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Cytotoxicity and Osteogenic Potential of Silicate Calcium Cements as Potential Protective Materials for Pulpal Revascularization

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Abstract

Objectives—In pulpal revascularization, a protective material is placed coronal to the blood clot to prevent recontamination and to facilitate osteogenic differentiation of mesenchymal stem cells to produce new dental tissues. Although mineral trioxide aggregate (MTA) has been the material of choice for clot protection, it is easily displaced into the clot during condensation. The present study evaluated the effects of recently-introduced calcium silicate cements (Biodentine and TheraCal LC) on the viability and osteogenic differentiation of human dental pulp stem cells (hDPSCs) by comparing with MTA Angelus.

Methods—Cell viability was assessed using XTT assay and flow cytometry. The osteogenic potential of hDPSCs exposed to calcium silicate cements was examined using qRT-PCR for osteogenic gene expressions, alkaline phosphatase enzyme activity, Alizarin red S staining and

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The authors do not have any conflict of interest related to this study.

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transmission electron microscopy of extracellular calcium deposits. Parametric statistical methods were employed for analyses of significant difference among groups, with $\alpha=0.05$.

Results—The cytotoxic effects of Biodentine and TheraCal LC on hDPSCs were time- and concentration-dependent. Osteogenic differentiation of hDPSCs was enhanced after exposure to Biodentine that was depleted of its cytotoxic components. This effect was less readily observed in hDPSCs exposed to TheraCal LC, although both cements supported extracellular mineralization better than the positive control (zinc oxide-eugenol-based cement).

Significance—A favorable tissue response is anticipated to occur with the use of Biodentine as a blood clot-protecting material for pulpal revascularization. Further investigations with the use of *in vivo* animal models are required to validate the potential adverse biological effects of TheraCal LC on hDPSCs.

Keywords

biocompatibility; cytotoxicity; calcium silicate cements; mineral trioxide aggregate; revascularization

1. Introduction

Treatment of immature teeth with non-vital pulps and apical periodontitis is fraught with challenges [1]. Traditionally, these teeth are managed by apexification to induce a natural calcific apical barrier [2], or by creating an artificial apical plug to facilitate conventional root canal treatment [3]. However, these procedures do not promote root development and result in thin canal walls that are susceptible to root fracture [4,5]. Revascularization of immature necrotic dental pulps has been proposed as a biologically-based alternative treatment strategy to conventional root canal treatment [6-13]. The revascularization process consists of meticulous asperification of the root canal space via the use of antibiotics or disinfecting irrigants, induction of bleeding to create a blood clot for homing of undifferentiated mesenchymal stem cells, insertion of a collagenous matrix over the blood clot for controlled placement of a biocompatible material to an optimal level to protect the blood clot, and placement of a leak-free restoration to serve as a coronal seal to prevent leakage and resulting reinfection [9,14-16].

Because of its excellent sealing properties and biocompatibility, mineral trioxide aggregate (MTA) has been the material of choice for covering the blood clot [6,9,17,18]. However, placing MTA over a soft blood clot can be a formidable task; MTA may collapse into the newly-formed clot during condensation [19]. This, in turn, may lead to islands of dystrophic calcification within the revascularized dental pulp [20]. Although placement of a collagen membrane between the clot and cement is a feasible alternative to reduce this displacement risk [9], it is legitimate to look for calcium silicate materials that are easier to handle than MTA for pulpal revascularization.

New calcium silicate cements have been commercialized because of MTA's clinical success. Biodentine (Septodont, Saint Maurdes-Fosses, France), a tricalcium silicate (Ca_3SiO_5)-based cement, is marketed as a dentin substitute [21]. Cell culture studies indicate that Biodentine has minimal cytotoxicity [22] and is biocompatible when placed in contact with

immortalized murine pulp cells [23]. The material's bioactivity was demonstrated by its ability to stimulate release of growth factor and to induce dental pulp mineralization [24]. TheraCal LC (Bisco Inc, Schamburg, IL, USA) is a light-cured resin-modified calcium silicate material designed for direct and indirect pulp capping [25]. The material was reported to be less cytotoxic than other resin-based light-cured liners [26] and released more calcium ions than the other dental cements [27].

These new tricalcium silicate cements appear to possess superior handling characteristics, making them potential candidates for pulpal revascularization procedures to prevent displacement of the cement into the freshly-formed blood clot. Calcium silicate cements should demonstrate excellent biocompatibility before they can be adopted as clot-protecting materials. Accordingly, the objective of the present study was to compare the *in vitro* biocompatibility and osteogenic potential of Biodentine and TheraCal, after exposure of these materials to human dental pulp stem cells (hDPSCs). Two null hypotheses were tested: (1) there is no difference in the survivability of undifferentiated hDPSCs after their exposure to Biodentine, TheraCal LC or a commercial MTA cement, and (2) Biodentine, TheraCal and a commercial MTA cement are equally adept at augmenting the osteogenic potential of the hDPSCs after the cements are rendered non-cytotoxic via elution of their cytotoxic components.

