In vitro osteogenic/dentinogenic potential of an experimental calcium aluminosilicate cement.

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IN VITRO
OSTEOGENIC/DENTINOGENIC
POTENTIAL OF AN EXPERIMENTAL CALCIUM ALUMINOSILICATE CEMENT

Thesis

Submitted in Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy

By

Ashraf Abulfotooh Mostafa Eid

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Chapter 1: Background and Objectives

Background

Over the course of the last three decades, advances in the field of biomaterials science have led to a paradigm shift in basic concepts in dentistry with consequent interesting applications that span the entirety of all dental specialties. Not only have those new applications helped save thousands of teeth and improve the quality of life of thousands of people, but they also contributed in making routine dental procedures more predictable, reliable, as well as more time and cost effective (1).

Of the numerous examples of such advances, the introduction of tricalcium silicate cements (TSCs) to the dental field remains one of the most successful to date, both academically and commercially. TSCs are Portland cement based hydraulic cements that react with water to form calcium silicate hydrate and calcium hydroxide (2). Owing to their reported bioactivity (3), the ever expanding list of applications of TSCs in dentistry includes, but is not limited to, retrograde root canal filling (4), perforation repair (5), vital pulp therapy including pulp capping (6) and apexogenesis (7), apexification of immature necrotic teeth (8), in regenerative endodontic procedures as a coronal plug (9), and more recently, as a root canal sealer (10).

Despite the aforementioned advantages of TSCs over conventional restorative materials, there are some hurdles that may limit their use to their full potential. For instance, commercial TSCs exhibit slow setting reaction which complicates final restoration procedures and necessitates multiple-visit treatments (11). Furthermore, the physical presence of water -a prerequisite for optimal setting of TSCs- can jeopardize the placement of a final bonded restoration atop unset TSCs. And thus, it will be of value to study the various setting conditions of TSCs and their effects on
their chemomechanical properties, as well as their effects on the structural interface when TSCs come in contact with adhesive filling materials.

Another aspect of TSCs that remains to be explored is their osteoactivity. TSCs are generally known to be biocompatible (3), and that they may favor hard tissue formation both in vivo and in vitro (12, 13). However, the exact mechanism by which they exert their osteogenic effect is not clear. Additionally, whether TSCs will stimulate odontoblasts or pre-odontoblasts to lay down dentin or bone matrix remains unknown. It is of significant clinical importance to investigate those points further in an effort to better understand the role of TSCs in hard tissue formation, both in vivo and in vitro.

Unlike TSCs, which enjoyed significant commercial and academic attention, other biomaterials which have been recently introduced to the dental field have not been sufficiently studied. A notable example is CPoint™ (EndoTechnologies, LLC, Shrewsbury, MA). CPoint™ is a point and paste polymer based gutta-percha substitute that expands on water sorption. It is believed that such property would help eliminate or reduce micoleakage, a common finding in failed root canal cases (14). Little is known about the chemical, mechanical and biological properties of CPoint™ at this point. It is our belief that since root canal filling materials can inadvertently extrude through the peri-radicular tissues during routine endodontic procedures, testing the biocompatibility of CPoint™ takes precedence over investigation of its expansion, water sorption and solubility properties.
**Objectives**

And so, in an effort to address the abovementioned queries, and to better understand the behavioral characteristics of dental biomaterials, the objectives of the current study were formulated to:

1. Investigate the effects of different setting conditions of a commercial TSC and different placement times of resin-modified glass ionomer cement on the hardness and structural interface of the 2 materials.
2. Analyze the in vitro cytotoxicity profile and osteogenic differentiation potential of CPoint™ root canal filling points.
3. Investigate the osteogenic/dentinogenic differentiation potential of TSC and Calcium Aluminosilicate cement in vitro.


Chapter 2: Characterization of the Mineral Trioxide Aggregate-Resin Modified Glass Ionomer Cement Interface in Different Setting Conditions

Introduction

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 (1). It was first used to seal off all pathways of communication between the root canal system and the external surface of the tooth (2). It is now widely used as a root-end filling material (1, 3), in vital pulp therapy including direct pulp capping and pulpotomy of immature teeth (apexogenesis) (4, 5), and as an apical barrier in immature teeth with necrotic pulps (apexification) (6). Lately, it has been successfully used in regenerative endodontic procedures in immature teeth with apical periodontitis (7, 8).

The advantages of MTA include high biocompatibility, radiopacity that is slightly greater than that of dentin, low solubility, and high alkalinity (pH = 12.5) that may impart some antimicrobial properties (9). The main disadvantages of MTA are its difficult manipulation and long setting time (10, 11). After the MTA powder is mixed with sterile water to make a thick creamy mix, it takes an average of 3 to 4 hours for the material to form a solid barrier (12). However, complete setting of MTA may take up to 21 days (13). The hydrophilic nature of MTA makes it an ideal material for different endodontic applications in which contact with blood, body fluids, and moisture is inevitable, but it complicates the same-visit application of the final adhesive restoration, which requires a relatively dry field. Consequently, the
application of the restoration usually requires a separate appointment after MTA has reached its initial setting stage.

The current literature contains few studies on glass ionomer cement (GIC)-MTA interactions. GIC was found to bond to MTA (14), although bond strength values seem comparatively inferior to that of resin composites (15). Placing GIC after 45 minutes, 4 hours, or 3 days of MTA placement did not affect the setting of MTA (16) or GIC (17). Nevertheless, placing GIC over freshly mixed MTA caused excessive interfacial cracking and void formation compared with calcium hydroxide paste (18). To our knowledge, a comparison between immediate and delayed placement of GIC over MTA in different setting conditions and the effect of that on the MTA-GIC structural interface and hardness was never attempted.

Therefore, the aim of the present study was to investigate the effect of MTA setting conditions and GIC placement time on the hardness and structural interface of the 2 materials. Two null hypotheses were tested: (1) GIC placement time does not affect the MTA-GIC structural interface and hardness and (2) moisture does not affect the MTA-GIC structural interface and hardness.
Materials and Methods

MTA (ProRoot MTA; Dentsply, Tulsa, OK) was mixed with sterile water according to the manufacturer’s instructions. Fifty transparent plastic cylinders (4-mm diameter, 7-mm long) were half filled with the mixture, and the surface of MTA was smoothed using a plastic plunger. The bases of all cylinders were in contact with sterile gauze wetted with distilled water to simulate natural setting conditions (tissue side). The specimens were then randomly divided into 5 equal groups (n = 10), and the other half of the cylinders were filled with resin modified GIC (Fuji II LC; GC Corporation, Tokyo, Japan) and cured for 20 seconds using a curing light (L.E.Demetron 1; Kerr Corp, Danbury, CT) as follows: group IMM: GIC was applied and cured immediately after MTA placement; group 1Dw: GIC was applied and cured after MTA was allowed to set for 24 hours in a wet condition (covered with a wet cotton pellet) and temporary filling was added (IRM; Dentsply De-Trey, Konstanz, Germany); group 1Dd: same as group 1Dw but in a dry condition (with no cotton pellet); group 7Dw: GIC was applied and cured after MTA was allowed to set for 7 days in a wet condition; group 7Dd: same as group 7Dw but in a dry condition. In the wet condition groups, IRM and cotton pellets were removed using a sharp excavator without touching the MTA surface. It was neither rinsed nor polished afterward. A gentle stream of air was used to remove excess moisture from the MTA surface before GIC placement. In the dry condition groups, IRM was applied on the outermost part of the plastic cylinders with no direct contact with the MTA surface. IRM was later removed using a sharp excavator, and any debris was gently blown using an air syringe. All the specimens were stored in an incubator at 95% humidity and 37°C during the entire procedure.
**Specimen Preparation**

The MTA-GIC specimens were embedded in transparent epoxy resin (SpeciFix-20; Struers, Ballerup, Denmark). The hardened epoxy resin blocks were sectioned perpendicularly to the MTA-GIC interface using a low-speed saw and polished with silicon carbide papers (500-2,000 grit) followed by a 0.3-μm alumina suspension on a rotary polishing cloth. The polished sections were then covered with a thin layer of carbon using a carbon coater (JEE-400 Vacuum Evaporator; JEOL Ltd, Tokyo, Japan).

