Differential Responses of Human Dental Pulp Stromal Cells to Bioceramic Materials: A Comparative In Vitro Study

Mohamed RW Ali¹, Manal Mustafa², Asgeir Bårdsen³, Maryam A Gharaei⁴, Inge Fristad⁵, Athanasia Bletsa⁶

ABSTRACT

Aim: The aim of this study was to compare the effects of white MTA-Angelus (wMTA), Biodentine® (Biodentine) and TotalFill® BC Root Repair Material™ putty (TotalFill) on human dental pulp stromal cells (hDPSCs) in vitro.

Materials and methods: hDPSCs were isolated from third molars of healthy young adults. Material elutes at different concentrations were prepared. Cells were exposed to the eluates for 1, 3, and 7 days. Cell proliferation was evaluated using MTT, vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor-1 (FGF-1), interleukin 6 (IL6), tumor necrosis factor alpha (TNFα), and interleukin-1-beta (IL1β) was determined by reverse transcription-polymerase chain reaction (RT-PCR). VEGF-A protein levels and ALP activity were quantified in the culture supernatant. Data were analyzed by two-way analysis of variance (ANOVA).

Results: hDPSC proliferation was decreased in a dose-related manner for all materials on day 3. The same effect was observed with wMTA and TotalFill on day 7. RT-PCR showed that Biodentine increased the expression of the osteogenic markers ALP, OPG, and OC. TotalFill decreased the ALP expression and activity, enhanced the production of angiogenic VEGF-A, and downregulated the inflammatory IL6 on day 7.

Conclusion: Although the tested materials are used interchangeably in vital pulp therapy, the findings showed varied hDPSC responses. Biodentine did not affect cell proliferation and increased the expression of osteo-/odontogenic markers compared to wMTA and TotalFill, whereas TotalFill decreased cell proliferation and exhibited enhanced angiogenic and anti-inflammatory effects over time.

Clinical significance: The clinical significance of the results needs further investigation in an attempt to provide recommendations on the selection of bioceramic pulp capping material under different scenarios of pulpal pathosis.

Keywords: Angiogenesis, Dental pulp calcification, Endodontic inflammation, Gene expression, Mineral trioxide aggregate, Pulpitis, Pulpotomy, Stem cells, Tricalcium silicates.

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INTRODUCTION

Vital pulp treatment (VPT) aims at maintaining the health of all or part of the pulp, including procedures from indirect pulp capping to full pulpotomy. As materials used in VPT come in direct or indirect contact with pulp tissue, their ability to set and to seal in a wet environment, as well as their anti-inflammatory and pulp healing potential are desired properties.¹⁻³ Tricalcium silicate-based cements (TSCs), also called hydraulic calcium silicate cements, have been a great advance in VPT. They are materials that can create a microenvironment that promotes dentin tissue formation by stimulating pulpal progenitor cells.⁴⁻⁶

The original mineral trioxide aggregate (MTA) still represents the golden standard in comparative studies between TSCs. Yet discoloration and handling properties have led to the development of new chemical formulations.⁷⁻⁸ Currently, several TSCs are interchangeably used in clinical practice, and operators' preferences rather than specific biological properties dictate their use. Biodentine® (Biodentine; Septodont; Saint-Maur-des-Fosses, France), and EndoSequence Root Repair Material (ERRM)/TotalFill® BC Root Repair Material™ putty (TotalFill; FKG Dentaire, La-Chaux-de-Fonds, Switzerland for the European market) are examples of bioceramic materials that do not cause discoloration, are easy to handle, and widely used in VPT.⁵⁻⁶ The composition and handling properties of these three TSCs according to their manufacturers are summarized in Table 1. Despite their biocompatibility, there are a few reports comparing pulpal cell responses. This is particularly true for TotalFill. When Biodentine was compared to MTA, it not only induced increased thickness of reparative dentin but also ectopic formation of osteodentin in the pulp.¹⁰⁻¹¹ The incidence of pulp canal obliteration after pulpotomy varies in clinical studies; Biodentine was associated with significantly

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more intracanal hard tissue formation compared to ProRoot MTA.12 Primary molar pulpotomy with Biodentine led to pulp canal obliteration in more than half of the teeth (54.1%) at 1-year follow-up.13 On the contrary, pulpotomy using MTA showed less frequent uncontrolled hard tissue deposition inside the root canal,14,15 thus distinguishing between the TSC materials in their hard tissue inducing abilities.