2. Materials and Methods

2.1 Materials

Biodentine, TheraCal LC and MTA Angelus (Angelus Dental Solutions, Londrina, PR, Brazil) were evaluated. The compositions of these calcium silicate-based materials are shown in Table I. Biodentine and MTA Angelus were mixed with deionized water or the liquid supplied, using the liquid/powder ratio recommended by the respective manufacturer. These 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 in a 100% humidity chamber for 24 h. TheraCal LC was supplied by the manufacturer in pre-mixed syringes and required no preparation before use. The resin-based calcium silicate cement was dispensed in increments into the Teflon mold and polymerized with a light-emitting diode-type light-curing unit for 20 sec per increment. The last increment was covered with a pre-sterilized Mylar sheet prior to light-curing to prevent the formation of an oxygen inhibition layer. Untreated cells were used as the negative control. Disks of similar dimensions to the test cements were prepared with zinc oxide-eugenol cement (Intermediate Restorative Material [IRM], Dentsply Caulk, Milford, DE, USA; one drop of liquid to one level scoop of powder) and used as the positive control. The set materials were sterilized with ultraviolet light for 4 h prior to testing.

2.2 Cell Culture

Previously characterized hDPSCs with CD90⁺/CD105⁺/CD34⁻/CD45⁻ immunophenotype [28] were employed in the present study. The cells were previously obtained from young healthy patients (18-25 years old; 10 teeth from 6 patients) according to a protocol approved by the Ethics Committee of the Fourth Military Medical University, Xi'an, China. The dental

pulp of each tooth was minced and digested in a solution containing 3 mg/mL type I collagenase and 4 mg/mL dispase (Gibco, BRL, Gaithersburg, MD, USA) at 37 °C for 2 h. Single-cell suspensions were then obtained by passing the cells through a 70-mm strainer (BD Falcon, Franklin Lakes, NJ, USA) and cultured in control medium in 5% CO₂ at 37 °C. The control medium consisted of α -modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin and 100 μ g/mL streptomycin. The medium was changed every 3 days.

In colony-forming test, the majority of the hDPSCs retained their spindle-shaped morphology, consistent with other mesenchymal stem cell populations [29]. The multipotency of those hDPSCs had been established using chondrogenic, adipogenic and osteogenic culturing conditions (Niu, unpublished results). The hDPSCs were seeded in 24-well plates at a density of 10⁴ cells/cm². The hDPSCs were cultured in complete growth medium consisting of α -modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO₂ at 37 °C. The growth medium was changed every 3 days; passages 2-4 hDPSCs were used for the present study.

2.3 Cell Viability

2.3.1 XTT assay—In pulpal revascularization protocols, materials are placed in close proximity to the blood clot from which hDPSCs are derived. Cytotoxic components derived from the set calcium silicate cements can readily diffuse into the blood clot. Thus, both materials and eluents were evaluated separately, using a previously-established cyclic regime [30].

An XTT Cell Viability Assay Kit (Biotium Inc, Hayward, CA, USA) was used to determine cell viability, based on the ability of mitochondrial enzymes in metabolically-active cells to reduce the yellow tetrazolium derivative 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to form a water-soluble orange-colored formazan product. The activities of those enzymes are inactivated shortly after cell death; hence, the assay enables determination of live cell numbers using spectrophotometric absorbance quantification at 475 nm. The viability of untreated hDPSCs was used as the negative control, the mean absorbance of which was adjusted to 100% for comparison with the relative dehydrogenase activities of the other groups (N = 12).

The XTT assay was performed on hDPSCs that had been directly exposed to test cements and on eluents derived from those materials. A weekly cycle consisted of direct evaluation of the toxicity of the set 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. Eluent collection was achieved by immersing cement disks in complete growth medium for 4 days after direct evaluation of the cement disks. During the first part of each weekly cycle, cement and control disks were placed individually in Transwell inserts with a 3- μ m pore size (BD Falcon, Franklin Lakes, NJ, USA) to prevent their direct contact with the hDPSCs. After the inserts were placed over the plated cells, an additional 2 mL of complete 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, each disk was retrieved and incubated at 37 °C with complete growth medium (1 disk/2 mL) for 4 days to collect the eluent from the set cement before using the same disk for the next cycle. For each disk, the same growth medium was used for eluent collection throughout the entire testing period. This recurrent cyclic aging protocol was continued until the material disks were rendered noncytotoxic (i.e. >85% of the mean dehydrogenase activity exhibited by the untreated control).

For indirect evaluation of the eluents, each eluent concentrate collected after completion of the aging cycles was diluted with fresh complete growth medium to 1:1, 1:10, and 1:100 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 hDPSCs. The XTT assay was performed in the same manner as previously described; absorbance was determined using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA).