**Microstructural Analysis of the Interface**

An electron probe microanalyzer (EPMA-1600e; Shimadzu, Kyoto, Japan) with a built-in scanning electron microscope (SEM) was used to analyze the MTA-GIC interface. Using the SEM, the following points were investigated on every specimen by 2 blinded observers: the adaptation of the 2 materials at the interface, the intrinsic crack pattern and propagation, the separation of the 2 materials and location, the presence or absence of the intermediate layer, and the distinctive morphologic features in MTA and GIC. The EPMA mapping analysis mode with preset settings (15-kV current, 1-μm beam size, and 1-μA sample current) was used to detect the elemental distribution of bismuth, fluorine, silicon, and calcium along the MTA-GIC interface. The area designated for the mapping analysis was 512 × 512 μm.

**Hardness Testing**

Vickers hardness testing was performed on half of the specimens in each group 24 hours after GIC placement and on the rest of the specimens 8 days after MTA placement using a hardness testing machine (MVK-H1; Akashi Co, Tokyo, Japan) with a 50 gram-force load and a 5-second dwell time. Ten measurements were made for each sample on the MTA side 100 μm away from the interface. The data were statistically analyzed using analysis of variance to investigate if there were significant
differences among the groups. If analysis of variance showed significant differences, a post hoc pair-wise comparison was made using the Tukey test. The level of statistical significance was set at $\alpha = 0.05$. 
Results

Microstructural Analysis of the Interface

The SEM showed that all the groups underwent adhesive separation and gap formation at the interface. Cohesive separation in MTA was also found in all groups, but it was observed more often in the dry condition groups (1Dd and 7Dd) compared with the wet condition groups (1Dw and 7Dw). Isolated island-like structures at the interface consisting of both materials were obvious in group IMM (Fig. 1A), with numerous voids and cracks evident at the interface. All groups exhibited vertical and horizontal cracks in GIC that interconnected with each other in the internal voids within the GIC. The changes observed were limited to the outermost interfacial layer of the MTA, and neither the deeper layers of MTA nor the GIC itself seemed affected. As for the EPMA elemental analysis, calcium appeared to be evenly dispersed as densely packed fine particles predominantly on the MTA side. In the wet condition groups, the growth of calcium crystals was evident at the interface, which appeared to increase in size with time. This observation was not made in the dry condition groups (Fig. 1C and D). Silicon was observed on both sides; on the GIC side, it was evenly distributed in small particles, whereas on the MTA side it took the form of large, widely dispersed clusters. Bismuth appeared as widely spaced, relatively large particles or aggregations of particles on the MTA side only. Fluorine was present as densely packed small particles exclusively on the GIC side. Because calcium and silicon were found in both materials, their migration could not be mapped. The bismuth migration to the GIC side was not detected in any of the specimens tested, whereas the migration of fluorine to the MTA side was detected in 1 group 1Dw specimen (Fig. 1B).
Hardness Testing

The mean and standard deviations for MTA hardness are summarized in Table 1. Hardness testing after 24 hours of GIC placement showed significant differences in hardness (P < .001) with increased temporization time (IMM < 1Dw < 7Dw and 1Dd < 7Dd) but not with the moisture condition (1Dw vs. 1Dd and 7Dw vs. 7Dd). Hardness testing after 7 days of GIC placement showed no significant differences among all the groups (P = .92). Nevertheless, there seemed to be a tendency toward higher hardness values (P = .059) in the wet condition groups compared with the dry condition groups (1Dw vs. 1Dd and 7Dw vs. 7Dd).
Figure 2-1 Representative EPMA mapping analysis images with their matching scanning electron microscopic micrographs.

Table 2-1 The Mean Vickers Hardness Values of MTA at the Interface

<table>
<thead>
<tr>
<th></th>
<th>IMM</th>
<th>1Dm</th>
<th>1Dd</th>
<th>7Dm</th>
<th>7Dd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean VHN ± SD</td>
<td>18.7 ± 7.3</td>
<td>42 ± 10.7</td>
<td>47.3 ± 13.9</td>
<td>67.4 ± 15.9</td>
<td>60.1 ± 18.6</td>
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<td>24 hrs after</td>
<td></td>
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<tr>
<td>GIC application</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean VHN ± SD</td>
<td>61.8 ± 9.8</td>
<td>65.2 ± 15.5</td>
<td>59.7 ± 8.7</td>
<td>67.4 ± 15.9</td>
<td>60.1 ± 18.6</td>
</tr>
<tr>
<td>8 days after</td>
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<tr>
<td>MTA application</td>
<td></td>
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</table>
Discussion

One of the most notable drawbacks of MTA is its prolonged maturation process that often continues past the manufacturer’s stated setting time of 3 to 4 hours (19). Earlier studies reported that the resistance to dislodgment (20, 21), push-out bond strength (13, 22), microbial leakage, and hardness of MTA were all affected over time. Consequently, the placement time of the final adhesive restoration on MTA is of clinical interest. This study did not reject the first null hypothesis. The results showed that even though there were significant differences in MTA hardness values among the groups tested 24 hours after GIC placement, the differences were not significant 8 days after MTA placement. This difference indicates that changes in the MTA hardness were transient, and the time of GIC placement did not influence the setting reaction of MTA. These observations agree with earlier reports using laser Raman spectroscopy and stereomicroscopy (16, 17) and also indicate that the changes may be attributed to the slow setting reaction of MTA rather than the interaction with GIC. The interfacial adaptation of MTA seemed to improve with the increased temporization time, which may be because of the high affinity of GIC to uptake the hydration water necessary for MTA setting (18). This fact might explain the high incidence of interfacial porosity and cracking observed in the IMM group. Nevertheless, adhesive separation and gap formation at the interface were commonly found in all groups. One reason for this phenomenon is the setting contraction of resin-modified GIC, which is comparable to the contraction of resin composites (23). Another reason is the vacuum-related dehydration shrinkage of GIC required for SEM-EPMA procedures, a factor that is not related to the setting reaction of either material (24).

The second null hypothesis was also not rejected because there were no
significant differences in hardness between the wet and dry condition groups. However, wet condition groups tended to have higher hardness values. In the literature, there are mixed results regarding the effect of moisture on the properties of MTA, which may be caused by the lack of consistency in the definition of the dry condition among researchers (13, 20, 25). A possible explanation for the current results is that MTA can acquire the water necessary for maturation from the tissue moisture or even through the root without the need of a wet cotton pellet (26, 27). Another factor might be the relatively small sample size because the P value between the 2 moisture conditions was 0.059. The cohesive separation on the MTA side of the dry condition groups may be related to the incomplete setting of interfacial MTA in the dry condition groups. This was also observed earlier and was attributed to the water withdrawal from the MTA into the GIC (18). However, this incomplete setting did not seem to significantly affect the hardness of these groups. A notable observation was the formation of calcium salt crystals at the interface in the wet condition groups (Fig. 1C). This can be attributed to the normal maturation process of MTA in the presence of sufficient moisture as described in previous studies (28, 29), a fact that might explain its absence in the dry condition groups. It was also reported earlier that the presence of calcium salts at the MTA-GIC interface was a result of the interaction of the negatively charged carboxylate anion (RCOO⁻) in the polyacrylic acid with the calcium in the MTA (16). It is not clear at this point if the presence of these crystals at the interface would affect the clinical performance of MTA or GIC, but this was beyond the scope of this study.

