser et al. 2013), giving push-out strength values in the region of 2.5–11.4 MPa. Resin-based sealers have also been studied. For instance, AH Plus has been shown to have push-out strength comparable to an MTA-based sealer (Assmann et al. 2012). However, no studies to date have evaluated the bond strength of antiwashout-type MTA or MTA-based resin composites to dentine.

The purpose of this study was to determine whether the novel MTA Plus-based formulations (i.e. MTA mixed with antiwashout gel, chemically curing resin or light-curing resin) bond to dentine as strongly as ordinary MTA Plus (mixed with water). The push-out strength of MTA root-end restorations using each of the four test materials after storage in physiological conditions for 28 days was used to assess the bond strength.

Materials and methods
The materials used in this study were based on MTA Plus (compounded by Prevest Denpro, Jammu, India for Avalon Biomed Inc. Bradenton, FL, USA) lot #2011022801, mixed with different liquids to obtain the following formulations:

- MTA-W: distilled water with a water-to-powder ratio of 0.35 by mass;
- MTA-AW: antiwashout gel (compounded by Prevest Denpro, Jammu, India for Avalon Biomed Inc. Bradenton, FL, USA) dosed by weight – 0.350 g gel per gram MTA Plus powder;
- MTA-Chem: chemical curing resin (Superbond C&B; Sun Medical, Shiga, Japan). The polymer powder and the MTA were dosed volumetrically using the scoops supplied in the Superbond kit. The proportions used are 0.4 mL (one scoop) clear L-type polymer, 0.136 g (8 drops) monomer and 0.01 g (2 drops) catalyst-V per 0.4 mL (one scoop) of MTA powder;
- MTA-Light: light-curing resin (Heliobond, Ivoclar-Vivadent, Schaan, Liechtenstein) filled with MTA Plus. The filler loading used was 1 g MTA per 0.3 g Heliobond.
Specimen preparation

Twenty single-rooted, extracted human teeth were selected and stored in sterile water until use. Each tooth was embedded in cold-curing resin (EpoFix, Struers, Ballerup, Denmark) and sectioned perpendicular to its long axis using a water-cooled diamond wafering disc (Buehler, Lake Buff, IL, USA) on a cutting machine (Struers Minitom, Ballerup, Denmark). Two 3 mm slices of mid-root dentin were obtained from each tooth. The root canal spaces were then drilled with a 1.5 mm hardened steel drill bit on a low-speed pillar drill (Fig. 1a) to obtain standardized cavities of 1.5 mm diameter. The sections were immersed in 17% EDTA (Glyde; Dentsply Maillefer, Ballaigues, Switzerland) for 60 s and immediately rinsed out and dried with a pneumatic syringe. The specimens were then randomly divided into four groups \( (n = 10) \). In group 1 and group 2, MTA-W and MTA-AW, respectively, were mixed as described and placed directly inside the root canal space. Filling was carried out on a flat glass plate, and excess material was trimmed from the specimens with a plastic instrument.

Additional preparation steps were applied to groups 3 and 4 before filling. The specimens were etched with 37% phosphoric acid for 60 s and rinsed and dried. A thin layer of bonding agent (Heliobond; Ivoclar-Vivadent, Schaan, Liechtenstein) was applied to the internal surface of the cavity using a
microbrush (Microbrush Intl., Waterford, Ireland) and light-cured for 30 s. MTA-Chem (group 3) or MTA-Light (group 4) was mixed as described and placed inside the cavity using the same method as for groups 1 and 2. No etch and bond was used in the control group (MTA-W) or in the MTA mixed with antiwashout gel because both these groups did not use resin-based systems.

All specimens were then immersed in Hanks’ Balanced Salt Solution (HBSS; H6648, Sigma Aldrich, St. Louis, MO, USA) and stored for 28 days in an incubator at 37 °C.

**Push-out test**

The push-out bond strength of the various test materials was measured using a cylindrical punch-and-die set-up manufactured from an austenitic stainless steel rod. The punch has a 1.30 ± 0.05 mm diameter, and the die has a through hole of 2.00 ± 0.01 mm diameter (Fig. 1b). An external sleeve was used to keep the hold and die aligned (Fig. 1c,d). The filled tooth slice under test was placed on the die, and the centre of the test material filled cavity was visually aligned with the centre of the punch. The punch was pushed against the test specimen at a speed of 1.0 mm min⁻¹ using a Testometric M350-10CT universal materials testing machine (Testometric Co. Ltd., Rochdale, UK), extruding the filling test material into the die. The maximum push-out force during the test was recorded. The shear strength of the bond (in MPa) was calculated according to the following formula: F/(π × d × t), where F is the peak force in Newtons, d is the hole diameter in mm (nominally 1.5 mm), and t is the thickness of the tooth slice (nominally 3 mm, but measured accurately with a micrometre prior to loading).

The slices were examined under a stereomicroscope (Nikon, Tokyo, Japan) at variable magnification to determine the mode of the bond failure. Each sample was classified into one of four possible failure modes (Fig. 2). These were as follows:

1. **adhesive failure** occurring at the material-to-dentine interface, characterized by a clean intact extruded cylinder and no material deposits on the walls of the tooth after push-out testing;
2. **cohesive failure** occurring within the test material, characterized by heavy deposits of test material on the dentinal walls and a significantly eroded extruded cylinder following push out;
3. **cohesive failure** within the dentine, characterized by the extruded cylinder emerging with a pieces of dentine still attached, and a correspondingly enlarged and rough hole in the tooth following push-out testing; and
4. **mixed-failure** mode which is a combination of adhesive failure and cohesive failure within the test material, characterized by holes and extruded cylinders having a combination of regions with the characteristics described in (1) and (2).