2.3.2 Flow cytometry—Flow cytometry was employed with differential staining for sorting and counting of individual cells within a cohort of hDPSCs that expressed changes in plasma membrane permeability induced by the toxicity of the tested materials. The hDPSCs were plated in 6-well plates at a density of 10^5 cells/cm² and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 24 h. The material disks placed in Transwell inserts as previously described. After 3 days of exposure to the materials, the hDPSCs were detached from the culture wells using 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA) (Mediatech, Inc., Corning, Manassas, VA, USA) and re-suspended in binding buffer derived from the Apoptosis and Necrosis Quantification Kit (Biotium). The hDPSCs were stained with fluorescein isothiocyanate–annexin V ($\lambda_{\text{abs}}/\lambda_{\text{em}}$: 492/514 nm) and ethidium homodimer-III ($\lambda_{\text{abs}}/\lambda_{\text{em}}$: 528/617 nm) as the respective fluorescence stains for cytoplasmic membrane phospholipids and nucleic acids (N = 3). During apoptosis, phosphatidylserine is translocated from the inner to the outer surface of the cell for phagocytic cell recognition [31]. Annexin V labeled with fluorescein identifies apoptotic cells by binding to phosphatidylserine exposed on the outer leaflet of the cytoplasmic membrane, resulting in the expression of green fluorescence within the cytoplasm. Ethidium homodimer III is a highly positive-charged nucleic acid probe impermeable to live cells or apoptotic cells, but stains necrotic cells with red fluorescence. The stained cells were incubated in the dark for 15 min and subjected to fluorescence-activated cell sorting (MACSQuant Analyzer, Miltenyi Biotec Inc., Auburn, CA, USA) to determine the percentage distribution of viable, early apoptotic, late apoptotic, and necrotic cells. Experiments were performed in triplicates.

2.4 Cell Differentiation and Mineralization

2.4.1 Gene expression—Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the relative expression of target markers of osteogenic differentiation among the test groups. The set cements were first rendered non-cytotoxic by aging in culture medium for 5 weeks. The hDPSCs were cultured in complete growth medium at a density of 10^4 cells/cm² to ~80% confluency. The culture medium replaced by osteogenic differentiation medium, consisting of the original complete growth medium supplemented

with 50 mg/mL ascorbic acid, 10 mmol/L β -glycerophosphate and 100 nmol/L dexamethasone (Sigma-Aldrich). The established hDPSCs were exposed to Transwells containing the 3 aged calcium silicate cements (MTA Angelus, Biodentine and TheraCal LC) for 7 days, with the osteogenic differentiation medium changed every 3 days.

The total RNA present in the hDPSCs (N = 3) from each group was isolated and purified using QIAshredder and RNeasy kit (Qiagen, Valencia, CA, USA). Quality control of the extracted RNA was performed with 2 μ L samples by measuring their UV absorbance (NanoDrop ND-1000 UV-Vis spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantity of extracted RNA was determined from the RNA absorbance at 260 nm; RNA purity was determined from the ratio of the absorbance ($A_{260\text{ nm}}/A_{280\text{ nm}}$ and $A_{260\text{ nm}}/A_{230\text{ nm}} > 1.8$ and < 2.0). Equal amounts of total RNA (0.1 mg RNA/mL) were reverse-transcribed into single-stranded complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific Inc.) in a 7300 real-time PCR system (Applied Biosystems). Reverse transcription was performed using thermocycling conditions recommended by the manufacturer: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min). The generated cDNA was stored at -20 °C until commencement of qRT-PCR.

For qRT-PCR, alkaline phosphatase (*ALP*), osteocalcin (OCN), bone sialoprotein (BSP), runt-related transcription factor 2 (*Runx2*), dentin sialophosphoprotein (*DSPP*) and dentin matrix protein-1 (*DMP-1*) were used as target markers of osteogenic/dentiogenic differentiation [32-35]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene. Untreated hDPSCs grown in osteogenic differentiation medium was assigned as the negative control. The cDNA (100 ng each) was mixed with Taqman® PCR Universal Master Mix II (Invitrogen, Thermo Fisher Scientific Inc.) and added in 20 μ L aliquots to 96-well sequence-specific TaqMan® array standard plates preloaded with the designated primers and housekeeping gene (*ALP*: Hs01029144_m1; *OCN*: Hs01587814_g1; *BSP*: Hs00173720_m1; *Runx2*: Hs00231692_m1; *DSPP*: Hs00171962_m1; *DMP-1*: Hs01009391_g1; *GAPDH*: Hs99999905_m1). The plate was loaded in the thermocycler and run in regular cycles. Each cycle consisted of 50 °C for 2 min, 95 °C for 10 min, 40 cycles of denaturing/annealing at 95 °C for 15 sec and 60 °C for 1 min. Experiments were conducted in triplicate. The comparative threshold cycle method (C_T) was employed for relative quantification of gene expression; the expression level of each target cDNA marker was normalized to the GAPDH cDNA endogenous control. Data output was expressed as fold changes of mRNA expression levels, given by 2^{-C_T} [36].

2.4.2 Alkaline phosphatase enzyme activity—Cells cultured in osteogenic differentiation medium were exposed to the 4 aged materials for 14 days (N = 12). Intracellular ALP activity was evaluated using the Quantichrom ALP assay kit (Bioassay Systems, Hayward, CA, USA). Colorimetric determination was based on hydrolysis of *p*-nitrophenyl phosphate by ALP into inorganic phosphate and *p*-nitrophenol, a yellow-colored product. After 14 days of exposure, the cement disks and their respective Transwell inserts were retrieved. The cells were washed with PBS and lysed with 0.2% Triton X-100 for 20 min. Assay buffer and *p*-nitrophenyl phosphate were added to a clear-bottomed 96-well plate (150 μ L/well). Following addition of the cell lysate, the optical density of *p*-nitrophenol at t

= 0 min was recorded at 405 nm using the Synergy microplate reader. The samples were shielded from direct light at room temperature for 4 min prior to recording the optical density of *p*-nitrophenol at $t = 4$ min. The ALP activity of the cell lysate (in IU/L = $\mu\text{mol/L} \cdot \text{min}$) was calculated from the difference in optical density readings.