In cases in which direct contact of MTA with the final restoration is inevitable, such as pulpotomy for both primary and immature permanent teeth and perforation repair, it is recommended by the manufacturer to perform a 2-visit procedure to place
the final restoration (30). In the first visit, a wet cotton pellet is applied over the MTA, and the tooth is temporized. In the second visit, the cotton pellet is removed, and the permanent restoration applied after the MTA has sufficiently hardened. It would be clinically beneficial for patients and dentists alike if the final adhesive restoration can be placed over MTA during the same visit. This way the cost and chairside time of the procedure will decrease significantly. Based on the current results, both null hypotheses were not rejected. The effect of GIC placement over MTA after different time intervals and setting conditions was transient. The GIC and deeper layers of MTA did not seem to be affected. Further research is required to assess the long-term clinical outcome of these interfacial reactions. In conclusion, resin-modified GIC can be successfully applied on freshly mixed MTA in a single visit with no expected adverse reactions between the 2 materials.
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Chapter 3: In Vitro Biocompatibility Evaluation of a Root Canal Filling Material That Expands on Water Sorption

Introduction

Prevention of recontamination after root canal treatment is traditionally achieved via 3-dimensional filling of the canal space (1–3). To date, there is little clinical evidence to support or reject any particular contemporary root canal filling technique (4, 5). Because no single approach can provide unequivocal, evidence based documentations of superior treatment outcomes (6–12), decisions regarding the choice of a root-filling technique may be based on other factors such as simplicity, speed, economics, or safety (5).

The CPoint system (EndoTechnologies, LLC, Shrewsbury, MA) is a point-and-paste root canal filling technique that consists of hydrophilic endodontic points and an accompanying sealer. CPoint is designed to radially expand as it absorbs water that is resident in the instrumented canal space and dentinal tubules (13). This lateral expansion is claimed to occur nonuniformly, with the expandability depending on the extent to which the hydrophilic polymer is pre-stressed (14). This non-isotropic lateral expansion is said to enhance the sealing ability of the root canal filling. The inner core of CPoint is a mix of 2 nylons, Trogamid T and Trogamid CX. The outer polymer coating is a cross-linked copolymer of acrylonitrile and vinylpyrrolidone, which is copolymerized and cross-linked by using allyl methacrylate and a thermal initiator. Radiopacity of both the core and polymer coating is provided with zirconium dioxide particles. Although the endodontic point is capable of achieving a relatively good fit of an irregular canal space, gaps may still remain between the walls of the canal and the expanded point. Consequently, an accompanying sealer has to be used to seal
those gaps.

Because root-filling materials may be inadvertently extruded through the apical foramen and lateral portal of exits, they should be biocompatible (15) and not adversely affect the ability of osteoblasts to regenerate hard tissues (16). Thus, evaluating the biocompatibility of CPoint takes precedence over investigations on its expansion properties, water sorption and solubility characteristics, or ability to create gap-free filling of the canal space. Accordingly, the objective of the present study was to compare the in vitro biocompatibility of CPoint with gutta-percha, after exposure of these materials to a rat odontoblast-like cell line. Two hypotheses were tested: 1) CPoint does not adversely affect the viability of odontoblast-like cells by inducing undue necrosis or apoptosis of those cells, and 2) after being rendered non-cytotoxic via elution of its cytotoxic components, CPoint does not adversely affect the in vitro osteogenic/mineralization potential of the odontoblast-like cells.
Materials and Methods

Size 30, 0.04 taper versions of CPoint and gutta-percha point (Brasseler USA, Savannah, GA) were evaluated in their original form. Teflon disks (4-mm diameter × 1-mm thick, similar surface area as the endodontic points) were used as the negative control (17, 18). Zinc oxide–eugenol cement disks (IRM; Dentsply International, Konstanz, Germany) of the same dimensions were used as the positive control. All set materials were sterilized with ultraviolet light before testing.

Cell Viability

MTT Assay

Rat dental papilla-derived odontoblast-like cells (MDPC-23) (18–22) were plated in 24-well format plate at 10^4 cells/cm^2 in complete growth medium and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 hours until they reached ≈ 70%–80% confluency. The growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Wakersville, MD) and 10% fetal bovine serum (Gibco, Invitrogen Corp., Carlsbad, CA), supplemented with 2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin. The cells were then subjected to a weekly cyclic regime (17) to evaluate their succinic dehydrogenase activity after exposure to the materials (direct evaluation) or their eluents (indirect evaluation) by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The weekly cycle consisted of a) direct evaluation of the toxicity of the materials over the plated cells for 3 days, and b) indirect evaluation of the effect of eluents derived from the root filling materials on the plated cells. The latter was achieved by immersion of the CPoints or gutta-percha points in growth medium for 4 days to collect eluents. Accordingly, during the first part of each weekly cycle, root filling material points
and control disks (N = 12) were placed individually in transwell inserts with 3 \( \mu \)m pore size (BD Falcon, Franklin Lakes, NJ) to prevent direct contact of cells by the specimens. After the inserts were placed over the plated cells, an additional 2 mL of 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 materials were exposed to the plated cells for 3 days, without further change in culture medium, prior to testing for succinic dehydrogenase activity. During the second part of each weekly cycle, the materials were retrieved and incubated at 37ºC with growth medium (one point/disk per 2 mL) for 4 days to collect the eluents (N = 12) derived from the experimental and control groups, prior to using the same materials for the next cycle. For each material, the same growth medium was used for eluent collection throughout the entire testing period. This cycling regime was repeated weekly until the material disks were rendered non-cytotoxic (i.e. > 90% of the mean succinic dehydrogenase activity exhibited by the Teflon negative control) (20). Each eluent concentrate collected after the 3-week aging period was diluted with fresh growth medium to 1:1, 1:10 and 1:10^2 of its original concentration to achieve a final volume of 2 mL. Each diluted, eluent-containing growth medium was then used as the respective culture medium for freshly plated MPDC-23 cells, for testing of cell viability.

Cell viability was evaluated by incubating 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with MDPC-23 cells that have been exposed to the materials and their eluents, respectively. The cells were incubated in MTT-succinate solution for 60 min and fixed with Tris-formalin. The purple MTT-formazan produced in the cells, as a result of mitochondrial succinic dehydrogenase activity, was dissolved \textit{in-situ} using DMSO-NaOH and the optical density was measured using a microplate reader at 562 nm. The optical density of blank DMSO-
NaOH was subtracted from all wells. The formazan content of each well was computed as a percentage of the mean of the Teflon controls, which was taken to represent 100% biocompatibility.

Statistical analysis of the logarithmically transformed data (to satisfy normality and homoscedasticity assumptions) was performed for both the root filling materials and their eluents by using separate two-factor analysis of variance (ANOVA) and Holm–Sidak multiple comparison procedures at $\alpha = 0.05$.

**Flow Cytometry**

Cells were plated as previously described to evaluate the effect of the materials on cell death–induced plasma membrane permeability. After 3 days of exposure to the materials, the cells were stained with fluorescein isothiocyanate–annexin V (FITC-AnV) (green fluorescence) and ethidium homodimer-III (Etd) (red fluorescence) and incubated in the dark for 15 minutes. 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 viable, early apoptotic, late apoptotic, and necrotic cells (23) Experiments were performed in triplicates. Statistical analysis was performed by using one-factor ANOVA and Holm–Sidak procedures at $\alpha = 0.05$.

