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Research Article

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Effect of Different Pulp Capping Materials on Proliferation and Odontogenic Differentiation of Human Dental Pulp Mesenchymal Stem Cells

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ABSTRACT

Aim: To examine the effects of Biodentine, TheraCal LC, nano-hydroxyapatite (NHA), and mineral trioxide aggregate (MTA) on proliferation and odontogenic differentiation of human dental pulp mesenchymal stem cells (hDP-MSCs). Materials and Method: Twelve-well plates containing hDP-MSCs were prepared and coated with each test material, and other 12 wells served as control. Cell viability was assessed. Odontogenic differentiation of hDP-MSCs was evaluated by analyzing mRNA expression of dentin sialophosphoprotein (DSPP) and collagen type I gene expression, alkaline phosphatase (ALP) activity, and calcium deposits on von Kossa staining. Results: Biodentine showed significantly higher cell viability (41.33%) than the control (35.00%), NHA (29.50%), MTA (29.00%), or TheraCal LC (20.83%) groups. There were significant differences in percentage expression of genes encoding DSPP and collagen type I (P<0.001); the mean rank of hDP-MSCDSPP and collagen type I gene expression was significantly higher in the Biodentine group. There were significant differences in ALP activity between the groups treated with Biodentine (43.58%), NHA (37.33%), MTA (32.25%), and TheraCal LC (25.83%). Biodentine showed the greatest amount of calcified mineralization and TheraCal LC showed the least. Conclusions: Biodentine had better effects on the proliferation and odontogenic differentiation of hDP-MSCs than TheraCal LC, NHA, or MTA.

Keywords: *Biodentine, Theracal LC, Nano-Hydroxyapatite, Mineral Trioxide Aggregate, Dental Pulp Capping, Dental*

INTRODUCTION

Direct pulp capping (DPC) has been the preferred treatment for an exposed vital dental pulp after physical or mechanical trauma to developing or mature teeth [1]. The success of a DPC procedure requires the successful removal of irritation and control of infection, and depends on the biocompatibility and bioactivity of the capping material [2]. Moreover, the dental pulp capping material used should be able to stimulate and modulate the healing process and regeneration of the dentine-pulp complex. Several materials have been commonly used for this purpose, including calcium hydroxide-based materials and mineral trioxide aggregate (MTA) [3, 4].

MTA has become the preferred dental pulp capping material because of its sealing properties, biocompatibility, and antimicrobial effects [5]. MTA does not cause inflammation of the pulp, and is conducive to the growth of dentine.

It promotes pulp healing and regeneration of dentin by promoting the release of cytokines and interleukins from white blood cells. However, the major drawbacks of MTA are its handling properties, long setting time, and discoloration of the remaining tooth structure. Calcium silicate-based materials, such as Biodentine and TheraCal LC, have been introduced in the dental field to overcome some of these drawbacks [6, 7].

Biodentine is a relatively new calcium silicate-based material that combines good mechanical properties with excellent biocompatibility and bioactive behavior [8]. Biodentine has been shown to induce proliferation and differentiation of dental pulp stem cells (DPSCs), and initiate early mineralization by releasing transforming growth factor-beta from pulpal cells to encourage pulp healing [9, 10]. In addition, Biodentine has a short setting time, high compressive strength, and excellent sealing ability, and is also easy to handle. Moreover, it has excellent antimicrobial properties because of its high pH of 12 [11, 12]. Therefore, Biodentine can be used safely and effectively as a pulp capping material.

TheraCal LC is a light-cured, resin-modified calcium silicate-filled liner designed to be used in direct and indirect pulp capping as a protective base/liner under other base materials. TheraCal LC gained attention when it was reported to be less cytotoxic than other resin-based light-cured liners, and to release more calcium ions than other dental cements [13, 14]. TheraCal LC was found to be conducive to formation of both apatite and a secondary dentine bridge [15]. The only known disadvantages of TheraCal LC are cosmetic. [16] compared the physicochemical properties of TheraCal LC, ProRoot MTA, and Dycal, and concluded that TheraCal had a higher calcium-releasing ability, and lower solubility than either ProRoot MTA or Dycal. TheraCal LC can be cured to a depth of 1.7 mm, so the risk of untimely dissolution maybe avoided. These properties offer major advantages in DPC treatment.

Nano-hydroxyapatite (NHA) was developed as a synthetic alternative to hydroxyapatite, a mineral that occurs naturally in human bones and teeth, and is currently widely used in dental implants and prosthetic hip joints [17]. NHA is superior to naturally occurring hydroxyapatite because it is more cyto compatible, in that the osteoblasts in tissues treated with NHA both adhere and proliferate better than those in tissues treated with conventional hydroxyapatite [18]. When used in DPC, NHA is effective in preserving an environment conducive to the formation of odontoblasts that promote formation of dentine bridges. A study by [19] showed that NHA was a viable alternative to calcium hydroxide, because both materials can be applied to the dental pulp without provoking an inflammatory response. The disadvantages of NHA include its tendency to cause inflammation and pulp necrosis. Furthermore, NHA is not able to prevent infection as effectively as MTA.

DPSCs play an important role in the healing process via odontoblast-like cell differentiation [20, 21]. DPSCs are clonogenic and capable of self-renewal and multi-lineage differentiation [22, 23]. The ability of a material to aid or induce this differentiation and maturation affects its bioactivity and biocompatibility. Tricalcium silicate-based cements have been shown to induce proliferation and differentiation of DPSCs [24, 25].

When used in the clinical setting, pulp capping materials are in direct contact with pulp tissue. The interaction of human dental pulp stem cells (hDPSCs) and pulp capping materials during a DPC procedure with exposed vital pulp affects the differentiation and proliferation of pulp cells [26].

It remains unclear which of the available DPC materials (Biodentine, TheraCal LC, NHC, and MTA) are the most conducive to the proliferation and odontogenic differentiation of dental mesenchymal stem cells (MSCs). Therefore, it is important to determine which of the capping materials in use today are best able to harness growth and proliferation of hDP-MSCs.