2.4.3 Extracellular mineralization—Cells cultured in osteogenic differentiation medium were exposed to the 4 aged materials for 21 days ($N = 6$). The hDPSCs were evaluated for calcium production by rinsing three times with phosphate-buffered saline. The cells were then fixed in 10% formaldehyde and stained with Alizarin red S (40 mmol/L, pH 4.2), a dye with affinity to calcium salts. Images of the specimens were taken for qualitative evaluation. Stained calcium deposits were incubated in 10% acetic acid for 30 min and neutralized with 10% ammonium hydroxide [37]. The supernatant was pipetted into a clear-bottom 96-well plate and the absorbance of the supernatant was determined at $\lambda = 550$ nm. The concentration of dye extracted from the specimens (mg/mL) was determined according to a linear regression equation derived from a calibration curve that correlated absorbance with known Alizarin red S concentrations. Because one mole of Alizarin red S binds to two moles of calcium to produce an Alizarin Red S-calcium complex [38], the extent of calcium deposition was expressed as molar equivalent of calcium.

Because Alizarin red S is not specific for calcium or alkali earth metals and can also react with a wide variety of cations [39], hDPSCs in various groups before and after addition of the osteogenic differentiation medium were examined by transmission electron microscopy (TEM) to confirm the formation of extracellular calcium deposits. The hDPSCs cultured in complete growth medium were detached by incubating in trypsin-EDTA for 2 min. Pooled dislodged cells were centrifuged, fixed with Karnovsky's fixative, post-fixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series (30%-100%), transferred to propylene oxide, and embedded in epoxy resin. Sections prepared by ultramicrotomy (90 nm thick) were stained with uranyl acetate and Reynold lead citrate. Stained sections were examined using a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV. After completion of the mineralization assay, hDPSCs from unstained wells were similarly processed and examined without further staining to identify the status of mineralization within the extracellular matrix.

2.5 Statistical analyses

Data from the IRM positive control was excluded from all statistical analyses to increase the robustness of the respective analysis. For XTT assay, results derived from weekly cycles were analyzed using two-factor repeated measures analysis of variance (ANOVA) and post-doc Holm-Sidak multiple comparison procedures to examine the effects of calcium silicate cements and aging cycle on hDPSC viability. Similar tests were used for analyzing the effect of eluent dilution and aging cycle on cell viability. For flow cytometry, one-factor ANOVA and Holm-Sidak multiple comparison procedures were used to examine the effects of calcium silicate cements on the percentage of vital, non-apoptotic, non-necrotic hDPSCs. For qRT-PCR, the effect of materials on expression of each osteogenic marker was analyzed separately by comparing with the GAPDH endogenous control, using a one-factor ANOVA and Holm-Sidak multiple comparison procedures. The results obtained from ALP enzymatic

activity and Alizarin red S staining were separately analyzed using one-factor ANOVA and Holm-Sidak multiple comparison procedures. For all statistical analyses, 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 nonlinearly-transformed to satisfy those assumptions prior to the use of parametric testing methods. Except for qRT-PCR, statistical significances for all analyses were set at $\alpha = 0.05$. For qRT-PCR, because the chance of finding one or more significant differences in testing six hypotheses on osteogenic markers with the same samples (type I error) is 26.49%, the Dunn–Šidák correction was used for controlling the familywise error rate. For each osteogenic marker, the critical value (α) was reduced to 0.0085, so that test results with $P < 0.0085$ were only considered significantly different from the GAPDH endogenous control.

3. Results

3.1 Cell Viability

The relative mitochondrial enzymatic activities of hDPSCs after their direct exposure to test cements in 4 weekly cycles are summarized in Figure 1A. Cell viability was significantly affected in the presence of materials ($P < 0.001$) and by the aging cycles ($P < 0.001$). The interaction of the two factors was also statistically significant ($P < 0.001$). For the factor material within the 1st cycle, all pairwise comparisons were significantly different except for the comparison between MTA Angelus and TheraCal LC ($P = 0.991$). Within the 2nd cycle, all pairwise comparisons were significantly different except for the comparison between MTA Angelus and Biodentine ($P = 0.804$). Within the 3rd cycle, all pairwise comparisons were significant except for comparisons between untreated cells and MTA Angelus ($P = 0.134$), and between MTA Angelus and Biodentine ($P = 0.388$). Within the 4th cycle, all pairwise comparisons were significantly different except for the comparisons between untreated hDPSCs and MTA Angelus ($P = 0.359$), between untreated hDPSCs and Biodentine ($P = 0.407$), and between Biodentine and MTA Angelus ($P = 0.774$). For the factor aging cycle within untreated hDPSCs, no difference could be detected among the four cycles ($P = 1.000$ for all six pairwise comparisons). Within MTA Angelus, all pairwise comparisons were significantly different except for the comparison between the 3rd and 4th cycle ($P = 0.733$). Similarly, within Biodentine, all pairwise comparisons were significantly different except for the comparison between the 3rd and 4th cycle ($P = 0.137$). Within TheraCal LC, each cycle was significantly different from one another ($P < 0.001$ for all six pairwise comparisons). For both MTA Angelus and Biodentine, 95% cell viability was achieved as early as the 3rd cycle. By contrast, TheraCal LC was considerably more cytotoxic, with >90% of hDPSCs exhibiting the capacity to reduce XTT only at the 4th cycle.