**Vital Cell Staining**

MDPC-23 cells were plated on coverslips at 400 cells/cm$^2$ and incubated for 3 days with the test materials. After that, they were triple-stained with Hoeschst 33342 ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 350/461$ nm; blue fluorescence), fluorescein isothiocyanate-Annexin V ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 492/514$ nm; green fluorescence) and ethidium homodimer-III ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 528/617$ nm; red fluorescence), and incubated for 15 min in the dark. Hoechst 33342,
a cell membrane-permeable, minor groove-binding DNA stain, stains the nuclei of both apoptotic and necrotic cells. However, healthy cells are stained by Hoechst stain only, but healthy cells are not stained by fluorescein isothiocyanate-Annexin V (a phosphatidylserine-binding cytoplasmic dye) and ethidium homodimer-III (a non-vital DNA dye). Apoptotic cells are stained both green and blue. Cells stained blue, green and red represent dead cells progressing from the apoptotic cell population. The morphology of cell death (apoptosis versus necrosis) was examined by using two-photon confocal scanning microscopy (LSM-510 META; Carl Zeiss, Thornwood, NY) coupled to a Ti:sapphire laser.

**Cell Differentiation and Mineralization**

**Quantitative Real-time Polymerase Chain Reaction**

The effects of CPoint and gutta-percha on messenger RNA (mRNA) expression of osteogenesis and mineralization markers in MDPC-23 cells were examined using qRT-PCR. As the MTT assay indicated that both materials were rendered non-cytotoxic after 4 immersion cycles, subsequent experiments were conducted using points that had been aged in culture medium for 4 weeks, using similarly aged Teflon as the negative control.

The MDPC-23 cells were plated at 1x10⁴ cells/cm², and cultured in growth medium until they exhibited ~70-80% confluence. The growth medium was then replaced by an osteogenic medium, consisting of the original growth medium, supplemented with 50 μg/mL ascorbic acid, 10 mM glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). The aged materials were placed in respective transwells and inserted into the culture wells. Cells were cultured for 7 days, with replacement of the osteogenic medium every 3 days.
The markers used for examination of osteogenesis/mineralization of the MDPC-23 cells were runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein II (BSP-II), dentin matrix protein-1 (DMP-1) and dentin sialophosphoprotein (DSPP) (24-28). The qRT-PCR was run using TaqMan® Probe-Based Gene Expression Assay. TaqMan® probes consist of an oligonucleotide with a 5’-reporter dye and a 3’-minor groove binder (MGB) quencher dye. FAM™ dye, the fluorescent reporter dye, is covalently linked to the 5’ end of the oligonucleotide and is quenched by TAMRA™ dye at the 3’ end or non-fluorescent quencher. The Passive Reference dye (ROX™) dye included in TaqMan® Universal PCR Master Mix provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume. During the PCR reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

The qRT-PCR procedures were performed by isolating total RNA from the MPDC-23 cells. The total RNA was used as templates for synthesis of single-stranded complementary DNA (cDNA), using QIAshredder and RNeasy kit (Qiagen, Valencia, CA). The manufacturer’s recommended protocol was followed for RNA purification from animal cells. The purity and quantity of the resultant RNA were then assessed from 2-[L samples using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE). Equal amounts of total RNA (0.1mg RNA/mL) were then reverse-transcribed into single-stranded cDNA, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), in a thermal cycler using the
recommended settings (25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and finally stored at 4°C until use). The resultant cDNA was then amplified in real-time using TaqMan® Universal master mix and Ready-to-go format TaqMan® assays for the designated target genes in a 7300 Real Time PCR system (Applied Biosystems). For each reaction (20 uL), 4 µL of cDNA (50 ng/reaction), 5 µL of RNase-free water, 1 µL 20X primer, well in a 96-well reaction plate. Each sample was run in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. Relative quantification of gene expression was performed using the comparative threshold cycle method (where the level of each target gene expression was normalized to the GAPDH endogenous control used as an active reference. The data output was expressed as fold-change or fold-difference of expression levels, given by 2^{ΔΔC_t} (29).

As the data were not normally distributed, changes in fold regulation of each target gene, after exposure of the MDPC-23 cells to CPoint, gutta-percha and Teflon were compared using Kruskal-Wallis ANOVA and Dunn’s multiple comparison tests, with statistical significance preset at \( \alpha = 0.05 \).

**ALP Activity**

Cells were cultured in osteogenic differentiation medium as previously described, and exposed to the 3 materials (CPoint, gutta-percha, Teflon negative control) for 14 days. Alkaline phosphatase activity was determined using a QuantiChrom ALP assay kit (BioAssay Systems, Hayward, CA). Colorimetric determination was based on hydrolysis of p-nitrophenyl phosphate by ALP into inorganic phosphate and p-nitrophenol, a yellow-colored product (30). After incubation, the cells were lysed with 0.2% Triton X-100 for 20 min, and the cell lysate was incubated with p-nitrophenyl phosphate at 37 °C for 1 h. Absorbance of p-nitrophenol at 405 nm was
determined consecutively after 1-8 min using a microplate reader. Alkaline phosphatase activity of the cell lysate from each well was calculated based on the difference in optical densities between the initial and the destined time period, and expressed in IU/L (17, 31).

The Wald test was employed for statistical comparison of the ALP activities among CPoint, gutta-percha and Teflon. Polynomial regression was used to model the relationship between ALP activity and time for each material. Simultaneous tests of linear contrasts were used to compare model coefficients among the three materials. A Bonferroni adjustment was used to control the overall error rate for appropriately defined “families” of tests. The half-life (time in min) required for 50% decline in ALP activity, denoted by $T_{0.5}$, was estimated for each material by using the interpolation method with the fitted curve. Polynomial regression was used to determine 95% confidence limits for the predicted activity at $T_{0.5}$, which permitted determination of the corresponding approximate 95% confidence limits for $T_{0.5}$ by drawing vertical lines to intersect the x-axis. These approximate confidence limits for $T_{0.5}$ were used to derive an approximate standard error for $T_{0.5}$ in each material. This approach facilitated a comparison of the $T_{0.5}$ values among the three materials. Two-tailed tests with a significance level of 0.05 were used for all comparisons except when a Bonferroni adjustment was used.

Alizarin Red S Assay

Cells were cultured in osteogenic differentiation medium as previously described in Section IV, and exposed to the 3 materials (CPoint, gutta-percha, Teflon negative control) for 21 days (N = 12). Extracellular mineralization was determined using the Alizarin red S assay (32). The cells were washed with phosphate-buffered saline, fixed in 10 % formaldehyde, and stained with alizarin red S (40 mM solution, pH 4.2). The stained cells
were incubated in 10% acetic acid for 30 min and neutralized with 10% ammonium hydroxide. Absorbance of the supernatants was determined at \( \lambda = 405 \) nm. The amount of Alizarin red S stain in the samples (in mg/mL) were determined using a calibration curve correlating optical density of known Alizarin red S concentrations to the optical density of AR-S stain in the samples.

As the data were not normally distributed (Shapiro-Wilk; \( P = 0.005 \)), they were statistically analyzed using Kruskal-Wallis ANOVA and Dunn’s multiple comparison tests at a significant level of 0.05.