The aim of this study was to examine the effects of Biodentine, TheraCal LC, NHC, and MTA on the proliferation and odontogenic differentiation of hDP-MSCs. Two null hypotheses were tested: (1) there is no difference in the viability of undifferentiated hDP-MSCs after their exposure to Biodentine, TheraCal LC, NHC, or MTA; (2) Biodentine, TheraCal LC, NHC, and MTA are equally adept at augmenting the odontogenic potential of the hDP-MSCs after the cements are rendered to non-cytotoxic via elution of their cytotoxic components.

MATERIALS AND METHOD

Materials

The materials used in this study were hDP-MSCs and four types of dental pulp capping materials, i.e., Biodentine (Septodont, Saint-Maur-des-Fossés, France), TheraCal LC (Bisco Inc., Schaumburg, IL), NHA (Nano Tech, Giza, Egypt), and ProRootMTA (Dentsply Sirona Endodontics, Tulsa, OK).

Methods

The laboratory work was implemented in seven phases according to a protocol approved by the Research Ethics Committee of the Faculty of Dentistry, King Abdulaziz University (approval number061-16).

Isolation and Culture of hDP-MSCs

After obtaining informed consent, hDP-MSCs were isolated from the permanent teeth that had been freshly extracted from healthy patients for orthodontic reasons. The following steps were carried out in the manner described by [22]. The isolated pulp tissues of each tooth were washed three times in phosphate-buffered saline (PBS). The specimens were placed on a small plate and cut into 1-mm 3 pieces using sterile scissors, and surgical blades. The cut pieces were digested in a solution of type I collagenase 3 mg/mL containing dispase4 mg/mL (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C.The solution was then passed through a 70-mm cell strainer to obtain single-cell suspensions (BD Biosciences, Franklin Lakes, NJ), which were seeded in culture dishes with culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mmol/L of L-glutamine (Gibco), antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and an antimycotic agent (Fungizone[PSF], Life Technologies Corporation, Carlsbad, CA) at 0.25 μg/mL in a humidified atmosphere of 5% CO2at 37°C. The medium was changed every 3 days.

Subculturing of hDP-MSCs

Passaging was performed when the primary culture of adherent cells reached 80% confluence. The primary culture of adherent cells was detached by treatment with a sterile solution of trypsin/ethylenediaminetetra acetic acid for 5–10 min at 37°C in the incubator with intermittent shaking. The primary cell culture was then propagated and expanded in repeated cell cultures. The cells were subcultured every other week, and the culture medium was replaced every 3 days over a 21-day period. All these procedures were conducted under aseptic conditions in an air-filtered laminar flow safety cabinet using sterile instruments.

Characterization of Isolated hDP-MSCs

The isolated hDP-MSCs were characterized as MSCs on the basis of their fusiform-shaped morphology according to the guidelines proposed by the International Society for Cellular Therapy [27]. The immunophenotypic characteristics of the hDP-MSCs were investigated using flow cytometric analysis. The expression of hDP-MSC markers was quantified. Adherent cells (at the end of the third passage) were treated with trypsin solution and adjusted to 1×105cells.The cells were incubated in PBS and 2% FBS with 10 μL of conjugated monoclonal antibodies against CD90 (cat#SC-53456), CD73 (cat#SC-550256), and CD34 (cat# SC- 51540; Beckman Coulter, Brea, CA) for 45 min at 4°C in the dark. The same isotopic species served as the negative control. After 20 min of incubation, 2 mL of PBS containing 2% FBS solution were added to each tube containing treated monoclonal cells. The mixtures were then centrifuged for 5 min at 2500 rpm. Next, the supernatant was discarded, and the cells were re-suspended in 500 μL of PBS containing 2% FBS. The cell analysis was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter), and analyzed using CXP software version 2.2.

Preparation of Materials and Cell Seeding

TheraCal LC, Biodentine, NHA, and MTA specimens were prepared according to the manufacturers' instructions. To standardize the material volumes, a sterile Teflon mold with standard holes (5 mm diameter, 4 mm depth) with sterile Mylar cover sheets was used, into which the freshly prepared materials were introduced under sterile conditions (Figure 1). Biodentine and MTA were allowed to set in a humidified 5% CO2 incubator at 37°C for 48 hours. No preparation was required for TheraCal LC because it was supplied in pre-mixed syringes. TheraCal LC was applied in increments into the Teflon mold and polymerized with a light-emitting, diode-type light-curing unit for 20s per increment. Next, it was covered with a pre-sterilized Mylar sheet before light-curing to prevent formation of an oxygen inhibition layer. NHA was formed by the wet chemical reaction of calcium nitrate with ammonium hydroxide ((NH4)2HPO4). The grain size was controlled by changing the time and temperature used for precipitation of NHA, keeping the pH values between 10 and 12, and allowing the reaction to proceed at room temperature according to the manufacturers' instructions. The materials were then applied to the bottom of a pre-sterilized multi-well plate (Nunc; Thermo Fisher Scientific, Waltham, MA), with 12 wells used for each material. The set materials were subjected to sterilization under ultraviolet light for 3 hours. Third passage DP-MSCs were seeded onto the tested materials at 3000 cells/well and incubated in a 5% CO2 atmosphere at 37°C for 48 hours. Twelve wells that were incubated with hDP-MSCs in tissue culture medium that was not treated with the capping materials, served as a negative control group (Figure 2).

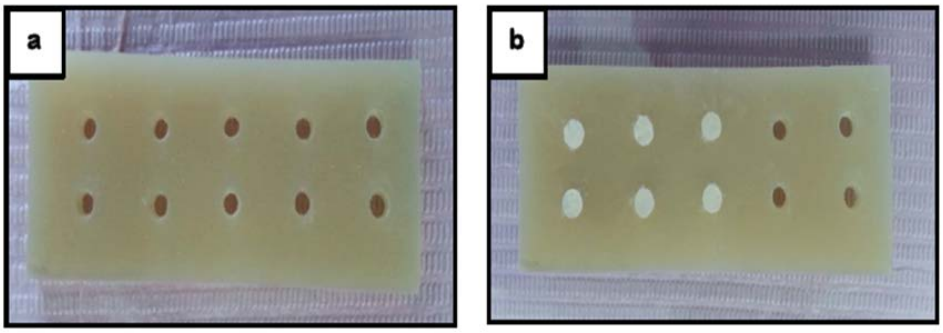

Figure 1. (a) Teflon mold with standard holes (5 × 4 mm). (b) Freshly prepared dental pulp capping materials with a standardized volume.