Results of direct evaluation of the materials were complemented by indirect evaluation of eluents released by the materials (Figure 1B). Cell viability was significantly affected in the presence of eluents ($P < 0.001$) and by the dilution factor ($P < 0.001$). The interaction of the two factors was also statistically significant ($P < 0.001$). For the factor eluent, all pairwise comparisons for the 1:1 and 1:10 dilution factors were significantly different except for the

comparison between elutents derived from Biodentine and MTA Angelus ($P = 0.124$ for 1:1 dilution and $P = 0.822$ for 1:10 dilution). For 1:100 dilution of the elutents, all pairwise comparisons were significantly different except for the comparisons among untreated hDPSCs vs Biodentine ($P = 0.446$), untreated hDPSCs vs MTA Angelus ($P = 0.446$), and MTA Angelus vs Biodentine ($P = 0.833$). For the factor dilution within untreated hDPSCs, no significant differences were detected among the three dilution factors (i.e. pure complete culture medium; $P = 0.996$ for all three pairwise comparisons). For all three calcium silicate cements, significant differences could be detected among the three dilutions ($P < 0.001$ for the dilution factors of elutents derived from each calcium silicate cement).

3.2 Flow Cytometry

Representative 2-dimensional dot plots of the distribution of vital, early apoptotic, late apoptotic and necrotic cells in untreated hDPSCs, and in hDPSCs after exposure to freshly-mixed MTA Angelus, Biodentine, freshly light-cured TheraCal LC and freshly-mixed IRM are shown in Figure 2 A-E, respectively. Significant difference in the percentage of healthy, non-apoptotic, non-necrotic cells was detected among untreated hDPSCs and in hDPSCs exposed to MTA Angelus, Biodentine and TheraCal LC ($P < 0.001$). Pairwise comparisons were all significantly different except for the comparison of MTA Angelus with Biodentine ($P = 0.799$). Under the same testing conditions, TheraCal LC was the most cytotoxic of the three calcium silicate cements in causing membrane permeability-related apoptosis and necrosis.

3.3 Expression of Osteogenesis-related Genes

Fold regulations of the expression of target mineralization markers after 7 days of osteogenic differentiation are summarized in Figure 3. Significant differences in gene expressions among untreated hDPSCs and cells exposed to the three silicate cements could be detected for *ALP*, *OCN*, *BSP*, *DSPP* and *DMP-1* ($P < 0.001$ for all comparisons) but not for *Runx2* ($P = 0.016$). For *ALP*, pairwise comparison with the GAPDH endogenous control showed that *ALP* expression was significantly upregulated after exposure to MTA Angelus or Biodentine (both $P < 0.001$); there was no significant difference between GAPDH and untreated hDPSCs ($P = 0.405$), and between GAPDH and cells exposed to TheraCal LC ($P = 0.358$). Expression of *OCN* was significantly upregulated after hDPSCs were exposed to MTA Angelus or Biodentine ($P < 0.001$); there was no significant difference between GAPDH and untreated hDPSCs ($P = 0.552$), and between GAPDH and cells exposed to TheraCal LC ($P = 0.536$). Expression of *BSP* was significantly upregulated after hDPSCs were exposed to MTA Angelus or Biodentine ($P < 0.001$); there was no significant difference between GAPDH and untreated hDPSCs ($P = 0.925$), and between GAPDH and cells exposed to TheraCal LC ($P = 0.294$). Expression of *DSPP* was significantly upregulated after hDPSCs were exposed to MTA Angelus or Biodentine ($P < 0.001$); there was no significant difference between GAPDH and untreated hDPSCs ($P = 0.182$), and between GAPDH and cells exposed to TheraCal LC ($P = 0.574$). Expression of *DMP-1* was significantly upregulated after hDPSCs were exposed to MTA Angelus or Biodentine ($P < 0.001$); there was no significant difference between GAPDH and untreated hDPSCs ($P = 0.153$), and between GAPDH and cells exposed to TheraCal LC ($P = 0.716$).

3.4 ALP Enzyme Production

Figure 4A represents the expression of ALP enzymatic activity in the five groups of hDPSCs that had undergone osteogenic differentiation for 14 days. Statistical analysis of the data derived from the untreated hDPSCs and cells exposed to the three calcium silicate cements detected significant difference among the four groups ($P < 0.001$). All pairwise comparisons were significantly different ($P < 0.001$ for Biodentine vs TheraCal LC, Biodentine vs MTA Angelus, Biodentine vs untreated hDPSCs; $P = 0.021$ for untreated hDPSCs vs TheraCal, and $P = 0.014$ for untreated hDPSCs vs MTA Angelus) except for the comparison between untreated hDPSCs and Biodentine ($P = 1.000$).