**Transmission Electron Microscopy**

Cells cultured in osteogenic medium for 21 days and exposed to the 3 aged materials were fixed, dehydrated in ethanol (30%–100%), transferred to propylene oxide, and embedded in epoxy resin. Seventy-nanometer-thick sections were examined by using a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at 110 kV to identify the status of mineralization within the extracellular matrix.
Results

MTT Assay

Both CPoint and gutta-percha took 4 immersion cycles to become non-cytotoxic (17) (Fig. 1A). Both materials (P = .000) and aging time (P < .001) significantly affected mitochondrial metabolic activity. Both materials exhibited reduced cytotoxicity with time, particularly for CPoint in which activities from the 4 time-periods were all significantly different (P < .05). CPoint (48.3% ± 1.7% of the Teflon negative control) was significantly different (P < .05) from gutta-percha (75.1% ± 1.7%) at baseline, but not for the subsequent weeks. For eluents (Fig. 1B), both elution materials (P = .002) and dilution factor (P < .001) significantly affected mitochondrial metabolic activity. The interaction of these 2 factors was significantly different (P = .006). Eluents derived from CPoint (12.0% ± 8.5% of Teflon) were significantly different from gutta-percha (3.6% ± 1.1%) only at 1:1 dilution (P < .05). Eluents from both materials at 1:1 dilution were more cytotoxic than the other dilutions (P < .05).

Flow Cytometry

Two-dimensional dot plots of test groups are shown in Figure 1C. Percentages of viable cells (AnV/Etd negative) were significantly different among the experimental and control groups (P < .001). CPoint (91.0% ± 1.8%), gutta-percha (89.7% ± 2.0%), and Teflon (92.5% ± 1.1%) were not significantly different from one another but were significantly different (P < .05) from IRM (0.9% ± 0.4%) (Fig. 1D).

Vital Cell Staining

Cells exposed to CPoint (Fig. 2A), gutta-percha (Fig. 2B), and the Teflon negative control (Fig. 2C) were mostly vital and exhibited blue fluorescent nuclei with minimal
signs of apoptosis/necrosis. Cells exposed to the IRM positive control were mostly apoptotic and necrotic, with pink nuclei (merging of blue and red fluorescence) and green fluorescent cytoplasm (Fig. 2D).

Quantitative RT-PCR

Fold regulation of osteogenic gene markers in MDPC-23 cells, after their exposure to CPoint, gutta-percha, and Teflon, are represented in Figure 2E. Compared with Teflon, expression of RUNX2, ALP, OCN, and DMP-1 gene markers was up-regulated in CPoint and gutta-percha. Up-regulation of OCN and DMP-1 was significantly different only between gutta-percha and Teflon (P > .05). There was no change in BSP after exposure to CPoint, but the marker was upregulated after exposure to gutta-percha; nevertheless, the difference was not significant (P > .05). DSPP was down-regulated after exposure to both CPoint and gutta-percha, but results were not significantly different from Teflon (P > .05).

ALP Activity

Decline in ALP activity occurred with absorbance recording time for each material (Fig. 3A). A quartic polynomial regression model provided an excellent fit for the relationship between ALP activity and recording time for each material. Simultaneous tests of linear contrasts with a Bonferroni-adjusted significance level of .05/5 = .01 comparing the coefficients of the quartic models yielded a significant difference only in the intercept term between CPoint and Teflon (P = .004) and between gutta-percha and Teflon (P = .003). None of the models differed in terms of quartic, cubic, quadratic, or linear terms or T0.5 value.

Alizarin Red S Assay

Significant differences were observed in the amount of alizarin red S staining (P
< .001). Gutta-percha and CPoint groups showed significantly higher AR-S values compared to control group (P < .05). There was no significant difference between CPoint and gutta-percha (Fig. 3B).

**Transmission Electron Microscopy**

Figure 3C is a representative unstained image of extracellular mineralization that was produced by MDPC-23 cells cultured in osteogenic differentiation medium. Mineralization was in the form of clusters of spherical electron-dense nodules. There was no difference in the morphology of the bone nodules among the 3 groups.
Figure 3-1 (A) MTT profile of MDPC-23 cells after exposure to test cements for 4 weeks. (B) MTT profile of MDPC-23 cells after exposure to eluents from aged test materials. Different letters indicate statistical significance.
Figure 3-2 (A) 2D plot of flow cytometry data of FITC AnV/ETD III stained cells after exposure to test materials. (B) Percentage of Viable cells in different test groups. Different letters indicate statistical significance.
Figure 3-3 Confocal laser scanning microscopy (CLSM) imaging of MDPC-23 cells triple-stained with Etd, FITC-AnV and Heoschst 33342 after exposure to (A) CPoint, (B) gutta-percha, (C) Teflon negative control and (D) IRM. The overall result after merging of the 3 channels is represented by the merged image. Bar = 20 mm.
Figure 3-4 Quantitative RT-PCR results showing fold regulation of gene markers after MDPC-23 cells were exposed to CPoint, gutta-percha point, and Teflon disk. Values > 1 indicate upregulation, and values < -1 indicate downregulation. Values between 1 and -1 indicate no fold change. Only genes with significant differences among the 3 groups are labeled. For each gene marker, columns labeled with the same descriptor designation (upper or lower case letters) are not significantly different (P > .05).

Figure 3-5 Plots of changes in ALP activity over absorbance recording time, when MPDC-23 cells were incubated with CPoint, gutta-percha point, or Teflon in osteogenic differentiation medium. Polynomial regression equations were used to model the relationship between ALP activity and recording time for each material.
Figure 3-6 Bar chart summarizing the extent of ex vivo mineralization in the 3 groups in the form of alizarin red S concentration. Groups designated by the same lower case letters are not significantly different (P > .05).

Figure 3-7 Unstained TEM image of extracellular bone nodules produced by MPDC-23 cells after they were cultured in osteogenic medium in the presence of Teflon, CPoint, or gutta-percha (scale bar = 500 nm).
Discussion

This study aimed to evaluate the biocompatibility of CPPoint from 2 distinct, clinically related aspects: the effect of the endodontic point on cell viability, necrosis, and apoptosis (33) of odontoblast-like cells and the effect on the mineralization potential of those cells. To examine the effects on cell viability, a cyclic regime (17) was used to test the effect of materials and varying dilutions of their eluents on succinic dehydrogenase activity of the cells. The effects of the materials on the apoptosis/necrosis of these cells were then evaluated, both quantitatively by using flow cytometry and qualitatively by using confocal microscopy imaging. During the first week, CPPoint was considerably more cytotoxic than gutta-percha. This may be due to the presence of an incompletely polymerized air inhibition component on the material’s surface. Gutta-percha, which is known for its bioinertness, was also more cytotoxic than the Teflon negative control during the first week, which may be attributed to the leaching of zinc ions from zinc oxide fillers (34). On the basis of these results, the cytotoxic effects of CPPoint were transient and comparable to gutta-percha, and they were likely to be attributed to mitochondrial dysfunction (necrosis) rather than translocation of intramembranous phosphatidylinerine (apoptosis) (35). Taken together, the combined results of the cell viability part of the study are supportive of the first hypothesis that CPPoint does not adversely affect the viability of odontoblast-like cells by inducing undue necrosis or apoptosis of those cells.

The effects of CPPoint on the mineralization potential of MDPC-23 cells were studied at the mRNA level, enzymatic level, and extracellular matrix mineralization level. The latter was evaluated both quantitatively by using alizarin red S staining and qualitatively by using transmission electron microscopy (TEM). At the mRNA level, CPPoint induces upregulation of most osteogenic gene markers examined, with the
exception of DSPP and BSP. The extents of up-regulation or down-regulation of these markers were predominantly nonsignificant on the basis of the results of statistical analysis. This may be attributed to the relatively short period (7 days) of culturing of the cells in osteogenic medium before mRNA extraction. Of particular interest was the up-regulation of RUNX2, because it has been shown that the transcription factor RUNX2 inhibits terminal differentiation of odontoblasts and induces transdifferentiation of odontoblasts into osteoblasts, with the formation of bone-like structure (27, 28). This could have explained why bone nodules were exclusively seen in the TEM image of MDPC-23 cells. Absence of epithelial-mesenchymal interactions may have caused the up-regulation of RUNX2 in vitro, which, in turn, inhibits the terminal differentiation of odontoblast-like precursors into true odontoblasts. Compared with quantitative RT-PCR, ALP enzymatic activities yielded more definitive results that were further validated by the increase in calcium content of the extracellular matrices. Taken together, the results are supportive of the second hypothesis that after being rendered non-cytotoxic via elution of its cytotoxic components, CPoint does not adversely affect the in vitro mineralization potential of the odontoblast-like cells.