A recent comparison of white MTA-Angelus® (wMTA; Angelus, Londrina, PR, Brazil), Biodentine® (Biodentine; Septodont, Saint-Maur-des-Fosses, France), and TotalFill® BC Root Repair MaterialTM putty (TotalFill; FKG Dentaire, La-Chaux-de-Fonds, Switzerland) revealed that the osteogenic and angiogenic responses of human bone marrow stem cells (hBMSCs) varied between these materials. Biodentine induced osteogenic whereas TotalFill induced angiogenic gene expression.16 In an attempt to provide evidence for clinical recommendations in VPT, we tested the same materials on human dental pulp stromal cells (hDPSCs). The aim of this study was to assess and compare the effects of wMTA, Biodentine and TotalFill on hDPSC metabolism and expression of osteogenic, angiogenic, and inflammatory markers in vitro. The null hypothesis was that there were no differences in the responses of hDPSCs to the tested materials.

### Materials and Methods

Flowchart 1 outlines the experimental set-up.

#### Material Eluates

MTA and Biodentine were mixed as indicated by the instructions-to-use whereas TotalFill was ready-to-use. Material eluates were prepared following the ISO Standards 10993-5 as described previously.16

Briefly, a standard set material volume was placed in Eppendorf tubes together with 1 mL of serum-free Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, Waltham, MA, USA) with 1% penicillin (HyClone, GE Healthcare, Logan, UT, USA) and then stored in an incubator at 37 °C with 6% CO₂ and 100% humidity (Heracell; ThermoFisher Scientific, Waltham, MA, USA) for 24 hours. The eluate was filtered with Acrodisc® syringe filters (pore size 0.2 µm diameter; Pall Life Sciences, New York, NY, USA) and the stock was stored at -80 °C until further use. Serial dilutions of the stock eluate (1:2, 1:4, 1:8, and 1:16) were made with DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin (HyClone) (Flowchart 1).
Cell Isolation and Culture
The hDPSCs used in this study had previously been isolated and characterized. Briefly, impacted third molars from healthy young adults, scheduled for routine surgical extractions at the Department of Clinical Dentistry, University of Bergen, Norway, were collected. The patients had signed an informed consent, and the protocol was approved by the Ethical Research Committee at the University of Bergen, Norway (2009/610REK Vest). The pulp tissue was subjected to enzymatic dissociation for isolation of the hDPSCs. The cells were then cultured in DMEM supplemented with 10% FBS (Sigma-Aldrich), 4 mM L-glutamine (ThermoFisher Scientific), 100 U/mL penicillin (HyClone), and 100 µg/mL streptomycin (HyClone) with media replenished twice a week.

Cells were trypsinized after reaching a confluence of approximately 80%, and a trypan blue assay was used to assess cell numbers and viability (Countess; Invitrogen, Carlsbad, CA, USA). All experiments were performed after allowing the hDPSCs to attach for 1 days. The cells were then exposed to the TSC eluates for 1, 3, and 7 days whereas hDPSCs exposed to growth medium only served as controls in all experiments (Flowchart 1). Cell morphology was evaluated by light microscopy (Nikon Eclipse E80i; Nikon Instruments, Tokyo, Japan).

Three different donors (mean age: 22 years) were used. All experiments were performed on hDPSCs at passages 3–7.