3.5 Extracellular mineralization

Figure 4B represents the calcium concentration of extracellular calcium deposits in the five groups of hDPSCs that had undergone osteogenic differentiation for 14 days. Statistical analysis of the data derived from the untreated hDPSCs and cells exposed to the three calcium silicate cements detected significant difference among the four groups ($P < 0.001$). All pairwise comparisons were significantly different ($P < 0.001$ for all comparisons). Representative Alizarin red S-stained calcium deposits observed in untreated hDPSCs, and in hDPSCs exposed to aged MTA Angelus, Biodentine and TheraCal LC are shown in Figure 4 C-F, respectively.

Figure 5A is a stained TEM image of untreated hDPSCs before switching to the osteogenic differentiation medium. A collagen fibril-containing extracellular matrix could be seen surrounding the cells. The newly-formed collagen fibrils had a diameter of approximately 25 nm and demonstrated only vague periodicity after staining (Figure 5B). Figure 5C is a stained section of established hDPSCs that had been cultured in osteogenic differentiation medium for 21 days; intact mitochondria identified from those cells were indicative of their healthy status. Extracellular mineral nodules could be identified. In a high magnification image of the unstained mineral nodules (Figure 5D), clusters of electron-dense, needle-shaped crystallites were identified within the 300-500 nm diameter nodules. These nodules did not resemble structures that are reminiscent of tubular dentin. Similar features were seen in hDPSCs that had been exposed to different calcium silicate cements (not shown).

4. Discussion

Biodentine and TheraCal LC are potential materials for blood clot protection in pulpal revascularization procedures due to their consistency, ease of manipulation and calcium silicate composition. Because of TheraCal LC's fluid-like consistency prior to light-curing, it is easy for the resin cement to be placed directly over the newly-formed blood clot. The material can be light-cured on demand, thereby preventing its apical migration into the blood clot. In addition, tooth restoration may be performed in the same session to seal the tooth coronally and save the patient time. However, there is a lack of literature regarding its cytotoxicity, and hence its efficacy as a pulpal revascularization material.

The XTT assay evaluates cell viability through colorimetric quantification of formazan produced via reduction of tetrazolium salts by mitochondrial dehydrogenases. 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. A modified cycling regimen was subsequently used to test the direct effects of calcium silicate cements on cell viability at different time points, as well as the indirect cytotoxic effects through eluents derived from the hDPSCs. Results from direct and indirect evaluations were in agreement and suggest that under the experimental conditions used in the present study, MTA Angelus and Biodentine are less cytotoxic than TheraCal LC. The results for the first two cements are supported by findings previously reported in the literature [40-42].

The cytotoxicity of TheraCal LC was also demonstrated by the results of flow cytometry. This analysis evaluated another aspect of cell viability by evaluating plasma membrane integrity and permeability induced by the toxicity of the materials. In the present work, the percentage of non-apoptotic healthy cells was low even for the untreated hDPSCs. This may be attributed to the sensitivity of the harvested cells to detachment by enzymatic digestion via the use of trypsin-EDTA. Most cell cultures are grown as a single-cell thick layer attached to the culture plate; intercellular and cell-to-substrate connections must be broken prior to subculture or retrieval for analysis. Trypsin is a proteolytic enzyme that breaks those bonds while EDTA is a chelator that helps bind calcium and magnesium, enabling trypsin access to the cell-cell and cell-substrate bonds. Because of the sensitivity of hDPSCs to trypsin-EDTA lift-off in the present work, coating culture plate surfaces with a thermoresponsive polymer such as N-isopropylacrylamide may be a better alternative in future studies [43,44]. Despite this limitation, the relative toxicity of the cements on plasma membrane permeability could still be evaluated by comparison with the untreated hDPSC negative control, under the same experimental conditions. All freshly-set calcium silicate cements were cytotoxic, though MTA Angelus and Biodentine had better results. This initial cytotoxicity may be attributed to the high pH values of the calcium silicate cements. Although TheraCal LC was the most cytotoxic among the three calcium silicate cements, it was better than the IRM positive control. Based on the results derived from two different aspects of cell viability evaluation (i.e. mitochondrial enzymatic activity and plasma membrane integrity), the first null hypothesis that “there is no difference in the survivability of undifferentiated hDPSCs after their exposure to Biodentine, TheraCal LC or a commercial MTA cement” has to be rejected.

According to the TheraCal LC's patent [25], the pulp capping and lining material comprises 40-50% by weight of Portland cement powder, 30-40% by weight of a neutral or mildly acidic hydrophilic resin monomer, 5-10% by weight of a hydrophobic resin monomer and 5-10% by weight of a hydrophilic filler. Photoinitiators and radiopacifiers are included to produce a single-component, light-curable miscible system without significant phase separation. Leaching of incompletely-polymerized resin monomers may accumulate over time to an overall toxic level, that irreversibly exhaust the cells' detoxifying glutathione metabolism and other defense mechanisms, resulting in cell apoptosis [45,46]. Unlike complex living systems, eukaryotic cells undergoing apoptosis in dissociated cell culture systems eventually die by secondary necrosis [47]; this accounts for the observation of necrotic cells in all groups. Mechanisms by which methacrylate resin components adversely affect cells include alteration of the lipid layers of plasma membranes, which, in turn,

increases the permeability of the membrane. The toxicity of BisGMA may be due to its incorporation into the lipid bilayer of phospholipid-containing cell membranes [48]. Reduction in cell metabolism and protein expression were found after contact of TheraCal LC with pulp cells [26]. Consequently, in the present investigation, it is not surprising that TheraCal LC exhibited substantial cytotoxicity when placed in contact with the hDPSCs throughout all evaluation periods. Although TheraCal LC also exhibited more severe cytotoxicity compared with Biodentine when examined with MTT assay after exposure to a rat MDPC-23 cell line [49], the results derived from the use of a human cell source, using two different aspects of cell viability evaluation, are conceivably more convincing and biologically relevant.