Within the limits of the present study, it may be concluded that CPoint is comparable to gutta-percha in terms of the effects on cell viability as well as the mineralization potential of MDPC-23 cells and possesses negligible toxicologic risks after elution of its cytotoxic components. Because these risks are relatively lower than those imposed by a zinc oxide–eugenol based cement, a favorable in vivo tissue response is likely to occur more rapidly. The material also possesses minimal risk in the repair of hard tissue demineralization in periapical lesions by osteogenesis. Although these biological objectives await further validation by in vivo animal
models, investigations on the expansion properties and water sorption/solubility characteristics of CPoint as well as its ability to create gap-free filling of the canal space are required to provide a sound rationale for its use as an alternative root-filling material.
References


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Chapter 4: In Vitro Osteogenic/Dentinogenic Potential of an Experimental Calcium Alumino-Silicate Cement

Introduction

Since its introduction in 1993 in the form of mineral trioxide aggregate (MTA), tricalcium silicate hydraulic cements have gained worldwide acceptance as the
material of choice for various endodontic applications (1). The biocompatibility, osteogenicity, and hydrophilicity of MTA made it ideal for use as a root-end filling material after surgical endodontics, perforation repair, vital pulp therapy including direct pulp capping and pulpotomy of immature teeth (apexogenesis), as an apical barrier in immature teeth with necrotic pulps (apexification), and as a coronal barrier in regenerative endodontic procedures in immature teeth with apical periodontitis (2). Despite the favorable clinical outcome of tricalcium silicate cements, some tricalcium silicate products possess major clinical deficiencies such as suboptimal handling characteristics, poor washout resistance, slow setting reaction (3), uncontrolled expansion (4), incompatibility with current adhesive restorative materials (5), and initial cytotoxicity (6). Although some of those drawbacks were addressed in more recent formulations of tricalcium silicate cements (7), the development of alternative biocompatible endodontic cements with overall improved properties is highly desirable.

Calcium aluminate hydraulic cements for biomedical uses are modifications of high-alumina cements used for refractory linings in furnaces and some civil engineering purposes. They were developed in the early 20th century as more chemically durable cements than Portland cement (8). The hydraulic calcium phases typically contain 30%–50% alumina, whereas Portland cements contain less than 5%. Commercial, non-biological, calcium aluminate cements are used as sewer pipe lining because of their inherent acid resistance to biogenic corrosion. This acid-resistant feature may be useful in infection-laden sites of the human body where the pH value drops significantly (9). In the biomedical field, these inert, biocompatible, and sufficiently strong cements have been successfully used as bone cements and in orthopedic implants (10). In dentistry, calcium aluminate powder has been
incorporated into direct restorative materials (DoxaDent; Doxa AB, Uppsala, Sweden) (11) and in glass ionomer–based luting cement (Ceramir C&B; Doxa AB) (12). Calcium aluminate with the addition of radiopacifier powders was mixed with water or salt solution to form an endodontic cement of similar indications as commercially available tricalcium silicate cements (EndoBinder; Binderware, São Carlos, SP, Brazil) (13).

Capasio (Primus Consulting, Bradenton, FL) is an experimental calcium aluminosilicate cement designed for endodontic use. It is composed primarily of calcium aluminosilicate powder, dental glass, and bismuth oxide as a radiopacifier and is supplied with a waterbased gel. This hydraulic calcium aluminosilicate cement was found to be slightly less basic (pH 10.9) than white MTA (WMTA) (pH 11.6) on final set, had a setting time of 9 minutes, penetrated dentinal tubules, and demonstrated improved acid resistance and washout resistance when compared with WMTA (3, 14). Its in vitro biocompatibility and bone nodule formation had also been tested in rat osteoblasts (15). Recently, Capasio powder has been refined and renamed as Quick-Set (Primus Consulting), and the cationic surfactant was removed from the liquid gel component, which was thought to interfere with its biocompatibility (15). A recent biocompatibility study that used a murine dental papilla-derived odontoblast-like cell line (MDPC-23) showed that Quick-Set exhibited similar in vitro cytotoxicity profiles as WMTA (performed with methyl-thiazol diphenyltetrazolium assay, flow cytometry, and vital cell staining) (6). However, it is not known whether this experimental aluminosilicate endodontic cement possesses osteogenic/dentinogenic properties that may promote periapical bone healing and pulpal calcific barrier formation; these properties are prominent features in commercially available tricalcium silicate cements (2). Thus, the objective of the present study was to
investigate the effect of Quick-Set on the osteogenic/dentinogenic differentiation potential of murine odontoblast-like cells. The null hypothesis tested was that no differences exist between the experimental calcium aluminosilicate cement and a commercially available tricalcium silicate cement on the osteogenic/dentinogenic differentiation potential of murine odontoblast-like cells.
Materials and Methods

Quick-Set and white ProRoot MTA (WMTA) (Dentsply Tulsa Dental Specialties, Tulsa, OK) were mixed with the proprietary gel or deionized water, respectively, in a powder/water ratio of 3:1. The mixed materials were placed in pre-sterilized Teflon molds (5-mm diameter and 3-mm thick), covered with pre-sterilized Mylar sheets, and allowed to set completely in a 100% humidity chamber for 24 hours. Teflon disks of the same dimensions were used as the control. All materials were sterilized with ultraviolet light for 4 hours before testing. Before evaluating the osteogenic/odontogenic potential of test cements, the cement and control disks were rendered noncytotoxic by immersing them in complete growth medium (Dulbecco modified Eagle medium) (Lonza, Walkersville, MD) for 2 weeks, as recommended by a previous study (6).

Similar to the previously reported biocompatibility study on Quick-Set (6), the osteogenic/dentinogenic potential of both cements was evaluated by using the previously reported MDPC-23 cell line (16). The MDPC-23 cells were plated at 1×10^4 cells/cm^2 and cultured in the aforementioned complete growth medium, supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corp, Carlsbad, CA), 2 mmol/L L-glutamine, and 100 U/mL penicillin-streptomycin until they exhibited ≈ 70%–80% confluence. The growth medium was then replaced by osteogenic differentiation medium, which consisted of the original growth medium, supplemented with 50 μg/mL ascorbic acid, 10 mmol/L β-glycerophosphate, and 100 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO). The aged materials were placed in respective Transwell inserts (Corning Inc Life Sciences, Tewksbury, MA) and inserted into the culture wells with osteogenic differentiation medium. The latter was changed every 3 days, and the effect of the test cements on the
osteogenic/dentinogenic differentiation potential of the MDPC-23 cells was evaluated from 3 aspects.

**Quantitative Real-time Polymerase Chain Reaction**

Expression of osteogenic/dentinogenic differentiation markers was examined by using quantitative reverse transcription polymerase chain reaction (qRT-PCR). This procedure was conducted after the MPDC-23 cells were incubated with aged cement disks in osteogenic differentiation medium for 1 week. Similarly aged Teflon disks were used as the control.