**Statistical analysis**

The data were evaluated using SPSS (Statistical Package for the Social Sciences) software (PASW Statistics 18; SPSS Inc., Chicago Illinois, USA). Parametric tests were performed as K-S tests on the results indicated that the data were normally distributed. The data were analysed using one-way ANOVA and Tukey’s post hoc tests with a 0.05 level of significance.

**Results**

The mean push-out bond strengths are shown in Table 1. MTA-AW gave significantly lower mean push-out strength than MTA-W (P = 0.022) and MTA-Light had significantly greater push-out strength than the other formulations tested (P < 0.05 in each case). All formulations except MTA-Chem exhibited...
predominantly mixed-failure mode, whilst MTA-Chem exhibited predominantly adhesive failure (Fig. 3). The percentage occurrence of each failure mode is shown in Table 1. None of the specimens exhibited purely cohesive failure of either test material or dentine; moreover, the partial cohesive failures exhibited in the mixed-failure specimens were confined to cohesive failure of the test material and not of the dentine.

**Discussion**

Different formulations based on MTA were assessed in this study. The control MTA was mixed with water as suggested by the manufacturer. This MTA has poor antiwashout characteristics (Formosa et al. 2013a,b). The antiwashout gel is a water-based solution containing minor quantities of chlorides and a water soluble polymer. These additives modify the behaviour of the freshly mixed material and also of the set MTA (Formosa et al. 2013b). The addition of antiwashout gel improved the washout resistance of the material (Formosa et al. 2013b). Such an improvement would enhance the properties of MTA used as root-end filling material. Two resin types were used to develop a resin-based MTA with the aim of reducing the setting time. These novel materials were aimed at

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation</th>
<th>Mean (MPa)</th>
<th>Median (MPa)</th>
<th>SD (MPa)</th>
<th>Adhesive (%)</th>
<th>Mixed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MTA-W</td>
<td>5.08</td>
<td>5.08</td>
<td>2.41</td>
<td>30.0</td>
<td>70.0</td>
</tr>
<tr>
<td>2</td>
<td>MTA-AW</td>
<td>4.34</td>
<td>4.02</td>
<td>1.16</td>
<td>30.0</td>
<td>70.0</td>
</tr>
<tr>
<td>3</td>
<td>MTA-Chem</td>
<td>4.68</td>
<td>4.84</td>
<td>1.26</td>
<td>55.6</td>
<td>44.4</td>
</tr>
<tr>
<td>4</td>
<td>MTA-Light</td>
<td>10.99</td>
<td>11.35</td>
<td>0.81</td>
<td>44.4</td>
<td>55.6</td>
</tr>
</tbody>
</table>

*No specimens failed by purely adhesive mode or by pure cohesive failure of dentine.

*This involved partial adhesive failure and partial cohesive failure within the test material.

Figure 3 Stereomicrographs of typical samples from the four materials showing the cavity topography and extruded test materials following push out testing. The failure modes for the test materials investigated are indicated (all extruded cement fillings shown exhibit mixed-mode failure).
utilization of MTA for pulp capping, and as a dressing over pulpotomies where the setting time of the material is an important property as a long setting time would jeopardize the success of treatment. The light-curing variant (MTA-Light) was based on bisphenol-A-glycidylmethacrylate (Bis-GMA) and triethylene glycol dimethacrylate (TEGDMA) with MTA filler, whilst the MTA-Chem was composed of polymethyl methacrylate polymer, methyl-methacrylate monomer and partially pre-oxidized tributylborane in acetone as the catalyst and MTA filler (Formosa et al. 2013c).

Push-out tests have been shown to be effective and reliable as a means of assessing bond strength to dentine (Goracci et al. 2004). Micropushout testing was found to be superior to microtensile testing in the case of resin composites as microtensile testing involves the risk of premature failure (Cekic-Nagas et al. 2008). This study was designed to assess the resistance to dislodgement of MTA Plus, and three novel formulations based on it.

Mixing technique was shown to have an insignificant effect on push-out bond strength of white MTA (Shahi et al. 2012), and hence, conventional mixing was employed for all samples. However, a number of other factors have been shown to affect push-out strength. The presence of humidity (under conditions which result in an effective humidity level of 100%) has been shown to increase the push-out strength of MTA in root fillings (Gancedo-Caravia & Garcia-Barbero 2006). Irrigation of the root canal with NaOCl provided significantly higher push-out strength with MTA than samples treated with chlorhexidine gluconate (Hong et al. 2010). The presence of chlorhexidine gluconate reduced the MTA strength (Guneser et al. 2013). Conversely, for resin-based materials, post-etching treatment with NaOCl has been shown to decrease bond strength and marginal adaption (Frankenberger et al. 2000a). To this effect, no NaOCl treatment was carried out on the teeth used in this study. For light-curing composite resins, push-out strength was found to increase whether pulse-delay or soft-start illumination methods were used (Cunha et al. 2008). However, a standard high-intensity illumination profile was adopted in this study to quantify the minimum (worst-case) bond strength that could be expected of the novel materials. Etching and application of bonding agent was only utilized for the resin-modified prototypes because resins bond by micromechanical retention. The dentine surface area available for bonding increases 156% after etching in the middle third of the root (Ferrari et al. 2000). The increased surface area of the etched surface could have affected the bonding of MTA-W and MTA-AW. This factor is one of the limitations of the study. The mixing solution was not resin-based; thus, it was considered unnecessary to include etch and bond procedure for MTA-W and MTA-AW more so because these materials are indicated for use as root-end fillers. Furthermore, etching the root-end is a difficult procedure when performed during surgery. The bonding mechanism of MTA-based materials is different to that of resin-based materials. Whilst the etching enhances bonding my micromechanical retention, MTA-based materials bond by deposition of hydroxyapatite, which is deposited within collagen fibrils, triggering the formation of an interfacial layer with tag-like structures at the interface with the dentine (Reyes-Carmona et al. 2009).