The effects of the cements on the mineralization potential of hDPSCs were investigated at the mRNA level, enzymatic level (ALP only) and extracellular matrix mineralization level. The latter was evaluated using Alizarin red S staining and TEM examination of extracellular mineralized nodule formation. At the mRNA level, both MTA Angelus and Biodentine upregulated most of the osteogenic gene markers examined, with the exception of *Runx2*. By contrast, osteogenic gene expressions of hDPSCs exposed to TheraCal LC were unaltered, similar to the extent expressed by untreated hDPSCs. The runt family transcription factor *Runx2* is a master regulator of osteogenic differentiation in bone formation and tooth development, determining the commitment of mesenchymal stem cells to the osteoblast or odontoblast lineage [51,52]. *Runx2* stimulates mesenchymal stem cells to become osteogenic cells through mediation of the major osteogenic target genes. This is achieved via binding of the transcription factor to Osteocalcin-Specific Element 2 (OSE2), the *Runx2* binding site present in the promotor region of those target genes [53]. It is interesting that *OCN* was upregulated in the present study after hDPSCs were exposed to MTA Angelus and Biodentine, while *Runx2* was neither upregulated nor downregulated. Because the process of osteogenic differentiation from mesenchymal stem cells occurs in different phases [54,55], each of which is characterized by a particular pattern of osteogenic marker genes, it is possible that MTA Angelus and Biodentine may upregulate other *Runx-2* dependent or *Runx-2* independent transcription genes, such as osterix, Wnt/b-catenin signaling, homeobox proteins DLX-3, DLX-5, MSX-2, or other less well-characterized downstream targets [52,55-60]. These factors have not been examined in the present work and require further investigation. Concomitant with the expression of osteogenic markers was the increased expression of odontoblasts markers such as *DSPP* and *DMP-1* in hDPSCs exposed to MTA Angelus and Biodentine. *DSPP* has a regulatory role in the mineralization of reparative dentin and is regarded as a specific marker of odontoblast differentiation [52,61]. *DMP-1* is present in the extracellular matrix of dentin and bone, and serves as an essential factor for mineralization of hard tissues [62,63].

Although qRT-PCR is a high throughput and rapid gene expression quantification technique, there is only modest correlation between mRNA and protein expressions in complex biological systems. This lack of correlation has been attributed to stochastic post-transcription and post-translation events, as well as experimental errors and noise interference [64,65]. Hence, the qRT-PCR results were supplemented by examination of ALP enzymatic activity and calcium deposition. Whereas *ALP* gene expression was not significantly downregulated in hDPSCs exposed to TheraCal LC, both ALP enzyme

expression and calcium deposition were significantly lower than the untreated hDPSCs. On the contrary, Biodentine induced significantly more mineralized nodule formation compared with the other groups, consistent with previous findings [23,42]. Accordingly, the second null hypothesis that “Biodentine, TheraCal and a commercial MTA cement are equally adept at augmenting the osteogenic potential of the hDPSCs after the cements are rendered non-cytotoxic via elution of their cytotoxic components” has to be rejected. Results at the enzyme and extracellular mineralization levels appear to suggest that the rate of hard tissue formation within a revascularized root may be hampered when TheraCal LC is employed for blood clot protection. Nevertheless, cell cultures involving a single cell type are incapable of reproducing the paracrine and exocrine signaling generated by cell-cell and cell-extracellular matrix interactions in living tissues or organs. These microenvironmental modulators of cell behavior are often missing in dissociated two-dimensional monolayer cell cultures, or even three-dimensional culture models [66]. Thus, data generated from the present study should be viewed upon as risk estimates that await validation with *in vivo* animal models.

Collagen fibrils secreted by undifferentiated hDPSCs were thin, immature fibrils that did not resemble the dentin extracellular collagen matrix. For all groups, the extracellular mineralization exhibited by hDPSCs that had undergone osteogenic differentiation was in the form of discrete circular clusters of needle-shaped crystallites instead of intrafibrillar crystallites within mineralized collagen fibrils. While it is reasonable to argue that dissociated hDPSCs will not secrete dentin in a two-dimensional cell culture system, others who had seeded hDPSCs on three-dimensional scaffolds reported that the secreted tissues resembled connective tissues more than dentin-like tissues [67]. It is important to point out that among the cell-cell paracrine interactions present in living tissues, ectodermal-mesenchymal interactions that occur during tooth development are critical for mesenchymal cells to commit to the odontoblast lineage [68]. Such paracrine cross-talks between the preameloblasts and preodontoblasts are lost following the senescence of the ameloblasts. Although a lot of work had been done to recapitulate those signaling pathways using discrete cytokines or chemokines, true dentin formation has not yet been convincingly demonstrated. Recent studies are of the consensus that tissues produced in direct pulp capping, pulpal revascularization or pulpal regeneration using *in-situ* stem cell homing approaches are reparative tissues instead of regenerated tissues, following the death of the primary odontoblasts and the lineage-committed daughter cells in the subodontoblastic layer [69,70]. It would be interesting to recapitulate the osteogenic differentiation part of the present study using hDPSCs that have been co-cultured with cells of ectodermal origin and seeding them in three-dimensional scaffolds, to see if dentin-like tissues can be formed. Research in this direction is in order.