The markers used for examination of differentiation of hard tissue forming cells and their mineralization were alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP1) (17, 18). The qRT-PCR procedures were performed by isolating total RNA from the MDPC-23 cells. The total RNA was used as templates for synthesis of single-stranded complementary DNA (cDNA) by using QIAshredder and RNaseasy kit (Qiagen, Valencia, CA). The manufacturer’s recommended protocol was followed for RNA purification from animal cells. The purity and quantity of the resultant RNA were then assessed from 2-μL samples by using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE). Equal amounts of total RNA (0.1 mg RNA/mL) were then reverse-transcribed into singlestranded cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a thermal cycler by using the recommended settings. The resultant cDNA was then amplified in real time by using TaqMan Universal master mix and Ready-to go format TaqMan assays (Life Technologies, Grand Island, NY) for the designated target genes in a 7300 Real Time PCR system (Applied Biosystems). Each sample was run in triplicate. Glyceraldehyde
3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Relative quantification of gene expression was performed by using the comparative threshold cycle method (ΔΔCT), where the level of each target gene expression was normalized to the GAPDH endogenous control used as an active reference. The data output was expressed as fold changes of expression levels, given by 2-ΔΔCT (19). Expression profiles of the target genes were compared among test groups. Because the data for each gene were normally distributed (Shapiro-Wilk test) and exhibited equal variance (modified Levene test), they were statistically analyzed by using one-factor analysis of variance, with statistical significance preset at α = 0.05. The Tukey method of multiple comparisons was used to perform pair-wise comparisons of the 3 groups at each gene level. A Bonferroni adjusted significance level of 0.05/3 = 0.017 was used for each pair-wise comparison so that the family-wise error rate for each gene expression level could be controlled at the 0.05 level.

**ALP Activity**

Cells were cultured in osteogenic differentiation medium as described previously and incubated with the 3 materials (Quick-Set, WMTA, and Teflon control) for 7 and 14 days (N = 6 wells; different MPDC-23 cells examined at the 2 time points). ALP activity was determined by using a QuantiChrom ALP assay kit (BioAssay Systems, Hayward, CA). Colorimetric determination was based on hydrolysis of p-nitrophenyl phosphate by ALP into inorganic phosphate and p-nitrophenol, a yellow-colored product (20). After incubation, the cells were lysed with 0.2% Triton X-100 for 20 minutes, and the cell lysate was incubated with p-nitrophenyl phosphate at 37°C for 1 hour. Absorbance of p-nitrophenol at 405 nm was recorded every minute for a maximum of 16 minutes by using a microplate reader. The level of ALP activity of the cell lysate in IU/L was calculated from the equation
Where \( OD \) is the optical density at 405 nm, and \( t \) is the time in minutes. \( ODSample \ 0 \) is the optical density at 0 minutes. The resultant data were plotted on a graph, and the peak value for each material was selected and compared with the other materials. Because the raw data obtained for the 3 materials and 2 time points were not normally distributed, they were logarithmically transformed and analyzed by using two-factor analysis of variance to examine the effect of materials and time points and the interaction of these 2 factors on ALP activity. The Tukey test was used for comparison among groups. For all tests, statistical significance was preset at \( \alpha = 0.05 \).

**Alizarin Red S Assay**

Cells were cultured in osteogenic differentiation medium as previously described and exposed to the 3 materials (Quick-Set, WMTA, and Teflon control) for 21 days (\( N = 18 \)). Extracellular mineralization was qualitatively and quantitatively determined by using the alizarin red S assay (21). Alizarin red S will stain calcium-rich deposits (nodules) produced by cells in culture. The dye can be extracted from the stained nodules and assayed by spectrophotometry. The tissue culture dishes were washed with phosphate-buffered saline; the cells and nodules were fixed in 10% formaldehyde and stained with alizarin red S (40 mmol/L solution, pH 4.2). Images of the specimens were taken for qualitative assessment of the stained nodules. The stained nodules were then incubated in 10% acetic acid for 30 minutes and neutralized with 10% ammonium hydroxide. Absorbance of the supernatants was determined at \( OD = 405 \) nm. The level of alizarin red S staining in the samples (\( \mu g/L \)) was determined according to a linear regression equation derived from the pre-equilibration standard curve. Because the data were not normally distributed, they
were analyzed by using Kruskal-Wallis analysis of variance and Dunn multiple comparison test at $\alpha = 0.05$. 
Results

Quantitative RT-PCR

Fold changes of osteogenic differentiation markers in MDPC-23 cells after 1 week of incubation with Quick-Set, WMTA, and Teflon are collectively represented in Figure 1. There was little or no change of RUNX2, OCN and DMP1 gene marker expression in both experimental groups; however, the results were not statistically significant from the Teflon control ($P = .583$ for RUNX2, $P = .263$ for OCN, and $P = .138$ for DMP1 ). Expressions of ALP among the 3 groups were significantly different ($P = .003$); however, there was no difference in the fold up-regulation between Quick-Set and WMTA ($P = .662$). Expression of DSPP among the 3 groups was also significantly different ($P = .003$); however, there was no significant difference in the fold up-regulation between Quick-Set and WMTA ($P = .072$). No significant difference in the fold changes between Quick-Set and Teflon could be detected after Bonferroni adjustment ($P = .044$). Expression of BSP appeared to have been down-regulated in both experimental groups, but the fold changes were not significantly different from Teflon control ($P = .385$).

ALP Activity

A summary of the ALP activities of MDPC-23 cells after 1 and 2 weeks of exposure to the test materials in osteogenic differentiation medium is presented in Figure 2. Both materials ($P < .001$) and time points ($P < .001$) significantly affected ALP activity. The interaction of these 2 factors was not statistically significant ($P = .177$). For each time point, ALP activities exhibited by Quick-Set and WMTA were not significantly different, but they were significantly higher than those exhibited by the Teflon control ($P < .05$).
**Alizarin Red S Assay**

Figure 3A–C shows representative images of alizarin red S–stained calcium deposits produced by the MDPC-23 cells cultured in osteogenic medium after exposure for 3 weeks to Quick-Set, WMTA, and Teflon, respectively. Nodular calcific deposits of varying stain intensities could be observed macroscopically. Significant differences were observed in the amount of alizarin red S staining among the 3 groups (P < .001). The amounts of dye expressed in Quick-Set and WMTA were not significantly different (P > .05), but they were both significantly higher than the amount of dye expressed in Teflon (P < .05). Concentrations (μg/ mL) of alizarin red S extracted from the stained calcium deposits in the 3 groups are summarized in Figure 3D.
Figure 4-1 qRT-PCR results showing expression of target osteogenic markers by MDPC-23 cells after exposure to test materials for 1 week. Gene markers examined were ALP, RUNX2, OCN, BSP, DSPP, and DMP1. Values >1 indicate upregulation, and values <-1 indicate downregulation. Any value between 1 and -1 indicates no fold change. Only genes with significant differences among the 3 groups are labeled. For each of those genes, columns labeled with the same descriptor are not significantly different (P > .05).