Sections from the mid-root of single-rooted teeth were used. This was carried out to standardize the diameter of the cavity prepared in dentine as sections taken further apically would result in the need to overcut with the drill resulting in a specimen with a very small diameter, whilst preparing specimens taken cervically could result in a wider cavity. The orientation of the dentinal tubules and the effect of the bonding were not considered an issue. Although it has been demonstrated that the thickness of the hybrid layer depended on the density of tubules with a thinner hybrid layer formed where tubule density was low, research has shown that tubule density varies in different locations within the same tooth (Ferrari et al. 2000). Thus, location of the section within the tooth was not considered an important parameter affecting bonding thus affecting the results of the study.

Increased plunger diameter was shown to give higher apparent strengths (Nagas et al. 2011), although it did not affect the comparative rankings of the materials tested in that study. As no recognized standard for push-out testing exists, several plunger sizes have historically been used for push-out testing, from 0.7 mm (Huffman et al. 2009) up to 1.5 mm (Hong et al. 2010). In this study, a 1.5-mm drill bit was used to produce a cavity with parallel sides (as opposed to being tapered). This ensures that the force placed on the material/dentine interface is purely shear force and is also a requisite for the equation used for calculating bond strength to be valid as it is based on the assumption of constant diameter along the entire height of the cavity. The use of a pillar drill ensured that the axis of the drilled hole was exactly perpendicular to the bottom surface of the tooth.
Other variations in methodology exist, for instance with regard to the aligning method (visual or aided by casting the tooth specimen in a cylindrical resin mould), hole profile (parallel-sided or tapered), thickness of root slices and the ratio between plunger diameter and the diameter of the hole drilled in the tooth. In this study, a method similar to that reported by other researchers (Reyes-Carmona et al. 2010, Saghiri et al. 2010) was adopted (with the exception of using a 1.5-mm parallel-sided drill bit and 3-mm section thickness). Regardless of the differences in methodology, the mean values found for MTA Plus in this study are consistent with the range of values reported in previous studies (Frankenberger et al. 2000a, Gancedo-Caravia & Garcia-Barbero 2006, Reyes-Carmona et al. 2010, Saghiri et al. 2010, Shokouhinejad et al. 2010) for ProRoot MTA and MTA Angelus. A novel nano-modified MTA claimed to have ‘similar composition to white (Angelus) MTA, but with very low particle size and high specific surface area of powder which may produce a faster and better hydration process’ (Saghiri et al. 2013) has been reported to exhibit a push-out strength of 138.48 MPa, which is an order of magnitude higher.

The use of antiwashout gel instead of water resulted in a decrease in push-out strength from 5.08 MPa to 4.34 MPa, which might be attributed to the increased viscosity the gel imparts to the cement paste, which may affect marginal adaptation. Although this decrease is statistically significant, it is not drastic enough to preclude the use of washout-resistant MTA in cases where traditional water-based MTA is indicated. MTA-Chem exhibited a push-out strength that was not statistically different to that of MTA-W, even though the teeth were etched and a bonding agent was applied only for the former. Care was taken to avoid application mistakes when preparing samples. In particular, one study (Frankenberger et al. 2000b) reports that prolonged etching and excessive drying after conditioning were found to significantly decrease the bond strength of resin composites to dentine. In this study, a non-self-etching resin (Superbond C&B) was used, and the etching was performed separately in a previous step. This was carried out to avoid the potential risk of self-adhesive sealers failing to self-etch and thus giving poor performance in the case of incomplete smear-layer removal (Babb et al. 2009).

As explained, the bonding mechanism of the water-based materials (MTA-W and MTA-AW) is different to that of the resin-based materials (MTA-Chem and MTA-Light). The high polymerization shrinkage of the resin causes it to pull away from the cavity walls, bringing about loss of adhesion. This explains the low strength observed and is supported by the fact that the failure mode of this material was predominantly adhesive. Qualitatively, similar results were reported in one study (Assmann et al. 2012) comparing the bond strength of an MTA/water-based sealer (Endo-CMP sealer) with an MTA/resin-based sealer (MTA Fillapex). The MTA/water-based sealer exhibited significantly higher strength than both the MTA/resin-based sealer and a purely epoxy resin-based sealer (AH Plus sealer).

MTA-Light on the other hand had more than twice the push-out strength of the other materials tested in this study. The resin used in this formulation (Helio-bond) contains a high content of Bis-GMA – a high molecular weight monomer that helps minimize polymerization shrinkage (Garg & Garg 2010). This lower polymerization shrinkage compared with the resin used in MTA-Chem is the likely reason for the results observed. The high strength of MTA-Light, coupled with the fact that it has the shortest setting time (as reported in previous studies), makes MTA-Light the most promising of the novel materials tested.

More studies of the materials’ chemical properties, particularly tests of their ability to promote remineralization, need to be undertaken. In addition, as the bond strengths of resin-based composites have been shown to decrease significantly over time periods of several years (Frankenberger et al. 2004), long-term studies of the novel materials are recommended.