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Highlights

- Cytotoxicity of Biodentine and TheraCal LC were time and concentration dependent
- Osteogenic differentiation of hDPSCs was enhanced after exposed to Biodentine
- Osteogenic differentiation of hDPSCs was adversely affected by TheraCal LC

Figure 1.

XTT assay. **A.** Direct evaluation of effect of calcium silicate cements (N = 12) on the mitochondrial enzymatic activity of human dental pulp stem cells (hDPSCs) after different aging cycles. Values (in means and standard deviations) are expressed as percentages of the dehydrogenase activities of untreated hDPSCs (negative control). For the factor material within each aging cycle, groups designated by horizontal bars at the same level are not significantly different ($P > 0.05$). For the factor aging cycle within each material, groups connected by dotted lines at the same level are not significantly different ($P > 0.05$). **B.**

Indirect evaluation of effect of eluents with different dilution factors ($N = 12$) on the mitochondrial enzymatic activity of hDPSCs. Values represent means and standard deviations. For the factor material within each dilution factor, groups designated by a horizontal bar are not significantly different ($P > 0.05$). For the factor dilution factor within each material, groups connected by dotted lines are not significantly different ($P > 0.05$).

Figure 2.

Flow cytometry. Representative two-dimensional dot plots of annexin V FITC and ethidium homodimer III-stained hDPSCs that were sorted into healthy (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left). **A.** Untreated hDPSCs (negative control). **B.** hDPSCs exposed to MTA Angelus. **C.** hDPSCs exposed to Biodentine. **D.** hDPSCs exposed to TheraCal LC. **E.** hDPSCs exposed to IRM (positive control). **F.** Bar chart comparing the percentage of healthy cells in the negative

control and the three calcium silicate cements ($N = 3$). Values represent means and standard deviations. Groups designated by a horizontal bar are not significantly different ($P > 0.05$).

Figure 3. mRNA expressions of target osteogenic markers in hDPSCs after exposure to different tricalcium silicate cements in osteogenic differentiation medium. Values represent means and standard deviations. For each osteogenic marker (N = 3), groups with the same colored asterisks are significantly different from the GADPH endogenous control (P < 0.0085).

Figure 4.

A. Intracellular ALP enzyme level in untreated hDPSCs and in hDPSCs exposed to the three calcium silicate cements and the IRM positive control ($N = 12$). Values represent means and standard deviations. For statistical comparison among the untreated hDPSCs and hDPSCs exposed to the calcium silicate cements, groups designated with horizontal bars are not significantly different ($P > 0.05$). **B.** Calcium molar concentration of extracellular mineral deposits secreted by hDPSCs that were stained by Alizarin red S in the five groups ($N = 6$). Values represent means and standard deviations. Pairwise comparisons among untreated

hDPSCs and hDPSCs exposed to the three calcium silicate cements indicate that all groups are significantly different from one another ($P < 0.05$). Representative images of Alizarin red S-stain mineral deposits prior to stain extraction and quantification in **C**. Untreated hDPSCs; **D**. MTA Angelus; **E**. Biodentine; **F**. TheraCal LC.

Figure 5.

TEM images of untreated hDPSCs before and after switching to the osteogenic differentiation medium. **A.** Low magnification of a stained section of virgin undifferentiated hDPSCs showing collagen fibrils deposited within the extracellular matrix. Bar = 500 nm. **B.** High magnification of stained, immature collagen fibrils secreted by undifferentiated hDPSCs. Bar = 100 nm. **C.** Low magnification of a stained section of hDPSCs after osteogenic differentiation with intracellular (arrowhead) and extracellular (pointer) mineral nodules. The minerals within the nodules were partially dislodged during sectioning. Bar = 1

µm. **D.** High magnification of unstained, extracellular mineralized nodules secreted by differentiated hDPSCs showing needle-shaped crystallites. Bar = 200 nm.

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Table I

Composition of the calcium silicate cements examined in the present study

Characteristics	MTA Angelus	Biodentine	TheraCal LC [26]
Liquid	water	water, CaCl ₂ , modified polycarboxylate (plasticizing agent)	
Powder:liquid ratio	1 spoon to 1 drop of distilled water	5 drops of CaCl ₂ -containing purified water to one capsule of powder	single syringe (no-mix)
Primary phases	3CaO.SiO ₂ 2CaO.SiO ₂ Bi ₂ O ₃ 3CaO.Al ₂ O ₃ CaSO ₄	3CaO.SiO ₂ CaCO ₃ ZrO ₂	Portland cement barium sulfate AeroSil 200 Bis-GMA, PEGDMA camphorquinone EDMAB MEHQ

Abbreviations: Bis-GMA - bisphenol A glycidyl methacrylate; EDMAB - ethyl-4-dimethyl aminobenzoate; MEHQ – hydroquinone mono methyl ether; PEGDMA - polyethylene glycol dimethacrylate