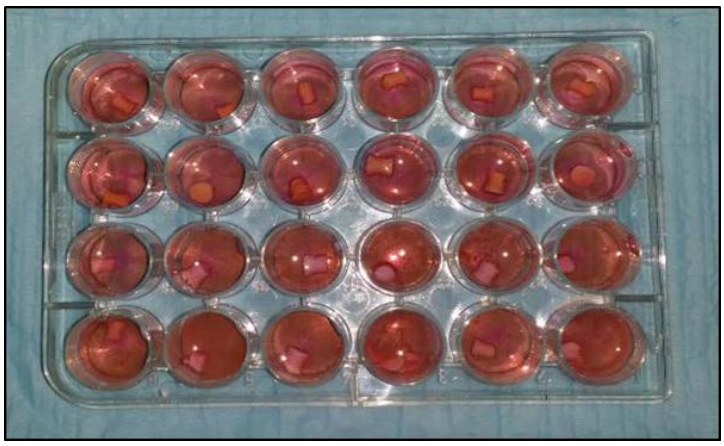

Figure 2. Seeding of human dental pulp mesenchymal stem cellson pulp capping materials in a 24-well plate at 3000 cells/well.

Evaluation of Cell Viability

After the hDP-MSCs had been incubated with the dental materials, cell proliferation was assessed using a TACS® MTT Cell Proliferation Assay kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. The MTT test is a standard colorimetric assay that evaluates cell viability by detecting the activity of mitochondrial enzymes (dehydrogenases) present in metabolically active cells. These enzymes reduce MTT to formazan, giving a purple-colored substance. Cells from the different groups were re-suspended in PBS at 1×106 per mL and diluted to1×104cells/mL. One hundred microliters of the dilutions were distributed per well. The cells were incubated at 37°C in 5% CO2for 24 h. Ten microliters of MTT Reagent (cat#4890-25-01; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were added to each well, and then returned to the cell culture incubator for 2–4 h until a purple precipitate was clearly visible under the microscope; 100 µL of Detergent Reagent (cat#4890-25-02; Sigma-Aldrich Chemie GmbH) was then added to all the wells. The plate was covered and incubated in the dark overnight at room temperature. The plate cover was then removed, and the absorbance of the converted dye in each well was read at a wavelength of 570 nm by a microplate reader (Bio-Rad Laboratories, Hercules, CA). The results were documented as percentage cell viability in relation to the untreated hDP-MSCs (negative control group) set at 100%.

Odontogenic Differentiation of hDP-MSCs

The confluent monolayers were incubated with odontogenic induction medium containing Dulbecco's Modification of Eagle's Medium (Cellgro, Manassas, VA) supplemented with 10% FBS, PSF, 2 mmol/L L-glutamine, 50 mg/mLascorbic acid (Sigma), and 2 mmol/L 2-glycerolphosphate(Sigma) for 21 days, with a medium change every other day.

Assessment of Effect of Capping Materials on Odontogenic Differentiation of hDP-MSCs

On day 21, the odontogenic differentiation of hDP-MSCs was assessed by detection of mRNA expression of odontoblastic markers, including dentin sialophosphoprotein (DSPP), collagen type I, intracellular alkaline phosphatase (ALP) activity, and extracellular calcified nodule structures.

Detection of DSPP and Collagen Type I Gene Expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the relative expression of odontogenic markers, such as DSPP and collagen type I, in the tested capping materials.

Extraction of RNA

Total RNA was isolated from the collected cultured cells using a cell/tissue extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA lysis buffer (175 µL) was added to the washed cells, and the pellet was dispersed and mixed by pipetting. Next, 350 µL of RNA dilution buffer was added to 175 µL of lysate and mixed. The mixture was placed in a water bath at 70°C for 3 min, and then centrifuged at 12,000–14,000 rpm for 10 min at 20°C–25°C. Two hundred microliters of 95% ethanol were added to the cleared lysate, and mixed by pipetting 3–4 times. This mixture was transferred to the spin column assembly, and centrifuged at 12,000–14,000 rpm for 1 min. RNA wash solution (600 µL) containing ethanol was added and centrifuged at 12,000–14,000 rpm for 1 min. The collection tube was emptied, and 250 µL of RNA wash solution containing ethanol was added and centrifuged at a high speed for 2 min. The spin basket was transferred from the collection tube to the elution tube, and 100 µL of nuclease-free water was added to the membrane. Finally, the sample was centrifuged at 12,000–14,000 rpm for 1 min. The spin basket was discarded, and the elution tube containing the purified RNA was stored at -70°C.

cDNA Synthesis

Total RNA was used for synthesis of cDNA using a high capacity cDNA reverse transcription kit (#K1621, Ferment as, Vilnius, Lithuania). First, 3 µL of random primers was added to 10 µL of RNA, and denatured for 5 min at 65°C in the thermal cycler. The cDNA master mix was prepared according to the kit instructions, and added to the test samples (19 µL each). This mixture was added to the 31-µL RNA-primer mixture resulting in 50 µL of cDNA. The final mixture was incubated in the programmed rmo-cycler for 1 h at 37°C followed by inactivation of enzymes at 95°C for 10 min, and finally cooling at 4°C. The RNA was converted into cDNA. The converted cDNA was stored at -20°C.