**Conclusion**

Through the results of this study, it can be concluded that from the aspect of push-out bond strength, each of the novel materials appears to be adequate replacements for traditional water-based MTA, whilst previous research has shown them to possess additional advantages with regard to setting time and washout resistance. MTA mixed with light-curing resin proved to have the strongest bond to dentine. Further studies should be conducted to quantify other physical and chemical properties of these promising new materials.

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Effects of Calcium Silicate–based Materials on the Flexural Properties of Dentin

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Abstract

Introduction: Prolonged exposure of root dentin to calcium hydroxide alters the fracture resistance of dentin. Calcium silicate–based materials (CSMs) used in endodontics release calcium hydroxide on setting. This study examined whether prolonged contact of dentin with CSMs adversely affects its mechanical properties. Methods: Dentin beams prepared from extracted human molars (7 × 3 × 0.3 mm) were divided into 3 groups on the basis of the material to which dentin was exposed (Biodentine, MTA Plus, and untreated control beams). Three-point flexure to failure was performed for each beam at designated exposure times (24 hours, 1, 2, and 3 months; n = 10). Data were analyzed with 2-factor repeated-measures analyses of variance to determine the effects of material and aging time on flexural modulus, flexural strength, and modulus of toughness (α = 0.05). Results: For flexural modulus, there was no significant difference for material (P = .947) or aging time (P = .064) when compared with baseline control. For flexural strength, significant differences were associated with aging time (P < .001) but not with material (P = .349). Flexural strength of dentin exposed to Biodentine decreased significantly after 2 and 3 months, whereas that exposed to MTA Plus decreased significantly after 3 months of aging (P < .05). For modulus of toughness, significant declines were observed for both material (P < .004) and aging time (P < .001). Conclusions: Both CSMs alter material toughness more than the strength and stiffness of dentin after aging in 100% relative humidity. Because dentin toughness is attributed to its collagen matrix, the amount of collagen extracted from mineralized dentin and changes in collagen ultrastructure should be further examined after exposure of dentin to CSMs. (J Endod 2012;38:680–683)

Key Words

Calcium silicate, dentin, flexural modulus, flexural strength, modulus of toughness

Calcium hydroxide (Ca(OH)₂) has been used for various endodontic procedures including interappointment antibacterial dressing, pulp capping, pulpotomy, and apexification (1–5). Previous studies have shown that Ca(OH)₂, on prolonged contact with dentin, adversely affects strength and fracture resistance (4–7). This is clinically relevant because endodontically treated teeth are generally thought to be weaker (8, 9). Moreover, teeth in need of apexification often have thin roots that are already prone to fracture (10).

Calcium silicate–based materials (CSMs) such as mineral trioxide aggregate (MTA) have largely replaced Ca(OH)₂ as endodontic repair materials because of their superior seal, biocompatibility, and regenerative capabilities (11–13). Their antibacterial properties are attributed to its release of Ca(OH)₂ on surface hydrolysis of the calcium silicate components (11, 14). When applied as a dentin substitute or during pulp capping, pulpotomy, and apexification, CSMs are left in place for the life of the tooth. There are also indications for obturating the entire canal with CSMs (15–18). An early study reported that prolonged contact of root dentin with Ca(OH)₂ or MTA resulted in similarly severe reductions (32% versus 33%) in dentin fracture resistance (6). The results of more recent studies are generally of the consensus that immature roots are less susceptible to fracture when Ca(OH)₂ is replaced by CSMs after prolonged contact with root dentin (19–22).

As new CSMs become commercially available, it is important to identify how the mechanical properties of dentin are affected by these materials. Thus, the purpose of the present study was to examine whether prolonged contact of dentin with 2 recently introduced CSMs, Biodentine (Septodont, Saint-Maur-des-Fossés, France) and MTA Plus (Prevest-Denpro, Jammu City, India), adversely affects flexural properties. Specifically, flexural modulus, flexural strength, and modulus of toughness (MOT) of dentin were tested by using a 3-point flexure design. Biodentine is recommended for use as both an endodontic repair material and a dentin substitute under resin composite restorations. It contains tricalcium silicate, dicalcium silicate, calcium carbonate and oxide, iron oxide, and zirconium oxide as its powder components and calcium chloride and a water-soluble polymer as its liquid components (23). MTA Plus has a finer particle size than other commercially available MTA versions (50% of the particles finer than 1 μm) and uses a salt-free water-soluble polymer gel as the mixing vehicle to improve its washout resistance (24). The null hypothesis tested was that there are no changes in...
flexural properties of dentin over time when the 2 CSMs are placed in direct contact with human dentin.

**Materials and Methods**

**Dentin Slabs and CSMs**

One hundred sixty extracted caries-free, nonrestored third molars were obtained after receiving patients’ consent under a protocol approved by the Georgia Health Sciences University Human Assurance Committee (age range of patients, 18–33 years). These teeth were stored at 4°C in 0.9% NaCl containing 0.02% NaN₃ to prevent bacterial growth and used within 3 months after extraction. A 0.3-mm-thick tooth slice was obtained from the mid-coronal portion of each tooth by using a slow-speed saw (Isomet; Buehler Ltd, Lake Bluff, IL) under water cooling (Fig. 1A). A dentin beam (3 × 7 × 0.3 mm) was prepared from each tooth slice (Fig. 1B); the use of 160 teeth resulted in 160 dentin beams. Dentinal tubules in each prepared beam were oriented perpendicular to the 3 × 7 mm surface, the surface that was subsequently used for contacting the CSMs. Eighty beams were randomly assigned to 8 experimental groups (n = 10) to be placed in contact with the set CSMs for a designated time period. The other beams were used as controls for each of the 8 experimental groups (n = 10).