Cell Proliferation
The hDPSCs were seeded on 96-well plates at a density of $0.1 \times 10^5$ cells per well and exposed to serial dilutions of the TSC eluates (1:2, 1:4, 1:8, 1:16) for 1, 3, and 7 days as described above whereas controls were not exposed to TSC eluates. We used the 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide assay (Sigma-Aldrich) to assess cell metabolism, and absorbance was recorded at 570 nm with FLUOstar OPTIMA (BMG LABTECH, Leicester, UK) microplate reader (Flowchart 1).

Three independent experiments carried out in a series of six were performed for each donor. Results were normalized in relation to control samples and presented as relative cell proliferation (control was set at 1).

Gene Expression Analysis
The hDPSCs were seeded on 96-well plates at a density of $0.3 \times 10^5$ cells per well and were stimulated with TSC extracts (1:4 dilution) for 3 and 7 days as described above. Unexposed cells served as controls. Total RNA was isolated from the cells using a Maxwell 16 instrument (Promega, Madison, WI, USA) with the Promega Maxwell 16 cell LEV total RNA purification kit (Promega) according to the manufacturer’s instructions. The RNA amount and quality were determined using a Nanodrop (Promega) according to the manufacturer’s instructions. The two-way ANOVA examined the effect of TSC and exposure time, as well as the interaction between those two factors on gene expression.

Gene Expression Analysis
The two-way ANOVA examined the effect of TSCs and exposure time, as well as the interaction between those two factors on gene expression levels. For OC and IL6, there was a statistically significant interaction between the effects of TSC and duration of exposure on relative fold mRNA expression ($p < 0.05$) as the exposure of hDPSCs to TSC changed the expression of these genes over time.

Table 2: Investigated genes in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Category</th>
<th>Amplicon length</th>
</tr>
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<td>GAPDH</td>
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<td>Control</td>
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<tr>
<td>ALP</td>
<td>Hs01029144_m1</td>
<td>Osteogenic</td>
<td>79</td>
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<td>COL1A</td>
<td>Hs00164099_m1</td>
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<td>OPG</td>
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<td>Hs01587814_m1</td>
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<tr>
<td>RUNX2</td>
<td>Hs01047973_m1</td>
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<td>Hs00900055_m1</td>
<td>Angiogenic</td>
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<td>IL6</td>
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<tr>
<td>TNFα</td>
<td>Hs00174128_m1</td>
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<td>80</td>
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</tbody>
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1TaqMan probes (Applied Biosystems, Foster City, CA, USA)

VEGF-A and ALP Quantification
Supernatants of hDPSCs exposed to 1:4 dilution of TSC eluates for 3 and 7 days were analyzed for VEGF-A protein levels and extracellular ALP with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA), and a colorimetric ALP assay (Sigma-Aldrich), respectively (Flowchart 1). The results were based on two independent experiments for each donor carried out in triplicate.

Statistical Analysis
The statistical analysis was performed with GraphPad Prism5 (GraphPad Software S, La Jolla, CA, USA). Data were analyzed using a two-way ANOVA followed by Dunnett’s multiple comparisons post-hoc testing to explore differences in cell responses in individual experiments. Statistical significance was set at $\alpha = 0.05$.

RESULTS

Cell Viability
Microscopic evaluation of the isolated hDPSCs showed no differences in morphology between donors. Furthermore, the hDPSCs’ morphology was not affected by exposure to the TSC eluates under the experimental conditions.

The two-way ANOVA showed that exposure to TSCs affected the viability of hDPSCs. However, the effects varied according to the material. After 1 day of exposure, only TotalFill enhanced cell viability at 1:4 dilution compared to controls ($p < 0.05$) (Fig. 1A). At 3 days, all tested TSCs showed an inhibitory effect on hDPSC viability in a dose-dependent manner (Fig. 1B). The same pattern of inhibition was apparent at 7 days for wMTA and TotalFill whereas the highest concentration of Biodentine (1:2 dilution) had the same inhibitory effect (Fig. 1C).