Figure 4-2 ALP enzyme level after 1 and 2 weeks of exposure to test materials. For each time point columns connected with a horizontal bar are not significantly different (P < .05). For each material, columns for the 2 time points that are labeled with different designators (Quick-Set, uppercase letters; WMTA, lowercase letters; Teflon control, numeric) are significantly different (P < .05).
Figure 4-3 Representative images of alizarin red S–stained calcium deposits produced by MDPC-23 cells cultured in osteogenic medium after exposure for 3 weeks to Quick-Set (A), WMTA (B), and Teflon (C). (D) Concentrations (μg/mL) of alizarin red S extracted from the stained calcium deposits in the 3 groups. For each material, columns labeled with different uppercase letters are significantly different (P < .05).
Discussion

Osteogenicity and dentinogenicity are highly desirable properties for any endodontic cement. Osteogenicity of commercially available endodontic cements has been demonstrated previously (2). However, reliable dentinogenesis after vital pulp therapy remains an elusive goal (22). In the present study, a cause/effect, time-dependent strategy was used to study the osteogenicity/dentinogenicity of the test cements. According to this strategy, the messenger RNA expression profiles of target genes were evaluated during the first week. This was followed by assessment of the enzymatic activity of ALP in the first and second weeks. Qualitative and quantitative expressions of calcific deposits and extracellular matrix mineralization were subsequently examined in the third week. Freshly mixed test cements were found in a previous study to affect mitochondrial activity and induce apoptosis and necrosis in MDPC-23 cells (6). These cytotoxic effects were transient and decreased with elution of the cytotoxic components from the materials with time. Our time-dependent strategy took into consideration the cytotoxicity profile of test cements and its inhibitive effects on the mineralization potential of such cements (6). Accordingly, all cement samples were rendered noncytotoxic before testing their mineralization potential by immersing them in complete growth medium for 2 weeks. The MDPC-23 odontoblast-like cells were used in the present study because their partially differentiated status enabled them to differentiate into either osteogenic or dentinogenic lineage (18).

On the gene expression level, several gene markers associated with osteogenic/dentinogenic differentiation were examined; among them was ALP, which has been shown to be expressed in the earlier stages of bone formation (23). Likewise, RUNX2 is up-regulated in immature osteoblasts but down-regulated in mature
osteoblasts (24). RUNX2 inhibits osteoblast maturation and mature bone formation, was also found to inhibit terminal differentiation of odontoblasts, and induces transdifferentiation of odontoblasts into osteoblasts (18). OCN is involved in bone remodeling by exerting a role in the resorption process and adhesion of osteoclasts (25). The function of BSP in mineralized tissues is unknown, although it may act as a nucleus for the formation of the first apatite crystallites (26, 27). Both Quick-Set and WMTA did not alter the gene expressions of RUNX2, OCN, or BSP to statistically significant levels when compared with the Teflon control. However, both cements significantly up-regulated the expression of ALP gene when compared with the Teflon control.

Both DSPP and DMP1 are gene markers that are most closely associated with dentinogenesis (28). Cleaving of DSPP into dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) occurs during dentin mineralization. Although DSP may be involved in the initiation of dentin mineralization but not in the maturation of this tissue (28), the functions of DSP are presently undefined (29). DPP, the most abundant extracellular matrix protein component in dentin, has been found to play a role in nucleation and growth of hydroxyapatite crystallites during dentin mineralization; it has a high affinity for type I collagen and has been shown to activate the SMAD pathway (30). DMP1 belongs to the SIBLING (small integrin binding N-linked glycoproteins) family, which are associated with mineralized tissues (31). After post-translational proteolytic cleavage, DMP1 exists within the extracellular matrix of bone and dentin as an NH2-terminal fragment, a COOH-terminal fragment, and the proteoglycan form of the NH2-terminal fragment (DMP1-PG) (32). Biochemical analysis of bovine teeth showed that predentin is rich in DMP1-PG, whereas mineralized dentin primarily contains the COOH-terminal
fragment (33), and thus, it is suggested that the C-terminal 57-kDa fragment (COOH terminal) of DMP1 is primarily responsible for the function of this protein in dentin biomineralization (33).

In the present study, both cements stimulated the up-regulation of DSPP. Although there was a tendency for DMP1 to be up-regulated by both cements compared with Teflon, the level of up-regulation was not statistically significant. The lack of statistically significant upregulation of DMP1 may be caused by small sample size that resulted in large standard deviations observed for the experimental groups. It has been suggested that DSPP may be up-regulated by DMP1 during dentinogenesis (34). This speculation is further supported by another study (35) that shows that the COOH-terminal end of DMP1, which is localized in the nucleus during early differentiation of the odontoblasts, is able to bind specifically with the rat DSPP promoter in the region between nt -450 and +80 and to activate the transcription of DSPP. The up-regulated expressions of DSPP in Quick-Set and WMTA suggest that both cements may favor dentinogenic differentiation of odontoblast-like cells. This is of clinical interest in cases such as direct pulp capping and vital pulpotomy, where deposition of osteodentin or bone-like tissues is the inevitable outcome (22). This conclusion is also supported by the decreased expression of the more osteoblast-specific differentiation markers. We speculate that inclusion of an epithelial component in the cell culture model may elicit a more profound dentinogenic response, because the resultant epithelial-mesenchymal interactions might induce a more dentinogenic-favorable crosstalk between both cellular components. Research in this direction is underway.

Compared with the qRT-PCR data, the ALP enzymatic activities yielded more definitive results that are indicative of increased matrix mineralization as a result of
exposure of MDPC-23 cells to aged Quick-Set and WMTA cements. ALP activity for both cements was significantly higher than the control, indicating increased osteoblastic/odontoblastic activity and increased mineralization turnover. Alizarin red S assay results also suggest increased extracellular calcium deposition by MDPC-23 cells after exposure to Quick-Set and WMTA.

Within the limits of the present study, it may be concluded that the in vitro osteogenic/dentinogenic differentiation potential of Quick-Set is not different from that of MTA after elution of their cytotoxic components. Thus, the null hypothesis that no differences exist between the experimental calcium aluminosilicate cement and a commercially available tricalcium silicate cement on the osteogenic/dentinogenic differentiation potential of murine odontoblast-like cells cannot be rejected. It will be of interest to see whether the favorable in vitro response exhibited by Quick-Set can be replicated in a large animal study to test the effect of Quick-Set aluminosilicate cement on healing of periapical lesions, as well as its effect on the quality and quantity of calcific barrier formation in vital pulp therapy.
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Chapter 5: Summary and Conclusions

Summary

This study aimed to assess the chemomechanical and biological properties of select biomaterials of significant impact in endodontics. In the first chapter we characterized the interface between MTA and resin-modified glass ionomer cement. In the second chapter we investigated the cytotoxicity profile of CPoint and commercial gutta-percha, and their effects on the osteogenic differentiation potential of MDPC-23 cells. In the third chapter, we analyzed the osteogenic/dentinogenic differentiation potential of an experimental calcium aluminosilicate cement and a commercial TSC.

Conclusions

The following conclusions can be drawn from the results of the current study:

1- The effect of RM-GIC placement over commercial TSC after different time intervals and setting conditions was transient.

2- The RM-GIC and deeper layers of commercial TSC did not seem to be affected.

3- RM-GIC can be successfully applied on freshly mixed TSC in a single visit with no expected adverse reactions between the 2 materials.

4- CPoint is comparable to gutta-percha in terms of the effects on cell viability as well as the mineralization potential of MDPC-23 cells and possesses negligible toxicologic risks after elution of its cytotoxic components.

5- Because these risks are relatively lower than those imposed by a zinc oxide–eugenol based cement, a favorable in vivo tissue response is likely to occur more rapidly.
6- The material also possesses minimal risk in the repair of hard tissue
demineralization in periapical lesions.

7- Calcium aluminosilicate cement and commercial TSC exhibit similar in vitro
osteogenic/dentinogenic differentiation potential after elution of their
cytotoxic components.

8- mRNA expression profile of osteogenic markers in both groups suggest that
both cements may favor dentinogenic differentiation rather than osteogenic
differentiation of MDPC-23 cells.

9- Calcium aluminosilicate cements may be a potential substitute for
commercially available tricalcium silicate cements.
Chapter 6: Acknowledgments

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