Biodentine and MTA Plus were mixed according to manufacturer’s instructions and placed in 3 × 7 × 2 mm silicone molds inside a 100% relative humidity chamber until set. For Biodentine, liquid from the single-dose container was emptied into the powder-containing capsule and triturated by using a capsule mixer for 30 seconds, with a final setting time of 10 minutes. MTA Plus was hand-mixed by using a 3:1 powder-liquid ratio to achieve a putty-like consistency, with a final setting time of 1.2 hours. Two dentin beams were placed in contact with a set CSM block, with only 1 side of each beam exposed to the CSM (Fig. 1C), to simulate contact of the material with crown/root dentin in a clinical scenario.

The dentin-CSM assemblies were aged at 37°C in 100% relative humidity chambers for 24 hours, 1 month, 2 months, or 3 months (ie, 2 materials × 4 aging times = 8 experimental groups). The control for each experimental group consisted of dentin beams that were aged similarly in the absence of CSM (ie, 2 materials × 4 aging times = 8 control groups). At each designated aging time, the beams were copiously rinsed with deionized water and immediately tested.

**Three-point Flexure**

Flexural testing was performed by using a miniature 3-point flexure device with a 5-mm support span (25). The side of the dentin beam that was in contact with the CSMs was subjected to tension, whereas the noncontacting side was subjected to compression during flexural testing (Fig. 1D). Each 7-mm-long beam was placed on top of the support span and loaded to fracture under water by using a universal testing machine (Vitrodyn V100, Burlington, VT) at a crosshead speed of 1 mm/min.

Flexural strength (megapascals [MPa]) was calculated by using the formula 3P/2bd². Flexural modulus (Gigapascal [GPa]) was calculated by using the formula LI²m/4bd³, where P = load at fracture, L = length of support span, m = slope of the initial straight-line portion of the load-deflection curve, b = beam width, and d = beam thickness. MOT (MPa) was calculated by converting the load-deflection data to stress-strain data and integrating the area under the stress-strain curve from the origin to the strain-to-fracture (26).

**Statistical Analyses**

For each variable (flexural modulus, flexural strength, and MOT), 1-way analysis of variance was first used to compare whether differences exist for the data obtained from the 8 control groups at the 4 designated aging times, after testing for the normality (Shapiro-Wilk test) and equal-variance assumptions (modified Levene test) of the data. Because there was no statistically significant reduction in flexural properties of the control specimens after aging in 100% relative humidity for up to 3 months (data not shown), the 24-hour control data obtained from the 2 CSMs were used as baseline data for comparison with the data derived from the 8 experimental groups. Data for each testing parameter were analyzed separately with 2-factor repeated-measures analysis of variance to determine the effects of material and aging time and the interaction of those 2 factors on flexural modulus, flexural strength, and MOT. Because the equal-variance assumption of the data set for MOT was violated, logarithmic transformation of the data was performed before analysis. Statistical significance for all analyses was preset at α = 0.05.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A schematic depicting (A) preparation of a tooth slice; (B) preparation of a dentin beam; (C) dentin beams in contact with set CSMs during aging in 100% relative humidity chamber; (D) flexing a beam to failure by using a uniaxial 3-point flexure design.
Results

For flexural modulus, there was no significant difference for material \((P = 0.640)\) or aging time \((P = 0.064)\) when compared with baseline control (Fig. 2A). The interaction of those 2 factors was also nonsignificant \((P = 0.947)\). For flexural strength, significant differences were associated with aging time \((P < 0.001)\) but not with material \((P = 0.349)\) (Fig. 2B). The interaction of those 2 factors was nonsignificant \((P = 0.585)\). Dentin flexural strength for dentin exposed to Biodentine decreased significantly after 2 and 3 months, whereas that exposed to MTA Plus decreased significantly after 3 months of aging \((P < 0.05)\).

For MOT, significant differences were observed for both material \((P < 0.004)\) and aging time \((P < 0.001)\) (Fig. 3). The interaction of those 2 factors was significant \((P = 0.02)\). For the factor “material,” MOT of Biodentine was significantly different from that of MTA Plus after 1 month and 2 months \((P < 0.05)\), but not after 3 months. For the factor “aging time,” MOT of dentin was significantly reduced after it was in contact with Biodentine after 1 month \((P < 0.05)\). By contrast, there was no significant reduction in MOT of dentin after it was in contact with MTA Plus for up to 3 months \((P > 0.05)\).

Discussion

Because there were significant reductions in the flexural strength and MOT of dentin after specimen beams were aged in direct contact with CSMs, the null hypothesis that there are no changes in flexural properties over time when the 2 CSMs are placed in direct contact with human dentin has to be rejected. The rationale for subjecting the CSM-contacting side of the dentin specimens to tensile stresses is that brittle materials are much weaker in tension than in compression (27). The advantage of a uniaxial flexure design is that a state of pure tension might be achieved on the lower side of the specimen, which is usually responsible for crack initiation in brittle materials (28). The values for strength and MOT reported in the present study are large with respect to values reported in the literature on flexure. This difference is due to (1) the 3-point load configuration and (2) the fact that the beam cross section (rectangular with large width-to-thickness ratio, versus square cross section) resulted in plane-strain stress distribution instead of pure uniaxial stress. Thus, the results generated from the present study are not directly comparable with those obtained from 3- or 4-point bending of specimens with square cross sections or those generated by using a biaxial flexural design.