Quantitative Real-time Polymerase Chain Reaction

TheqRT-PCR amplification and analysis were performed using Step One™ version 3.1 software (Applied Biosystems, Foster City, CA). Relative expression of DSPP and collagen type I mRNAs was analyzed using the SYBR Green reverse transcriptase PCR method. Table 1 shows the sequence of the PCR primers. The q-PCR reaction master mix component was prepared by adding the reagents (1 µL of forward primer, 1 µL of reverse primer, 12.5 µL of Syber Green mix, 5µLof cDNA template, 5.5 µL of RNAse-free water) in a final volume of 20 µL for each sample. The GAPDH gene was used as the reference housekeeping gene for normalization of the data. The well plate was loaded in the thermo cycler and run in regular cycles. Each cycle consisted of hold at 50°C for 2 min, one cycle of denaturation 95°C for 15 min, annealing at 60°C for 1min, and extension at 72°C for 40 1-min cycles. Final relative quantitation was calculated according to the Applied Biosystems software analysis using the delta CT (cycle threshold) method.

Table 1. Sequence of polymerase chain reaction primers

Gene	Primer Sequence
GAPDH	F 5'-TAT CGTGGAAGGACTCA-3' R 5'-GCAGGGATGATGTTCTGGA-3'
DSPP	F 5'-GACATCGCCTACCAGCTCAT-3′ R 5'-TCACGTTGTTCCTGTTCAGC-3'
Collagen type I	F 5'-BCAGTCGATTCACCTACAGCACG-3' R 5'-GGGATGGAGGGAGTTTACACG-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DSPP, dentin sialophosphoprotein

Intracellular ALP Enzyme Activity

After 21 days, 25 µL of hDP-MSC lys ate was mixed with 75 µL. An ALP commercial reagent (Randox Laboratories, Crumlin, UK) was added to the well plates followed by incubation for 1 h. ALP activity was examined by measuring absorbance at 15-min intervals using a plate reader (Bio-Tek Instruments, Winooski, VT).

Detection of Extracellular Mineralization

A Von Kossa staining kit (Bio-optica, Milano, Italy) was used for histologic visualization of extracellular calcium deposits in differentiated hDP-MSCs in each group. After 21 days of incubation with odontogenic differentiation medium, the cells were fixed with 2% paraformaldehyde at 4°C for 30 min. Silver nitrate solution (5%) was added to cell sections and incubated for 1 hour with exposure to ultraviolet light. The sections were rinsed in distilled water 3 times. Each cell section was covered with 5% sodium thiosulphate, and incubated for 5 min, then rinsed for 2 min in running tap water, followed by 2 changes of distilled water. The cell sections were then counter-stained with Nuclear Fast Red and incubated for 5 min, followed by rinsing for 2 min in running tap water, and 2 changes of distilled water. Cell sections were dehydrated in three changes of fresh absolute alcohol, after which they were cleared, and a coverslip was added. Images of the specimens were taken for qualitative evaluation of calcified mineralization, and observed using a phase-contrast light microscope (Nikon TS100, Tokyo, Japan).

Statistical Analysis

The data analysis was performed using SPSS version 20.0 (IBM Corp., Armonk, NY). The data were ranked and analyzed using a rank-based nonparametric (Kruskal-Wallis) test. Post-hoc pairwise comparisons using the Dunn-Bonferroni approach were produced for dependent variables for which the Kruskal-Wallis test was significant. A P-value <0.05 was considered statistically significant.

RESULTS

Characterization of Isolated hDP-MSCs

Cell Morphology Characteristics

Cells were identified as MSCs based on their morphology. Isolated hDP-MSCs had a spindle (fusiform)-shaped morphology after the third passage.

Immunophenotypic Characteristics

Flow cytometric analysis of the immunophenotypic characteristics of the hDP-MSCs revealed that the isolated cells were positive for cell surface antigens CD90 and CD73, and negative for CD34, which confirmed the mesenchymal origin of the pulp stem cells (Figure3).

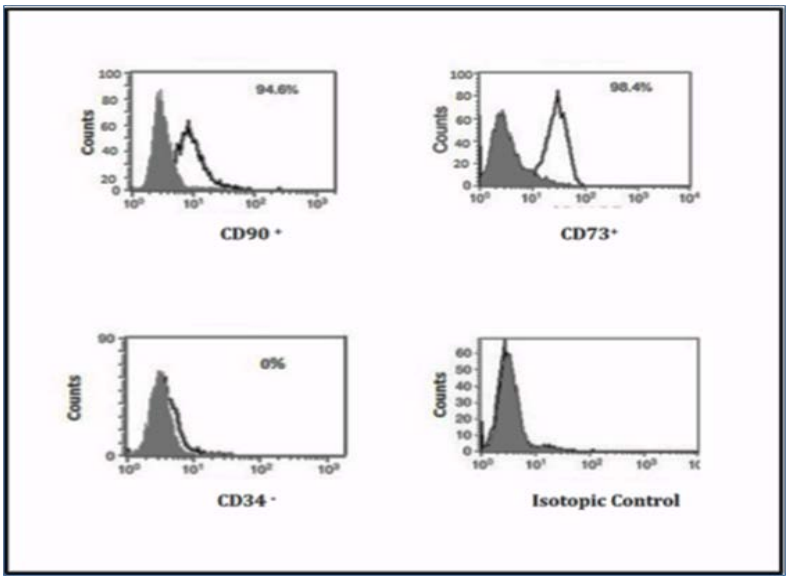

Figure 3. Immunophenotypic characteristics of human dental pulp mesenchymal stem cells.

Effect of Tested Dental Capping Materials on Cell Viability

After 48 hours of direct contact between the hDP-MSCs and the tested capping materials, the viability of the hDP-MSCs was evaluated using the MTT cell proliferation assay. The analysis showed that the number of attached cells varied in the wells containing the different capping materials. The Kruskal-Wallis test revealed statistically significant differences in cell viability between the groups ($\chi 2$ (4) = 9.950, P = 0.041). The Biodentine group showed the highest mean rank percentage of viable cells (41.33), followed by the negative control (35.00), NHA (29.50), ProRoot MTA (29.00), and TheraCal LC (20.83) groups (Table 2). Pairwise comparison revealed a statistically

significant difference in viability of the hDP-MSCs between the TheraCal LC and Biodentine groups (P= 0.037; Table 3, Figure 4).