Gene Expression Analysis
The two-way ANOVA examined the effect of TSCs and exposure time, as well as the interaction between those two factors on gene expression levels. For OC and IL6, there was a statistically significant interaction between the effects of TSC and duration of exposure on relative fold mRNA expression ($p < 0.05$) as the exposure of hDPSCs to TSC changed the expression of these genes over time.
Simple main-effect analysis showed that exposure to TSCs altered the expression of OC, OPG, ALP, VEGF-A, and FGF1 ($p < 0.05$). In addition, exposure time to TSC had an effect on the expression levels of IL6, ALP, OC, and OPG as shown for the simple main-effect of time ($p < 0.05$).

The responses of hDPSCs were not uniform for the TSC materials tested as shown in Figure 2. Specifically, the osteogenic ALP expression was increased ($p < 0.001$) compared to control after 3 days of exposure to Biodentine whereas TotalFill led to the decreased expression of ALP ($p < 0.001$) at 7 days compared to the control (Fig. 2A). Biodentine increased OPG at 3 days whereas exposure to MTA was followed by a decrease of OPG at 7 days ($p < 0.01$) (Fig. 2A). OC expression was not changed at 3 days compared to the control, but it was increased at 7 days after exposure to Biodentine only ($p < 0.05$) (Fig. 2A). There were no differences in the expression of RUNX2 and COL1A compared to the control cells at both time points (data not shown).

The expression of VEGF-A was increased for TotalFill at 3 and 7 days ($p < 0.001$ for both incubation times) (Fig. 2B). Similarly, increased FGF1 expression was observed for Biodentine ($p < 0.05$ at 3 days and $p < 0.01$ at 7 days, respectively) (Fig. 2B). Exposure to all TSCs for 3 days increased the relative gene expression level of IL6 compared to the control (Fig. 2B). However, after 7 days, only TotalFill had an inhibitory effect on IL6 gene expression ($p < 0.01$) (Fig. 2B). IL1β and TNFα were not detected under the experimental conditions.

VEGF-A and ALP Quantification

VEGF-A production increased over time. However, only exposure to TotalFill exhibited a significant enhancement at 3 and 7 days compared to the control ($p < 0.05$) (Fig. 3A). On the contrary, ALP activity decreased over time. All TSCs decreased the ALP activity compared to controls at 3 days, but only TotalFill maintained this inhibitory effect at 7 days ($P < 0.001$) (Fig. 3B).

Discussion

The present study was designed to investigate the biological responses of hDPSCs to the application of different TSCs used in VPT and compare these responses between the tested TSCs. As previously mentioned, TSCs are used interchangeably in VPT. Having in mind that MTA may cause discoloration, other bioceramic materials such as Biodentine and TotalFill are preferable in VPT. To
The relatively young age of the donors may also have contributed to a larger pool of undifferentiated cells as the provided third molars might have been not fully root developed. Nevertheless, the isolated hDPSCs were more likely to maintain their molecular phenotype throughout in vitro conditions when compared to cell lines which may undergo phenotypic “drifting” in culture. Thus, the use of isolated hDPSCs is clinically relevant and the current results may indicate that the pulp does not respond in the same manner when those materials are used in VPT.

None of the materials affected the morphology of the hDPSCs under the experimental conditions. However, there were apparent differences in cell proliferation; MTA and TotalFill significantly reduced cell metabolism over time whereas Biodentine was the most inert material among the three. We explored the expression...
of mineralization-associated genes as markers of differentiation to detect the osteogenic/odontogenic potential of hDPSCs after exposure to the TSCs. Odontoblast-like cell differentiation shares similarities with the osteogenic cell differentiation in the bone marrow. It appeared that Biodentine influenced to a greater extent the expression of osteogenic markers. Biodentine upregulated both early (e.g., ALP, OPG) and late markers (e.g., OC) of odontoblast differentiation. On the contrary, certain mineralization markers were downregulated with MTA and TotalFill after longer exposure (OPG and ALP, respectively). A recent study using human stem cells from the apical papilla (SCAP) showed that Biodentine induced significant odontoblast differentiation compared to ProRootMTA and RetroMTA in vitro. Ideally, the materials used for RET and VPT should stimulate stem cell proliferation and differentiation leading to restoration of the pulp–dentine complex. The current findings indicate that Biodentine exhibited a higher osteogenic/odontogenic potential on hDPSCs compared to MTA and TotalFill. However, we cannot conclude that Biodentine has superior properties in clinical practice as excess or uncontrolled hard tissue deposition may be at the expense of pulp tissue and result in pulp obliteration. It is worth investigating further in a clinical trial on the occurrence of pulp obliteration after use of those three bioceramics in VPT.