Grigoratos et al (4) previously reported that contact of dentin with saturated Ca(OH)$_2$ reduced the flexural strength but not the modulus of elasticity of dentin. Similar trends were observed in the present study when dentin was placed in contact with the CSMs. Although we had evaluated the effect of Ca(OH)$_2$ on dentin flexural properties, those results were not directly comparable with the data in this work and were not reported. This is because when the same experimental design was used, the fluidity of a Ca(OH)$_2$ paste (Calesept; Nordiska Dental AB, Angelholm, Sweden) caused sink-in of the dentin beams, producing double-sided instead of single-sided contact.
Human dentin contains 70% mineral, 20% organic materials, and 10% water. Carbonated apatite is the inorganic component of dentin, whereas the organic phase is predominantly type I collagen fibrils. This composition makes dentin more compliant than enamel, with a typical modulus of elasticity of 11–20 GPa versus 86 GPa for enamel (29, 30). The inorganic phase provides strength, whereas the organic phase is responsible for the toughness of dentin (31, 32). Overall, MOT of dentin was altered more (37.3% reduction for Biodentine, 22.3% reduction for MTA Plus) than its flexural strength (17.6% reduction for Biodentine, 14.0% reduction for MTA Plus). MOT should not be confused with fracture toughness. Fracture toughness is a property that describes the ability of a material containing a crack to resist fracture and is usually determined by subjecting compact tension specimens with a crack to a quasi-static tensile load (33, 34). Conversely, MOT is defined as the amount of energy per volume that a material can absorb before rupturing. The larger the area under a stress-strain curve, the tougher the material. A high MOT is important when a material is subject to stresses that exceed the elastic limit or during impact loads (26).

On the basis of the results, it might be concluded that the CSMs investigated in the present study reduce dentin’s ability to resist deformation (strength) and to absorb energy without fracturing (toughness). This is clinically significant because CSMs such as MTA were found to reduce the risk of root fractures by avoiding long-term Ca(OH)₂ treatment. Placement of these CSMs will probably not adversely affect the fracture resistance of roots with a short apical CSM plug or a thin layer of CSM as pulp-capping material. However, the practice of completely obturating root canals with these new CSMs or their use as dentin substitutes should be carefully considered. Hatibović-Kofman et al (21) attributed the improved fracture resistance of extracted immature sheep teeth treated with MTA over Ca(OH)₂ to the ability of MTA to induce expression of tissue inhibitor of metalloproteinase-2 (TIMP-2). The latter prevents destruction of the collagen matrix by matrix metalloproteinases MMP-2 and MMP-14. However, such an explanation is questionable because it is difficult to justify how TIMP-2 expression could be altered in extracted sheep teeth in the absence of live cellular components. It is known that MTA-like materials can dissolve bioactive matrix components from mineralized dentin because of its high pH after setting (35). This could also have resulted in dissolution of collagen from the surface of the mineralized dentin. Because dentin toughness is attributed to its collagen matrix, the amount of collagen extracted from mineralized dentin and changes in collagen ultrastructure should be further examined after exposure of these materials to dentin.

Acknowledgments

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References

Effects of Calcium Silicate–based Materials on Collagen Matrix Integrity of Mineralized Dentin

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Abstract

Introduction: Calcium silicate–based materials (CSMs) are used in various endodontic procedures. The present study examined whether prolonged contact of mineralized dentin with recently commercialized versions of these materials adversely affects dentin collagen matrix integrity. Methods: Dentin slabs prepared from extracted human third molars (7 × 3 × 0.3 mm) were divided into 3 groups on the basis of the material to which dentin was exposed (MTA Plus, Biodentine, untreated control dentin slabs) and the time period of exposure (24 hours, 1, 2, and 3 months; n = 6). Hydroxyproline assay was performed on each group’s supernatant to quantify the collagen extraction amounts of each group per time period. Data were analyzed with two-factor repeated-measures analysis of variance and Holm-Sidak pair-wise comparisons (α = 0.05) to determine the effects of material and aging time on collagen extraction. Dentin slabs from the 3 months of aging group were demineralized for transmission electron microscopy examination of collagen matrix ultrastructural changes. Results: Material (P = 0.002), aging time (P < .001), and their interactions (P = 0.007) significantly affected the amount of hydroxyproline (pg/mg of mineralized dentin) extracted from mineralized dentin and were significantly correlated by power regression models. Collagen degradation was identified from the surface of dentin slabs that were in direct contact with CSMs. Conclusions: Prolonged contact of mineralized dentin with CSMs has an adverse effect on the integrity of the dentin collagen matrix. However, the amount of collagen extracted was limited to the contact surface. Clinicians can continue to apply CSMs in endodontic procedures; however, caution is advised when these materials are applied to thin dentinal walls. (J Endod 2012;38:829–833)

Key Words

Calcium silicate, collagen, hydroxyproline, transmission electron microscopy

Use of calcium silicate–based materials (CSMs) in dentistry became popularized (1–5) with the advent of mineral trioxide aggregate (MTA) in 1993 as a root-end filling material (6). Other developed clinical applications of MTA include root perforation repair (2–4, 7), pulp capping (2–4, 8–11), pulpotomy (3, 4), dens in dente cases (12), internal resorption (12), apexification or as an apical barrier for teeth with necrotic pulps and open apices (2–4, 9, 12–14).