Table 2. Comparison of the effect of the different capping materials on viability of human dental pulp mesenchymal stem cells

Dental Capping Material	n	Mean Rank, %	Chi-square	P-value
TheraCal LC	12	20.83	9.950	0.041*
Biodentine	12	41.33		
NHA	12	29.50		
ProRoot MTA	12	29.00		
Negative Control	12	35.00		

+Kruskal-Wallis test*Significant at P <0.05.

Table 3. Post-hoc pairwise comparison of the effect of the different capping materials on viability of human dental pulp mesenchymal stem cells

Dental Capping Materials	P-value
TheraCal LC – Biodentine	0.037*
TheraCal LC – NHA	1.000
TheraCal LC – MTA	1.000
TheraCal LC – Negative Control	0.443
Biodentine – NHA	0.374
Biodentine – MTA	0.835
Biodentine – Negative Control	1.000

+Dunn-Bonferroni test. *Significant at P <0.05.

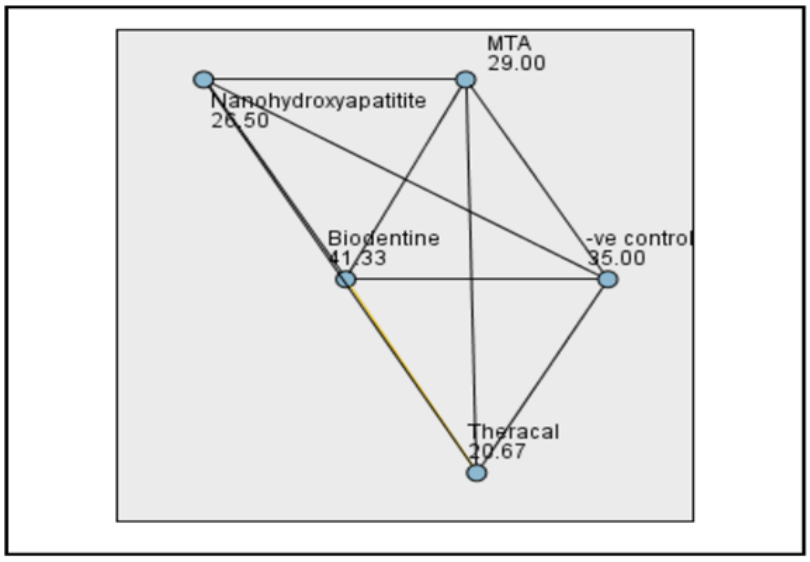

Figure 4. Pairwise comparisons of the viability of human dental pulp mesenchymal stem cells between the tested capping materials using the Dunn-Bonferroni test. Each node shows the average rank of the tested material sample.

Expression of Odontogenic Target Genes

The effects of the cements on the mineralization potential of hDP-MSCs were investigated at the mRNA level, enzymatic level (ALP only), and the extracellular matrix mineralization level. After incubation of hDP-MSCs with the tested capping materials in odontogenic differentiation medium for 21 days, qRT-PCR for DSPP, collagen type 1 gene expression, and ALP enzyme production produced the following results:

Relative Expression of the DSPP GeneinhDP-MSCs

There were statistically significant differences in relative levels of DSPP gene expression by the hDP-MSCs between the tested materials ($\chi 2(4) = 39.091$, P<0.001, Table 4). The mean rank of DSPP gene expression by hDP-MSCs was significantly higher in the Biodentine group (44.50), followed by the NHA and MTA groups (42.75 and 36.75; respectively). The TheraCal LC group showed the lowest gene expression (20.50, Table 4). Post-hoc pairwise

comparisons using the Dunn-Bonferroni approach revealed a statistically significant difference in the relative level of DSPP gene expression by hDP-MSCs between the TheraCal LC, Biodentine, and NHA groups. Significant differences were also found between the negative control, Biodentine, NHA, and MTA groups but not between the TheraCal LC and negative control groups (Table 5, Figure 5).

Table 4. Comparison of the effects of the different capping materials on relative levels of DSPP gene expression in human dental pulp mesenchymal stem cells

Dental Capping Materials	n	Mean Rank,%	Chi-square	P-value
TheraCal LC	12	20.50	39.091	<0.001*
Biodentine	12	44.50		
NHA	12	42.75		
ProRoot MTA	12	36.75		
Negative Control	12	8.00		

+Kruskal-Wallis test. *Significant at P <0.05.

Table 5. Post-hoc pairwise comparison of the effect of the different capping materials on relative levels of DSPP gene expression in human dental pulp mesenchymal stem cells

Dental Capping Materials	P-value
TheraCal LC – Biodentine	0.008*
TheraCal LC – NHA	0.018*
TheraCal LC – MTA	0.225
TheraCal LC – Negative Control	0.792
Biodentine – NHA	1.000
Biodentine– MTA	1.000
Biodentine – Negative Control	<0.001*
NHA – MTA	1.000
NHA – Negative Control	<0.001*
MTA – Negative Control	<0.001*

+Dunn-Bonferroni test. *Significant at P <0.05.

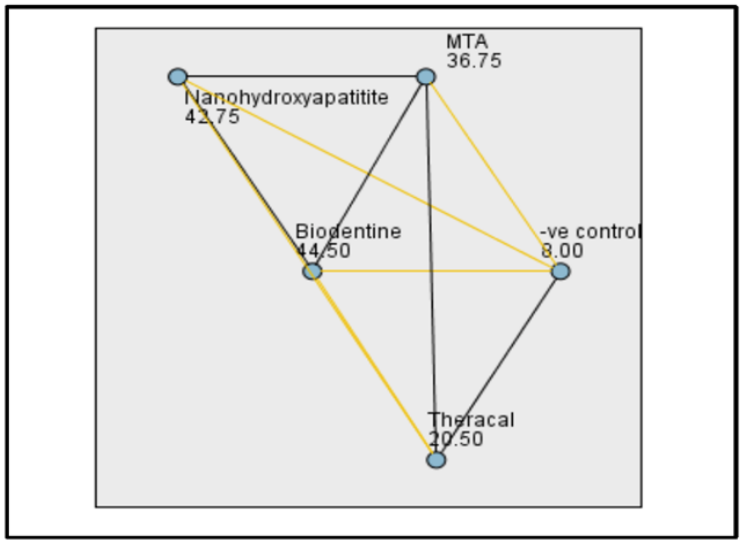

Figure 5. Pairwise comparisons of DSPP gene expression in human dental pulp mesenchymal stem cells between the tested capping materials using the Dunn-Bonferroni test. Each node shows the average rank of the tested material sample.