Moreover, the results of VPT are also related to the effects of TSCs on angiogenic and inflammatory responses in DPSCs. Angiogenesis is decisive in the healing and repair of the dental pulp and dentin bridge formation. Inflammation is a prerequisite for healing and regeneration. However, intense, or long-term pulpal inflammation precludes regeneration. Therefore, the ideal pulp capping material should promote angiogenesis and have an anti-inflammatory effect. This study showed that TotalFill enhanced the VEGF-A expression compared to MTA and Biodentine. A similar response was observed in hBMSCs. Previously, ERRM/TotalFill induced similar levels of VEGF-A compared to ProRoot MTA in mouse DPCs, suggesting that ERRM was equally effective in angiogenic responses to MTA. Similarly, both ProRootMTA and Biodentine upregulated VEGF-A in isolated hDPSCs. When it comes to inflammatory markers, the current findings indicated that all three materials induced an initial inflammatory response that did not trigger a significant adverse reaction as shown by the initial upregulation and thereafter downregulation of IL6. Notably, TotalFill exhibited an anti-inflammatory effect in terms of significant downregulation of IL6. Initial pulpal inflammation subsiding over time has previously been reported with MTA-type products. Low production of proinflammatory mediators by dental pulp cells was seen after exposure to Biodentine and MTA-type products. ERRM and MTA induced similar proinflammatory cytokine expression in osteoblast-like MG-63 cells whereas, TotalFill and Biodentine exhibited comparable results in terms of pulpal inflammation when used as capping materials. Although it is difficult to compare the results of previous studies due to variations in experimental set-ups, it is apparent in the literature that the tested materials influence angiogenesis and inflammation. In the current study, TotalFill delivered superior angiogenic and anti-inflammatory effects compared to MTA and Biodentine. It is interesting to test in a clinical setting if use of TotalFill in VPT is beneficial in cases of severe pulpal inflammation.

The current results warrant further evaluation. The application of TSCs in VPT is a long-term process and therefore longer observation times combined with functional assays such as deposition of calcium nodules might have given more information regarding the osteogenic potential of these bioceramic materials. Furthermore, investigation of dentinogenic markers expressed during late stages of odontoblast differentiation, as well as other molecules involved in the resolution of the inflammatory process such as transforming growth factor beta family proteins and IL10, may have been useful. Another limitation of the study is the use of a monolayer two-dimensional culture as it does not accurately reflect the complex structure within human tissue. Lately, there has been an increase in the application of three-dimensional (3D) cell culture techniques as the 3D models simulate closely the in vivo conditions. Finally, the differentiation profile observed in the current study should be confirmed in prospective long-term clinical trials using the three bioceramic materials in VPT under different pulpal conditions.

**Conclusion**

Dental pulp stromal cells respond in a different way to TSCs. Under the experimental conditions, Biodentine showed higher cell viability and increased expression of osteogenic markers compared to MTA and TotalFill. On the contrary, TotalFill had the highest angiogenic and anti-inflammatory potential.

**Clinical Significance**

TSCs are used interchangeably in vital pulp therapy based on operators’ preferences. However, there are differences in the biological responses of isolated hDPSCs. The clinical relevance of the current findings is worth investigating further to provide information for the proper selection of TSCs as pulp capping agents under different scenarios of pulpal pathosis, for example, trauma vs. caries.

**References**