These numerous clinical applications of MTA are due to its significant inherent advantages, which include biocompatibility (2, 4), sealing ability (4, 6), regenerative capabilities (15, 16), and antibacterial characteristics (3). The antibacterial properties of CSMs are due to the release of calcium hydroxide (Ca(OH)2) on surface hydrolysis of the calcium silicate components (3, 17). However, on prolonged contact with dentin, Ca(OH)2 has been shown to adversely affect strength and fracture resistance (18–21). White et al (18) also found that bovine root dentin was weakened after 5 weeks of exposure to MTA. Further research is needed to determine the biochemical effects of CSMs on the collagen ultrastructure because dentin toughness is attributed to its collagen matrix (22, 23).

Other noted shortcomings of MTA such as difficult handling characteristics (24), long setting time (25, 26), high cost (2), and potential of discoloration (2) led to the development of new CSMs such as Biodentine (Septodont, Saint Maur des Fossés, France) and MTA Plus (Prevest-Denpro, Jammu City, India). Biodentine is a bioactive (27) dentin substitute composed of powder components of tricalcium silicate, calcium carbonate, and zirconium oxide and a water-based liquid containing calcium chloride as the setting accelerator and water-reducing agent (28). MTA Plus has a finer particle size than other commercially available MTA versions (50% of the particles finer than 1 μm) and comes with a salt-free water-soluble polymer gel as the mixing vehicle to improve its washout resistance (29).

With the development of these new commercially available products and the potential for decreasing dentin toughness, this study was designed to analyze the effects of Biodentine and MTA Plus on the collagen matrix integrity of mineralized dentin. The null hypothesis tested was that there are no changes in the amount of hydroxyproline extracted from mineralized dentin over time when the 2 CSMs are placed in direct contact with human dentin.
Materials and Methods

Mineralized Dentin Slabs and CSMs

One hundred forty-four extracted, caries-free, nonrestored third molars were obtained after receiving patients’ consent under a protocol approved by the Georgia Health Sciences University Human Assurance Committee. A 0.3-mm-thick tooth slice was obtained from the midcoronal portion of each tooth by using a slow-speed saw (Isomet; Buehler Ltd, Lake Bluff, IL) under water cooling. A dentin slab (3 × 7 × 0.3 mm) was prepared from each tooth slice. Ninety-six slabs were randomly assigned to 8 experimental groups to be placed in contact with either of the 2 CSMs for 4 designated aging times. The other 48 slabs were used as controls for each aging time.

MTA Plus and Biodentine were mixed according to manufacturers’ instructions and placed in 3 × 7 × 2 mm silicone molds inside a 100% relative humidity chamber until set. For Biodentine, liquid from the single-dose container was emptied into the powder-containing capsule and triturated by using a capsule-mixer for 30 seconds, with a final setting time of 10 minutes. MTA Plus was hand-mixed by using a 3:1 powder-liquid ratio to achieve a putty-like consistency, with a final setting time of 1.2 hours.

Aging of Dentin Slabs in Contact with Set CSMs

Each mineralized dentin slab was first placed in anhydrous CaSO₄ (W. A. Hammond Drierite Co Ltd, Xenia, OH) and weighed to constant mass (weighed half-hourly for 8 hours) by using a XP2U ultra-microbalance (Mettler Toledo, Hightstown, NJ; repeatability at nominal load 0.000025 mg). Each slab was then rehydrated in a solution of 0.9% NaCl and 0.02% NaN₃. Two rehydrated dentin slabs were placed in contact with a set CSM block with only one side of each slab exposed to the CSM to simulate contact of the material with crown/root dentin in a clinical scenario. Each dentin-CSM assembly was aged in a polypyrrole microcentrifuge tube containing 400 μL of storage medium at 37°C for 24 hours, 1 month, 2 months, or 3 months. Twelve dentin slabs were placed into 6 vials (n = 6) for each material per aging time (2 materials × 4 aging times = 8 experimental groups). The storage medium consisted of 50 mmol/L HEPES buffer, 5 mmol/L CaCl₂ · 2H₂O, 0.001 mmol/L ZnCl₂, 150 mmol/L NaCl, and 3 mmol/L NaN₃ to prevent bacterial growth (pH 7.2) (30). There were 4 control groups, 1 for each designated aging time (n = 6). No CSM was used in the control groups; dentin slabs were placed in the storage medium and aged as previously described.

Hydroxyproline Assay

Assessment of solubilized collagen peptides from the mineralized dentin slabs was performed by using hydroxyproline assay (31) to determine the amount of hydroxyproline present in the storage medium (30, 32, 33). The rationale for using hydroxyproline as a relative index of the amount of solubilized collagen peptide fragments is that 90% of the dry mass of demineralized dentin collagen consists of type 1 collagen, and that type 1 collagen contains 9.6 mass% hydroxyproline, whereas most other proteins contain no hydroxyproline (34). At the end of each designated aging time, the pH values of the storage media were measured by using a pH meter. Then, 200 μL of vortexed storage medium was collected from each vial and placed in an individually labeled ampule and diluted with an equal volume of 12 N HCl to give a final concentration of 6 N HCl. The ampules were flame-sealed (Ampulmatic; Biosciences Inc, Allentown, PA), and the media were hydrolyzed at 120°C in an oil bath for 18 hours. After hydrolysis, the ampules were opened and placed in glass desiccators containing Drierite and NaOH pellets to trap the water and HCl vapor released from the hydrolysates as they were evaporated to dryness. The hydroxyproline content of each hydrolysate was analyzed spectrophotometrically at 558 nm by using a Shimadzu Model UV-A180 spectrophotometer (Tokyo, Japan) in the transmission mode. The amount of hydroxyproline released from solubilized collagen peptide fragments was determined from a regression equation derived from absorbance values obtained from known concentrations of HCl-hydrolyzed hydroxyproline standards. The amount of hydrolyzed hydroxyproline was expressed as pg/mg of dehydrated mineralized dentin.