Relative Levels of Collagen Type I Gene Expression in hDP-MSCs

Collagen type I gene expression by hDP-MSCs after direct contact with the tested capping materials was evaluated using the Kruskal-Wallis test. There were statistically significant differences in hDP-MSC expression of the

collagen type I gene between the tested materials ($\chi 2(4) = 28.049$, P<0.001). The mean rank of collagen type I gene expression in the hDP-MSCs was significantly higher in the Biodentine group (39.67), followed by the NHA and TheraCal LC groups (38.83 and 33.50, respectively). The MTA group showed the lowest gene expression (33.25, Table 6). Post-hoc pairwise comparisons using the Dunn-Bonferroni approach revealed a statistically significant difference in relative expression of the collagen type I gene by hDP-MSCs between the negative control group and the respective TheraCal LC, Biodentine, NHA, and MTA groups (Table 7, Figure 6).

Table 6. Comparison of the effect of the different capping materials on relative levels of collagen type I gene expression in human dental pulp mesenchymal stem cells

Dental Capping Material	n	Mean Rank, %	Chi-square	P-value
TheraCal LC	12	33.50	28.049	<0.001*
Biodentine	12	39.67		
NHA	12	38.83		
ProRoot MTA	12	33.25		
Negative Control	12	7.25		

+Kruskal-Wallis test. *Significant at <0.05 level.

Table 7. Post-hoc pairwise comparison of the effect of the different capping materials on relative levels of collagen type I gene expression in human dental pulp mesenchymal stem cells

Dental Capping Materials	P-value
TheraCal LC – Biodentine	1.000
TheraCal LC – NHA	1.000
TheraCal LC – MTA	1.000
TheraCal LC – Negative Control	0.002*
Biodentine – NHA	1.000
Biodentine– MTA	1.000
Biodentine – Negative Control	<0.001*
NHA –MTA	1.000
NHA – Negative Control	<0.001*
MTA – Negative Control	0.003*

+Dunn-Bonferroni test. *Significant at <0.05 level.

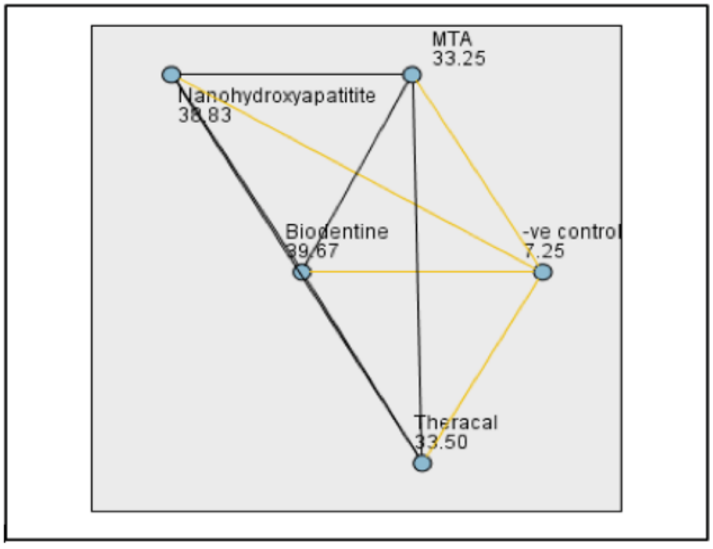

Figure 6. Pairwise comparison of collagen type I gene expression in human dental pulp mesenchymal stem cells between the tested capping materials using Dunn-Bonferroni test. Each node shows the sample average rank of the tested material.

Level of ALP Activity in hDP-MSCs

There was a statistically significant difference in the cell ALP activity level between the tested materials ($\chi 2$ (4) = 20.979, P<0.001). The mean rank of ALP activity in the hDP-MSCs was significantly higher in the Biodentine

group (43.58), followed by the NHA and MTA groups (37.33 and 32.25; respectively). TheTheraCal LC group showed the lowest ALP activity (25.83; Table 8). Post-hoc pairwise comparisons using the Dunn-Bonferroni approach revealed a statistically significant difference in ALP activity between the negative control group, and the respective Biodentine and NHA groups (Table 9, Figure 7).

Table 8. Comparison of the effect of the different capping materials on alkaline phosphatase activity in human dental pulp mesenchymal stem cells

Dental Capping Material	n	Mean Rank, %	Chi-square	P-value
TheraCal LC	12	25.83	20.979	<0.001*
Biodentine	12	43.58		
NHA	12	37.33		
ProRoot MTA	12	32.25		
Negative Control	12	13.50		

+Kruskal-Wallis test, *Significant at <0.05 level.

Table 9. Post-hoc pairwise comparison of the effect of the different capping materials on alkaline phosphatase activity in human dental pulp mesenchymal stem cells

Dental Capping Materials	P-value
TheraCal LC – Biodentine	0.127
TheraCal LC – NHA	1.000
TheraCal LC – MTA	1.000
TheraCal LC – Negative Control	0.832
Biodentine – NHA	1.000
Biodentine–MTA	1.000
Biodentine – Negative Control	<0.001*
NHA –MTA	1.000
NHA –Negative Control	0.008*
MTA – Negative Control	0.085

+Dunn-Bonferroni test. *Significant at <0.05 level.