Statistical Analyses

One-way analysis of variance was first used to examine whether differences existed among the data obtained from the 4 control groups (24 hours, 1 month, 2 months, 3 months) and those obtained from the 2 experimental groups at 24 hours. This analysis was performed after...
validating the normality (Shapiro-Wilk test) and homoscedasticity assumptions (modified Levene test) of the data sets.

Because there were no statistically significant differences in the amount of extracted hydroxyproline among the 4 control groups and the 2 experimental groups after 24 hours of aging, the 24-hour results of the 2 CSM groups were used for statistical evaluation of the 8 experimental groups. Data were analyzed with two-factor repeated-measures analysis of variance to examine the effects of material and aging time and the interaction of those 2 factors on hydroxyproline content (normality assumption satisfied, $P = .787$; homoscedasticity assumption satisfied, $P = .787$).
P = .058). Post hoc pair-wise multiple comparisons were performed by using the Holm-Sidak method. For each CSM, regression analysis was further performed to seek potential correlation between the amounts of hydroxyproline release and aging times. Statistical significance for all analyses was preset at α = 0.05.

Transmission Electron Microscopy

Two dentin slabs from each of the 3-months-of-aging groups (control, no CSM; MTA Plus; and Biodentine) were completely demineralized in 0.5 mol/L ethylenediaminetetraacetic acid. Demineralized slabs were fixed in Karnovsky’s fixative, post-fixed with 1% osmium tetroxide, dehydrated in an ascending ethanol series (30%–100%), transitioned through propylene oxide, and embedded in epoxy resin. Ninety-nanometer-thick sections of the dentin slabs were prepared, stained with 2% uranyl acetate and Reynolds’ lead citrate, and examined by using a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at 110 kV.

Results

The pH values of the storage media were 12.75 ± 0.09 and 12.49 ± 0.06 for MTA Plus and Biodentine, respectively, after 3 months of aging in the presence of the respective CSM (n = 6). Both material (P = 0.02) and aging time (P < .001) significantly affected the amount of hydroxyproline extracted from mineralized dentin (Fig. 1). The interaction of these 2 factors was also statistically significant (P = .007). For both CSMs, the amount of hydroxyproline extracted after 1 month of aging was significantly higher than that extracted after 24 hours (P < .05); the amount extracted after 3 months was also significantly higher than that extracted after 1 month (P < .05). For each CSM the increase in the amount of extracted hydroxyproline with aging time was significantly correlated by a power regression model (Fig. 2; P < .05).

Minimal collagen degradation was observed from the surface of demineralized dentin slabs in the control group after 3 months of aging (Fig. 3A and B). By contrast, a zone of collagen degradation could be identified from the surface of dentin slabs that was in direct contact with CSMs. For MTA Plus, partially degraded collagen fibrils could be identified from the 1- to 2-μm-thick degradation zone (Fig. 3C and D). For Biodentine, collagen fibrils were completely broken down into microfibrils within the 5-μm-thick degradation zone (Fig. 3E and F).

Discussion

The results of this study do not support the null hypothesis that there are no changes in the amount of hydroxyproline extracted from mineralized dentin over time when the 2 CSMs are placed in direct contact with human dentin. The alkaline pH of both CSMs’ storage media after 3 months is most likely due to the production of Ca(OH)₂ because this by-product is formed on reaction of CSMs with water and even with atmospheric humidity.

The composition of human dentin is 70 wt% mineral, 20 wt% organic, and 10 wt% water, with carbonated apatite as the inorganic component and type I collagen fibrils primarily as the organic constituent (23). The results shown in Figures 1 through 3 could be due to a disruption in the link between the carbonated apatite crystallites and the collagen fibrils in dentin because of the Ca(OH)₂ released in CSMs (20).

Usually, organic matters such as collagen molecules, fossilized growth factors, and endogenous matrix metalloproteinases within mineralized collagen fibrils in dentin are protected from degradation by extracellular and intrafibrillar apatite crystallites. Such a protective mechanism enables these organic matters to be preserved for thousands of years. For example, collagen could be detected by biochemical means from archaeological remains of human and animal bones (35). Both collagenases (molecular weight, 68–130 kDa) and matrix metalloproteinases associated with collagen degradation (molecular weight, 42–105 kDa) are bulky, high-molecular-weight molecules; their sizes preclude them from penetrating collagen molecules that are protected by intrafibrillar apatite crystallites. Apparently, both sodium hypochlorite (molecular weight, 74.4 Da) and Ca(OH)₂ (molecular weight, 56.1 Da) alter the elastic modulus of the mineralized collagen fibrils and alter the 3-dimensional conformation of tropocollagen. Conceivably, a diffusion gradient of these molecules must have existed, which prevents their further inward diffusion into the underlying heavily mineralized dentin. Thus, the integrity of the subsurface collagen fibrils was preserved.

Even though increased amounts of hydroxyproline-containing collagen fragments were collected in the assays over time with both CSMs, these amounts are very small in respect to the amount of dentin (picograms of hydroxyproline versus milligrams of dentin). In accordance with these miniscule amounts of hydroxyproline, only surface degradation of collagen was observed by transmission electron microscopy for dentin exposed to the CSMs.

In conclusion, CSMs are still valid materials for clinical use in dentistry. However, caution is advised when CSMs are used to obturate root canals with thin dentinal walls and in filling the full length of the canal to prevent collagen degradation that might lead to root fracture.

Acknowledgments

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