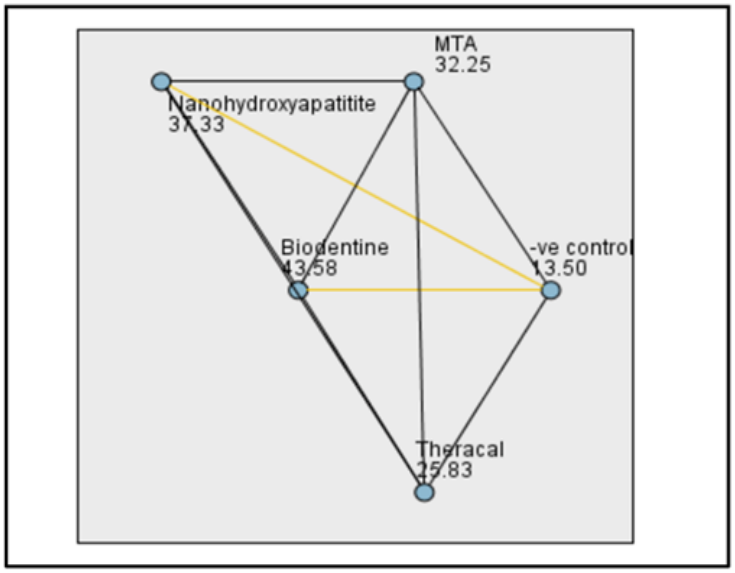

Figure 7. Pairwise comparison of alkaline phosphatase activity in human dental pulp mesenchymal stem cells between the tested capping materials using Dunn-Bonferroni test. Each node shows the sample average rank of the tested material.

Extracellular Remineralization of hDP-MSCs Coated with Different Capping Materials

A phase-contrast light microscope was used to observe the von Kossa staining of extracellular calcium deposits in the test groups after odontogenic differentiation of hDP-MSCs. The calcified mineralization effect was the highest in the Biodentine group, and the lowest in the TheraCal LC group (Figure 8).

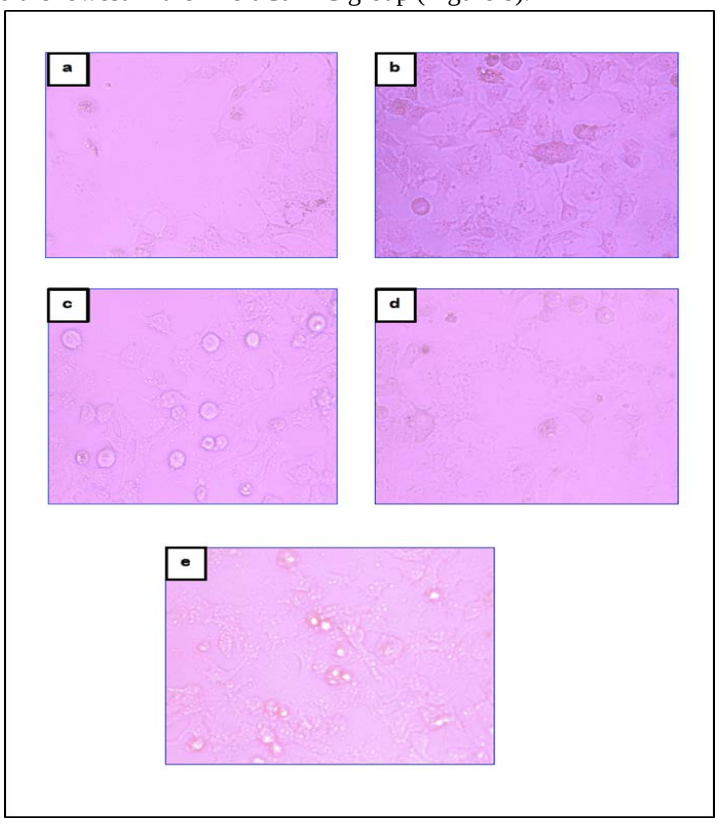

Figure 8. Mineralization effect of the tested dental capping materials using von Kossa staining. (a) TheraCal LC. (b) Biodentine. (c) NHA. (d) MTA. (e) Negative control. Images were captured at 10× magnification using an inverted electronic microscope.

DISCUSSION

The current study was performed in vitro, because dental pulp stem cells are easy to handle, and they grow rapidly, which increases the reproducibility of the results, and the need for animal testing can be avoided. The cell line used has been recommended by the International Organization for Standardization to assess biological responses to dental materials [28]. hDP-MSCs were also used because of their many clinical applications, and the feasibility of placing different dental capping materials in direct contact with these cells, such as during DPC.

Dental pulp stem cells can differentiate into odontoblast-like cells, which play an important role in the healing process and formation of reparative dentin [29]. Hence, the bioactivity and biocompatibility of the capping materials depend on their ability to induce proliferation and odontogenic differentiation of hDP-MSCs. Therefore, the aim of this in vitro study was to evaluate and compare the effects of commercially available pulp-capping materials (Biodentine, TheraCal LC, NHA, and MTA) on the proliferation and odontogenic differentiation of hDP-MSCs following the direct contact between the tested materials and pulp cells. All the materials selected for the present study had passed appropriate safety tests.

The effects of dental pulp-capping materials on hDP-MSCs were assessed using cell viability and proliferation tests and detecting the expression of odontogenic differentiation gene markers encoding DSPP and collagen type I, the intracellular ALP activity level, and extracellular calcified mineralization.

Dental pulp stem cells were withdrawn from extracted human teeth and maintained in tissue culture medium. The hDP-MSCs was confirmed to have a spindle-shaped morphology. Flow cytometric analysis established that membrane protein markers (CD90+, CD73+, and CD34-) were expressed, confirming the mesenchymal origin of the isolated cells [30]. After the hDP-MSCs were characterized, they were cultured directly on prepared Biodentine, TheraCal LC, NHA, and ProRoot MTA, with untreated hDP-MSCs cultured as a negative control group.

After the hDP-MSCs had been incubated with the test pulp capping materials for 48 hours, the effect of the test materials on cell viability was assessed using the MTT cell proliferation assay. This assay was based on the ability of mitochondrial (dehydrogenase) enzymes in metabolically active cells to reduce tetrazolium to formazan, which resulted in a blue/purple color. This method was straightforward; the relevant enzymes were inactivated shortly after the cell death, so the formation of a highly colored formazan dye was indicative of a metabolically active cell population. The assay could also reveal the number of live cells [31].

The Biodentine group showed a significantly high percentage of viable cells, followed by the negative control, NHA, MTA, and TheraCal LC groups. This result was in agreement with the findings of two in vitro studies by [14, 32] who compared the biocompatibility of seven different types of pulp-capping materials (Dycal, Calcicur, Calcimol LC, TheraCal LC, ProRoot MTA, MTA-Angelus, and Biodentine) on murine odontoblasts. These studies found that Biodentine and MTA-based products had low cytotoxicity. In contrast, TheraCalLC decreased the percentage of viable cells in 72 hours. The findings of this study were also supported by two further studies that showed Biodentine and ProRoot MTA to have similar biocompatibility [33, 34].

Based on the results for the cell viability evaluation, the first null hypothesis that there was no difference in the viability of undifferentiated hDP-MSCs after their exposure to Biodentine, TheraCal LC, NHC, orProRoot MTA was rejected.

Differentiation of hDPSCsinto odontoblasts is essential for the success of a pulp capping procedure. Odontoblasts are capable of depositing secondary/reparative dentine, secreting and mineralizing the fibrillar extracellular matrix of dentine, and expressing proteins such as DSPP and collagen type I. These proteins play a regulatory role during the mineralization of reparative dentine, so are considered to be specific markers for the odontoblast phenotype. Odon oblasts also had ALP activity [35, 36].

In this study, to examine the effects of Biodentine, TheraCal LC, NHC, and ProRoot MTA on the odontogenic differentiation of hDP-MSCs; the relative expression of target gene markers, DSPP and collagen type I, in the test groups were detected by a reverse-transcription reaction to the complementary DNA template, and then quantified by qRT-PCR [37]. Statistically significant differences were detected between the tested groups. Biodentine was the most efficient in increasing the relative expression of mRNA for the odontogenic markers (DSPP and collagen type I), followed by NHA, MTA, and TheraCal LC. The relative expression of the mRNA for these markers in Biodentine, NHA, and MTA groups differed significantly from that in the negative control group; in contrast, there was no significant difference in this respect between the negative control group and the TheraCal LC group. This demonstrated that TheraCal LC was the least efficient in increasing the relative expression of mRNA for the odontogenic markers, and in subsequent odontogenic differentiation.

Intracellular ALP activity is another marker of odontogenic differentiation of hDP-MSCs following direct contact with tested capping materials. In the present study, all the test groups stained positive for ALP, with maximal staining for the Biodentine group, followed by the NHA group. Although, there were significant differences in ALP activity between the test groups, only the ALP activity levels of hDP-MSCs in the Biodentine and NHA groups were significantly higher than the level in the untreated negative control group.

The current study's findings were consistent with those of [38] who showed that the cell viability was the highest for Biodentine followed by MTA. Viability on glass ionomer cement and dentin disks was significantly lower. ALP activity was lower in cells in contact with Biodentine compared to those in contact with MTA, but the expression patterns for the marker genes were similar.

Extracellular matrix mineralization has been another indicator of odontogenic differentiation of hDP-MSCs. Following von Kossa staining, the extracellular calcium deposits in the tested groups were observed by phase-contrast light microscopy. The data obtained indicated that culturing of hDP-MSCs cells on the tested capping materials enhances their differentiation and mineralization ability. Of these materials, Biodentine showed the greatest induction, and TheraCal LC showed the least.

According to the results of this study, the second null hypothesis that Biodentine, TheraCal LC, NHC, andProRoot MTA had an equal ability to enhance the induction of the odontogenic potential of hDP-MSCs after the direct contact was also rejected.

The results of this study were in agreement with those of [39] and [9] who showed an ex vivo whole human tooth culture models in that the application of Biodentine directly onto the pulp induced the early formation of reparative dentine. Furthermore, [10] investigated the response of hDPSCs to the direct contact with Biodentine, and showed that Biodentine is a bioactive and biocompatible capping material.

[40] compared the effects of Biodentine and TheraCal LC on the viability and osteogenic differentiation of hDPSCs with those of MTA-Angelus. TheraCal LC was found to be the most cytotoxic of the three calcium silicate cements tested. Moreover, Biodentine and MTA-Angelus produced better osteogenic gene expression and ALP enzyme activity than TheraCalLC. The authors stated that although, MTA and Biodentine had better results, the three freshly set tested capping materials showed some degrees of initial cytotoxicity, which might be explained by their high pH values. Moreover, the pH of these pulp capping materials is time-dependent [41], so, these results might reflect the fact that the cell viability was examined over a short period of time. TheraCal LC consists of a neutral or mild acidic hydrophilic resin monomer component, and a hydrophobic resin monomer component. Incompletely polymerized resin monomers may leach and accumulate to an overall toxic level with an irreversible effect on the detoxifying glutathione metabolism and other defense mechanisms of the cell, resulting in apoptosis [42]. Cells may adversely be affected by methacrylate resin components by alteration of the lipid layers in the cell membrane, leading to an increase in its permeability. Hence, the direct contact of Theracal LC with the pulp cells result in decreased cell metabolism and protein expression. [40] provided a foundation for further investigations using in vivo animal models to validate the potentially adverse biological effects of TheraCal LC on hDPSCs.

Biodentine did not show cytotoxicity or genotoxicity. Biodentine has been reported to be a suitable alternative to ProRoot MTA for DPC because of its short setting time, [43] which was supported by the findings of this study that Biodentine has high odontogenic ability with low toxicity in comparison with TheraCal LC, NHC, and ProRoot MTA.

CONCLUSIONS

In this study, Biodentine had better effects on the proliferation and odontogenic differentiation of hDP-MSCs than TheraCal LC, NHA, or MTA. The findings of this study supported the use of Biodentine as a pulp capping material to increase therapeutic efficiency. Further in vitro studies are needed to examine the viability of these capping materials over a longer period of time.

Conflict of Interest

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

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